Tight Coupling between Glucose and Mitochondrial Metabolism in Clonal β-Cells Is Required for Robust Insulin Secretion

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The biochemical mechanisms underlying glucose-stimulated insulin secretion from pancreatic β-cells are not completely understood. To identify metabolic disturbances in β-cells that impair glucose-stimulated insulin secretion, we compared two INS-1-derived clonal β-cell lines, which are glucose-responsive (832/13 cells) or glucose-unresponsive (832/2 cells). To this end, we analyzed a number of parameters in glycolytic and mitochondrial metabolism, including mRNA expression of genes involved in cellular energy metabolism. We found that despite a marked impairment of glucose-stimulated insulin secretion, 832/2 cells exhibited a higher rate of glycolysis. Still, no glucose-induced increases in respiratory rate, ATP production, or respiratory chain complex I, III, and IV activities were seen in the 832/2 cells. Instead, 832/2 cells, which expressed lactate dehydrogenase A, released lactate regardless of ambient glucose concentrations. In contrast, the glucose-responsive 832/13 line lacked lactate dehydrogenase and did not produce lactate. Accordingly, in 832/2 cells mRNA expression of genes for glycolytic enzymes were up-regulated, whereas mitochondria-related genes were down-regulated. This could account for a Warburg-like effect in the 832/2 cell clone, lacking in 832/13 cells as well as primary β-cells. In human islets, mRNA expression of genes such as lactate dehydrogenase A and hexokinase I correlated positively with HbA1c levels, reflecting perturbed long term glucose homeostasis, whereas that of Slc2a2 (glucose transporter 2) correlated negatively with HbA1c and thus better term glucose homeostasis, whereas that of Slc2a2 (glucose transporter 2) correlated negatively with HbA1c and thus better term glucose homeostasis, whereas that of Slc2a2 (glucose transporter 2) correlated negatively with HbA1c and thus better term glucose homeostasis, whereas that of Slc2a2 (glucose transporter 2) correlated negatively with HbA1c and thus better term glucose homeostasis, whereas that of Slc2a2 (glucose transporter 2) correlated negatively with HbA1c and thus better term glucose homeostasis, whereas that of Slc2a2 (glucose transporter 2) correlated negatively with HbA1c and thus better term glucose homeostasis, whereas that of Slc2a2 (glucose transporter 2) correlated negatively with HbA1c and thus better term glucose homeostasis, whereas that of Slc2a2 (glucose transporter 2) correlated negatively with HbA1c and thus better term glucose homeostasis, whereas that of Slc2a2 (glucose transporter 2) correlated negatively with HbA1c and thus better metabolism (Uppsala, Sweden) for providing human islets.

Pancreatic β-cells regulate whole body metabolism by secreting the hormone insulin in response to raised levels of blood glucose. However, the mechanisms underlying glucose-stimulated insulin secretion (GSIS) are not completely understood. The main signaling event is believed to be the rise in the ATP:ADP ratio, mainly accounted for by mitochondria. This closes ATP-dependent K+ (KATP) channels, depolarizing the plasma membrane. In turn voltage-gated Ca2+ channels open, allowing Ca2+ to enter the cell, initiating exocytosis of insulin-containing vesicles (1, 2). In addition, there seem to be several other metabolic events that affect insulin secretion independent of KATP channel activity (3, 4); among them are anaplerotic rate, levels of mitochondrial glutamate, and several other metabolic intermediates (1, 5–7).

Pyruvate carboxylase (PC), catalyzing the carboxylation of pyruvate to oxaloacetate, is a key regulator of cellular anaplerosis, providing a two-carbon net addition to the tricarboxylic acid cycle, thereby replenishing the cycle with intermediates. PC can be found in higher levels in the pancreatic β-cells than in most tissues (5, 8), and inhibition of the PC enzyme has been shown to lower the ATP:ADP ratio in rat islets correlating with reduced GSIS (6).

To gain further insight into the mechanisms controlling insulin secretion, we have examined two clonal cell lines derived from the INS-1 cell line, which was initially established from cells isolated from an x-ray-induced rat transplantable insulinoma (9). The parental INS-1 cell line responds to a rise in glucose concentration with at best a 4-fold increase in insulin release, which is low compared with the 15-fold release that is observed when freshly isolated rat islets are stimulated with glucose (10). The 832/2 and 832/13 subclones express similar levels of Ca(V)1.2, Ca(V)1.3, and Ca(V)2.3 Ca2+ channels, which are involved in GSIS in β-cells. This suggests that altered calcium channel activity is not responsible for discrepant insulin secretion responses (11). Furthermore, these clones are characterized by similar insulin content (12). However, significant differences in tricarboxylic acid cycle fluxes are evident that could be attributed to an accelerated anaplerotic pyruvate cycling pathway in glucose-responsive 832/13 cells (13). These characteristic differences were found to correlate with the altered GSIS in 832/2 versus 832/13 cells. However, the molec-

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The abbreviations used are: GSIS, glucose-stimulated insulin secretion; HbA1c, glycated haemoglobin; LDA, low density array; LDH, lactate dehydrogenase; PC, pyruvate carboxylase; SAB, secretion assay buffer.
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ular mechanisms by which the different metabolic performances translate into altered GSIS have not been resolved.

In the present study, we have examined specific metabolic parameters and the expression of a number of relevant genes involved in cellular metabolism in these two clonal cell lines. Our main aims were to determine: 1) insulin secretion stimulated by glucose under K_{ATP}, channel-dependent and -independent conditions; 2) rates of glucose utilization and hence glycolysis; 3) rates of respiration reflecting electron transport chain activity; 4) rates of mitochondrial ATP production; 5) activities of complexes I, III, and IV of the electron transport chain; 6) proteins expressed in the electron transport chain; and 7) expression profile of genes encoding enzymes in glycolysis, the tricarboxylic acid cycle, and the respiratory chain in clonal β-cells and human islets. Using these approaches, we found that the glucose-responsive 832/13 cells exhibited a tight regulation of mitochondrial metabolism in response to changes in ambient glucose; this regulation was lost in the glucose-unresponsive 832/2 cell line, which instead favored glycolytic metabolism and production of lactate. In support of these different phenotypes, a low density array (LDA) revealed that mRNA levels of a number of genes involved in mitochondrial metabolism were highly expressed in glucose-responsive 832/13 cells, whereas expression of genes for enzymes of glycolysis were up-regulated in glucose-unresponsive 832/2 cells. When examining expression of the same genes in human pancreatic islets, they correlated with long term glucose control, i.e., HbA\textsubscript{1c} levels, in a fashion similar to that found in glucose-responsive and -unresponsive 832/2 cells. Thus, genes (i.e., mRNA levels) correlating with higher HbA\textsubscript{1c} levels were up-regulated in unresponsive 832/2 cells, whereas genes correlating with lower HbA\textsubscript{1c} levels, i.e., better glycemic control, were up-regulated in glucose-responsive 832/13 cells.

EXPERIMENTAL PROCEDURES

Reagents—All of the reagents and solutions were obtained from Sigma unless otherwise indicated. HbA\textsubscript{1c} was determined by the mono-S procedure (the current Swedish national standard) (14).

Cell Culture—The 832/13 and 832/2 cells are two clonal lines isolated following transfection of the parental INS-1 cell line with a plasmid containing the human insulin gene under control of the cytomegalovirus promoter and a neomycin resistance gene. The 832/13 cells are robust secretors of insulin (10-fold) when stimulated by raised glucose (12). The 832/2 cell line, however, fails to release insulin in response to increases in ambient glucose. 832/13 and 832/2 cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO\textsubscript{2}. RPMI 1640 culture medium containing 11 mM D-glucose was supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 50 μM β-mercaptoethanol (12).

Human Pancreatic Islet Culture—All of the islets were from nondiabetic deceased donors (13 females, 10 males; body mass index, 17.6–29.0 kg/m\textsuperscript{2}; ages, 26–73 years) obtained from the Nordic Islet Transplantation Programme by the courtesy of Professor Olle Korsgren (Uppsala University, Uppsala, Sweden). The islets were processed at the Human Tissue Laboratory at Lund University Diabetes Centre. Purity varied from 13 to 90%. The islets were culture in CMRL 1066 culture medium (ICN Biomedicals, Costa Mesa, CA) containing 10 mM HEPES, 2 mM L-glutamine, 50 μg/ml gentamicin, 0.25 μg/ml fungizone (Invitrogen), 20 μg/ml ciprofloxacin (Bayer Healthcare, Leverkusen, Germany), and 10 mM nicotinamide at 37 °C in a humidified atmosphere of 95% air and 5% CO\textsubscript{2} for 1–9 days prior to RNA preparation. All of the islet donors had given consent to donate organs for medical research. All of the procedures were approved by the ethical committees at Uppsala and Lund Universities.

Insulin Secretion—832/13 and 832/2 cells were cultured in 24-well plates (Sarstedt, Nümbrecht, Germany) to reach ≥90% confluency. Prior to assay the cells were washed in secretion assay buffer (SAB; 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1.16 mM MgSO\textsubscript{4}, 20 mM HEPES, 2.5 mM CaCl\textsubscript{2}, 25.5 mM NaHCO\textsubscript{3}, 0.2% bovine serum albumin, pH 7.2) supplemented with 2.8 mM glucose. After that the cells were incubated in 2.8 mM glucose SAB for 2 h at 37 °C. Fresh buffer was then added, and GSIS was measured after incubation for 1 h at 2.8 or 16.7 mM glucose. Insulin concentration in supernatants was measured using the Coat-a-Count kit (Simens Medical Solutions Diagnostics, Los Angeles, CA), which recognizes human insulin and cross-reacts ~20% with rat insulin (12).

Glucose Utilization—The cells were cultured in 24-well plates overnight and washed in phosphate-buffered saline prior to assay. The first step was 1.5 h of incubation of cells in SAB containing 2.8 mM glucose at 37 °C. After incubation, two blanks for each condition were created by adding 100 μl of 10% trichloroacetic acid to the wells. After removing the SAB, 500 μl of SAB containing D-[5-\textsuperscript{3}H]glucose (specific activity, 19.63 Ci/mmol; PerkinElmer Life Sciences) and glucose to a final concentration of 2.8 or 16.7 mM glucose, respectively, were added to each well where cells were incubated for 30 min at 37 °C. 100 μl of 10% trichloroacetic acid were added to prevent further metabolism of [5-\textsuperscript{3}H]glucose. The cells were harvested from the wells, and 500 μl of lysate were transferred to 1.5-ml tubes, which were placed inside scintillation vials containing 500 μl of H\textsubscript{2}O. The scintillation vials were then sealed and incubated at 56 °C overnight to permit \textsuperscript{3}H]OH formed by the cells to evaporate and equilibrate with water in the vials. The vials were then cooled to room temperature. After removing the tubes, \textsuperscript{3}H]OH content was measured by liquid scintillation spectrometry (15). The average number of disintegrations/min in blank tubes was subtracted from experimental measurements. Glucose utilization was calculated as \[ ([\textsuperscript{3}H]OH formed (disintegrations/min))/([specific radioactivity of D-[5-\textsuperscript{3}H]glucose (disintegrations/min/μmol)])].

Lactate Production—For the measurement of lactate production, the cells were prepared as described for insulin secretion. Lactate released from cells incubated at 2.8 and 16.7 mM glucose was measured in supernatants, using a colorimetric lactate assay kit (Biovision, Mountain View, CA).

Respiration—The Seahorse Extracellular Flux Analyzer XF24 (Seahorse Bioscience) measures oxygen consumption rate in a 24-well format by sensing changes in oxygen content in a 7-μl
volume above the plated cells with a fluorescence biosensor. The measurements are noninvasive and made in short and repeated intervals. An assay medium composed of 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.16 mM MgSO₄, 20 mM Hepes, 2.5 mM CaCl₂, 0.2% bovine serum albumin, pH 7.2, and supplemented with 2.8 mM glucose was used in the XF analysis. The cells were seeded in an XF24 24-well cell culture microplate at 250,000 cells/well (0.32-cm² growth area) in 500 µl of growth medium and incubated overnight at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Prior to assay, RPMI 1640 medium was removed and replaced by 750 µl of assay medium. The cells were preincubated under these conditions for 2 h at 37 °C in air. The experiments were designed to determine respiration in low (2.8 mM) glucose and for 60 min following the transition to high (16.7 mM) glucose. The proportions of respiration driving ATP synthesis and proton leak were determined by the addition of oligomycin (4 µg/ml). After a further 30 min, 4 µM of the uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone was added to determine maximal respiratory capacity. After a further 10 min, 1 µM rotenone was added to block transfer of electrons from complex I to ubiquinone.

**ATP Production by Mitochondria in Digitonin-permeabilized Cells**—The cells were cultured in 24-well culture plates overnight and collected by trypsinization and centrifugation at 750 x g for 3 min at 4 °C. The pellet (700,000–900,000 cells) was then resuspended in 100 µl of Tris-HCl (pH 7.5) containing 0.1 g/liter digitonin (Calbiochem) and incubated for 10 min on ice to permeabilize the cells. The cells were collected by centrifugation at 750 x g for 3 min at 4 °C and resuspended in 100 µl of Tris-HCl (pH 7.5). The oligomycin-sensitive ATP production rate was determined with a luminescence system similar to that previously described for isolated mitochondria (16). An aliquot of permeabilized cells was diluted 1:2500 to a final volume of 250 µl in a mixture consisting of ATP monitoring reagent (BioThema AB, Handen, Sweden), 150 mM sucrose, 15 mM KH₂PO₄, 2 mM (CH₃COO)₂ Mg₄H₂O, 0.5 mM EDTA, 0.6 mM ADP, 1 mM AMP, 25 µM pentalithium P₃P₅-di(adenosine-5') pentaphosphate, and a substrate combination of 15 mM glutamate and 15 mM succinate. After a 5-min incubation at 25 °C, ATP production was monitored for 4 min. Oligomycin (0.6 mg/ml) was added to inhibit mitochondrial ATP synthase, and any further ATP production (e.g. from residual glycolysis and/or adenylate kinase) was monitored for another 4 min. Finally, 0.5 µM ATP was added, and the subsequent increase in signal was used to calibrate the amount of ATP produced during the measurements. Oligomycin-sensitive ATP production was established as the difference in ATP production before and after the addition of oligomycin. The measurements were performed in triplicate on TECAN Infinite M200 equipped with Magellan software.

**Isolation of Mitochondria**—Intact mitochondria were isolated from clonal cells using differential centrifugation as described (17).

**Complex I, III, and IV Activities in Mitochondrial Extracts**—Complex I activity was determined by measuring the rate of oxidation of NADH at 340 nm using ubiquinone₁ as the electron acceptor. The rate sensitive to rotenone was taken to be complex I activity (18). Complex III activity was measured by monitoring the oxidation of ubiquinol₁ with cytochrome c (III) as the electron acceptor at 550 nm (18). Complex IV activity was measured by monitoring the oxidation of cytochrome c (II) at 550 nm (18).

**Immunoblotting**—Isolated mitochondria were homogenized in a homogenization buffer containing 50 mM Tris, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 1:100 protease inhibitor mixture. Protein content was determined by the BCA method, the samples were mixed with Laemmli buffer, and 10 µg protein was run onto a 12% SDS-PAGE gel and subsequently blotted onto polyvinylidene difluoride membranes. Proteins for ND6, Core 2, COX I, and F₆F₁ ATP synthase subunit α were detected with a primary mouse antibody mixture (MitoSciences, Eugene, OR) in dilution of 1:1000. Horseradish peroxidase-coupled anti-rabbit IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used as a secondary antibody (1:8000). Enhanced chemiluminescence was used to detect protein on the blots. The protein levels were determined by densitometry.

**LDA in Clonal Cells**—An LDA was used to screen for changes in gene expression of metabolic enzymes. TaqMan LDAs (Applied Biosystems, Foster City, CA) are created on a 384-well microfluidic card, which is arrayed with TaqMan gene expression assays. We designed an array of 46 essential genes in glycolysis, the tricarboxylic acid cycle, and the respiratory chain³ (13); the assays were predesigned and validated by Applied Biosystems. 832/13 and 832/2 cells were cultured at 11.1 mM glucose for 48 h, and RNA was extracted using an RNeasy RNA purification kit (Qiagen) according to the manufacturer’s instructions. RNA concentration was measured by 260-nm absorbance. Reverse transcription was carried out using RevertAid™ First-Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) in reactions containing 500 ng of total RNA, 50 mM Tris-HCl (pH 8.3), 1 mM dNTPs, 200 ng of random hexamer primers, 50 mM KCl, 4 mM MgCl₂, 10 mM dithiothreitol, 200 units of RevertAid™ Moloney murine leukemia virus reverse transcriptase, and 20 units of RiboLock™ ribonuclease inhibitor in a final volume of 20 µl. The template was denatured by heating (65 °C for 5 min), and annealing was performed at 25 °C for 5 min. The reverse transcription reaction was run at 42 °C for 60 min followed by enzyme inactivation at 72 °C for 10 min. 50 ng of cDNA and TaqMan Universal PCR Master Mix (AmpliTaq Gold DNA Polymerase, Passive Reference 1, buffer, dNTPs, AmpErase UNG) were then loaded to each port on the microfluidic card, and the card was run on a 7900HT fast real time system. After 2 min at 50 °C and 10 min at 94.5 °C, the templates were co-amplified by 40 repeated cycles of which one cycle consisted of a 30-s denaturing step at 97 °C and a 1-min annealing/extension step at 60 °C. Cycle threshold (Ct) values were calculated by the ABI PRISM software, and relative gene expression levels were expressed as the difference in Ct values (ΔCt) of the target gene and the mean Ct of the hypoxanthine-guanine phosphoribosyl transferase housekeeping gene. ΔΔCt values were calculated for each sample using the mean of its ΔCt.

³ U. Bryborn, O. Kotova, P. Spégel, T. Köck, E. Hallgard, M. Sugden, and H. Mulder, manuscript in preparation.
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was carried out with the GeneChip® H23041 and stained in a GeneChip® hybridization oven 6400 using standard procedures. The arrays were washed and stained in a GeneChip® Fluidics Station 450. Scanning was carried out with the GeneChip® Scanner 3000, and image analysis was performed using GeneChip® Operating Software. The array data were summarized and normalized with the robust multi-array analysis method using Expression Console software (Affymetrix).

Protein Measurement — Protein content was determined in the obtained cell lysates by the bicinchoninic acid protein assay (Thermo), measuring the absorbance at 590 nm and comparing it with a bovine serum albumin standard curve.

Data Analysis — All of the results are presented as the means ± S.E. Statistical comparisons of the mean values were performed with Student’s t-test. Correlations were analyzed using nonparametric Spearman’s tests. Differences between mean values were considered significant when p < 0.05.

RESULTS

Insulin Secretion — The data depicted in Fig. 1A show that GSIS differed greatly between the two cell lines. The glucose-responsive 832/13 cells displayed an 11.9-fold increase in insulin secretion when incubated in 16.7 mM glucose compared with 2.8 mM glucose (Fig. 1A; p < 0.01), whereas the glucose-unresponsive 832/2 cells did not respond. Additionally, activation of the K<sub>ATP</sub> channel-independent pathway by adding 35 mM KCl and 250 μM diazoxide (20, 21) lead to a 9.6-fold increase of basal insulin secretion (2.8 mM glucose) in 832/13 cells (p < 0.001). Stimulation with 16.7 mM glucose further increased insulin secretion in 832/13 cells under this condition. K<sub>ATP</sub> channel-independent glucose sensing in 832/2 cells; however, increased basal insulin secretion only 4.2-fold (p < 0.01) and additional glucose stimulation had no effect. (Fig. 1A). The fact that KCl + diazoxide increased insulin secretion of 832/2 cells from ~10 to ~60 ng/mg protein/h shows that the exocytic response to plasma membrane depolarization is still intact.

Glucose Utilization — Glycolysis was estimated from the rate of [3H]OH production from [5-3H]glucose; one molecule of [3H]OH is formed when 2-phosphoglycerate is converted to phosphoenolpyruvate by enolase. Remarkably, basal D-[5-3H]glucose utilization (2.8 mM glucose) by glucose-unresponsive 832/2 cells was already 11.6-fold greater than that by 832/13 cells (Fig. 1B; p < 0.001). In the 832/2 cells, however, glucose stimulation did not further increase the glucose utiliza-
Mitochondrial metabolism is crucial for insulin secretion, respiration after stimulation with 16.7 mM glucose compared with basal oxygen consumption rate in the presence of 2.8 mM glucose (Fig. 1A; \( p < 0.05 \)). In contrast, glucose-unresponsive 832/2 cells showed a 1.6-fold higher respiration at the basal glucose level (\( p < 0.05 \)) but failed to increase their respiratory rate in response to elevation of the glucose concentration (Fig. 2B).

The proton circuit generated by the electron transport chain is completed by parallel re-entry via both the ATP synthase and the inherent inner membrane proton leak. To distinguish between these two pathways, oligomycin was added to inhibit proton re-entry via the ATP synthase. Both clonal cell lines showed a drop in respiration upon the addition of oligomycin; however, only in glucose-responsive 832/13 cells was the extent of this drop dependent on the availability of glucose. This indicates that ATP synthesis (and hence turnover) was responsive to the glucose concentration. It is notable that the proton leak in 832/13 cells (but not 832/2 cells) was also higher in the presence of elevated glucose. Although mitochondrial membrane potentials were not determined, it is likely that this increase in leak reflects the voltage dependence of the pathway because the mitochondria are known to hyperpolarize in response to increased substrate availability.

Glutamate- and Succinate-induced ATP Production by Mitochondria in Permeabilized Cells—In the \( \beta \)-cell, an important purpose of mitochondrial metabolism is to generate ATP, the main trigger of insulin secretion. To evaluate the capacity of the two cell lines to produce ATP in a way other than by determining respiration and to bypass respiratory limitations caused by substrate availability, mitochondrial ATP production was measured in cells permeabilized with digitonin and in the presence of a combination of glutamate and succinate. Thus, state 3 ATP synthesis in glucose-unresponsive 832/2 cells was 2.5-fold lower than that in glucose-responsive 832/13 cells (Fig. 3; \( p < 0.05 \)).

Complex I, III, and IV Activities—To elucidate the difference in rates of mitochondrial ATP production in the two cell lines, activities of complexes I, III, and IV in the electron transport chain were measured in purified mitochondria. In agreement with the impaired respiratory response to glucose, Complex I, III, and IV activities in glucose-unresponsive 832/2 cells were 2.7-, 3.0-, and 2.9-fold lower than those in glucose-responsive 832/13 cells, respectively (Fig. 4; \( p < 0.05, p < 0.001, \) and \( p < 0.01 \), respectively), when expressed per mg of mitochondrial protein.

Protein Expression of Subunits in Electron Transport Chains—To further investigate altered mitochondrial function in glucose-unresponsive 832/2 cells, we examined proteins expressed in the electron transport chain: mitochondrial DNA encoded ND6 (complex I) and COX I (complex IV) subunits and nuclear DNA encoded Core 2 (complex III) and \( \alpha \) (complex V) subunits. Protein expression of ND6, Core 2, COX I, and \( \alpha \) subunits in glucose-unresponsive 832/2 cells was 3.4-, 1.4-, 2.5-, and 1.4-fold lower than in glucose-responsive 832/13 cells (Fig. 5; \( p < 0.01, p < 0.05, p < 0.01, \) and \( p < 0.05 \), respectively).

Gene Expression Analysis in Clonal \( \beta \)-Cells—Next, we proceeded to analyze which changes in gene expression of metabolic enzymes may account for the metabolic switch from predominant mitochondrial metabolism to glycolytic metabolism and the subsequent lack of GSIS in 832/2 cells. To this end, heat treatment did not cause the expression pattern of these genes to resemble that of glucose-responsive 832/13 cells (Fig. 6).

**FIGURE 2. A**, the \( O_2 \) consumption rate is accelerated in 832/13 cells by increases in glucose concentration. The data are presented as the means \( \pm S.E. \) for four independent experiments (\( *, p < 0.05 \) relative to 2.8 mM glucose). **B**, the oxygen consumption rate is not changed in 832/2 cells by changes in glucose concentration. The data are presented as the means \( \pm S.E. \) for four independent experiments.
comparative expression of the candidate metabolic genes in glucose-responsive 832/13 cells and glucose-unresponsive 832/2 cells was assessed by an LDA. Genes for glucose transport, enzymes in glycolysis, the tricarboxylic acid cycle, and electron transport were chosen for the transcript profiling. Of the 46 genes, 26 were differentially expressed in 832/13 and 832/2 cells, respectively (Table 1). Sixteen genes were down-regulated, whereas six were up-regulated on the mRNA level in 832/2 cells compared with 832/13 cells. Specifically, the expression of the Slc2a2 encoding the facilitative glucose transporter 2 and the high Michaelis-Menten constant (Km) glucose-phosphorylating enzyme glucokinase mRNA was markedly lower in 832/2 cells than in 832/13 cells. In contrast, the low Km enzyme hexokinase I mRNA was expressed in 832/2 cells but could not be detected in 832/13 cells. Additionally, in accordance with the observation that the nonoxidative glucose metabolite lactate was released from 832/2 cells, but not 832/13 cells, lactate dehydrogenase A mRNA was expressed in 832/2 cells but not in 832/13 cells.

Gene Expression Analysis in Human Pancreatic Islet—Finally, we proceeded to examine whether any of the metabolic perturbations found in glucose-unresponsive 832/2 cells occur in human islets. We performed a global expression analysis in pancreatic islets from human donors and correlated the expression of the genes analyzed in glucose-unresponsive 832/2 cells with HbA1c levels. The HbA1c plasma level is a robust indicator of mean glycemia in human subjects in the preceding 2 or 3 months and is widely used in clinical practice. In addition, there is a negative correlation between HbA1c levels and GSIS in the human islets.4 Indeed, in human islets, we found
that expression of eight of the genes that were altered in the clonal β-cells also correlated with HbA1c levels. This suggests that they are regulated by or regulate glucose levels. Thus, down-regulation in 832/2 cells was reflected by a negative correlation of human islet mRNA levels with HbA1c levels. This held true for mRNA levels of, for example, Slc2a2 encoding the facilitative glucose transporter 2, malic enzyme 1, glycerol-3-phosphate dehydrogenase 2 (mitochondrial), and acetyl-CoA carboxylase α (Fig. 6), which were low in 832/2 cells (Table 1). On the other hand, up-regulation in 832/2 cells was reflected by a positive correlation of said gene expression with HbA1c levels; for example, hexokinase I, triosephosphate isomerase 1, lactate dehydrogenase A, and citrate synthase mRNA levels (Fig. 6), which were high in 832/2 cells (Table 1), were higher in human islets with long term elevation of plasma glucose levels.

DISCUSSION

The rationale for undertaking the current study was that we have access to two INS-1-derived clonal β-cell lines, 832/2 and 832/13, that were initially generated upon stable expression of a plasmid containing the cDNA for human insulin (12). Remarkably, despite having a common parental ancestor cell line, i.e. INS-1, these isolated subclones exhibit a spectrum of different characteristics; for example, the glucose-stimulated insulin response ranges from nonexistent (832/2) to very strong (832/13; >10-fold). These circumstances provided an opportunity to explore how it is possible that closely related clonal cells still may exhibit such different secretory performances. This is, in part, analogous to the function of β-cells in healthy and diabetic individuals; studying the differences between closely related clonal β-cell lines may increase our understanding of molecular mechanisms of β-cell function and, possibly, the pathogenesis of Type 2 diabetes.

Indeed, we have previously shown that the robust insulin secretory response in 832/13 cells compared with 832/2 cells, in part, can be attributed to increased cAMP/protein kinase A signaling caused by a substantially higher expression of the catalytic subunit of cAMP/protein kinase A (22). However, neither expression of critical calcium channels nor insulin content is different in the two subclones (11). Furthermore, Newgard and co-workers (13) showed that glucose responsiveness in different INS-1 subclones correlates with an increased flux in an anaplerotic pathway, termed pyruvate cycling. To investigate which processes distal to enhanced pyruvate cycling are responsible for glucose responsiveness, we have previously used phenylacetic acid, an irreversible inhibitor of PC, as a tool to inhibit anaplerosis in rat islets. The most striking finding in the study was that abrogated anaplerosis impairs the glucose-induced rise in the ATP:ADP ratio, and this inhibits both first and second phase insulin secretion (6).

In the present study, we focused on differences in glucose metabolism between 832/2 and 832/13 cells to determine whether and how an altered metabolic response to glucose may contribute to the differential glucose responsiveness in β-cells. Following the pathway of glucose metabolism, we first examined glycolytic activity by determining glucose utilization. Already here, a profound difference between 832/13 and 832/2 cells was observed. In the glucose-responsive 832/13 line, the rate of glycolysis was proportional to ambient glucose. In contrast, glycolysis was highly up-regulated in the glucose-unresponsive 832/2 line, reaching very high rates already at a low glucose concentration, and exceeded that in 832/13 cells. These results could be explained by several alterations in glucose metabolism of 832/2 cells affecting glycolysis. One potential contributor to the 832/2 phenotype is a change in substrate affinity of glucose transport and/or phosphorylation. This has previously been reported for the m14 subclone of the insulin-secreting mouse β-cell line MIN6 that exhibits poor GSIS (23). Indeed, we found that 832/2 cells, like MIN6-m14 cells, exhibited a high expression level of low K_m hexokinase I, whereas 832/13 cells exclusively expressed the high K_m glucokinase. This would explain the maximal glycolytic rate in 832/2 cells already at low ambient glucose, because of the saturation of glucose transport and phosphorylation, whereas the same process would be dependent on ambient glucose concentrations in 832/13 cells. The observed accelerated glycolytic rate in 832/2 cells would require provision of NAD^+ for the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase. This
TABLE 1
Genes differentially expressed between glucose-responsive 832/13 and glucose-unresponsive 832/2 cells

Gene expression was measured by low density array from four independent mRNA isolations for 832/13 and 832/2 cells. The cells were cultured for 48 h at 11 mM glucose, and gene expression was then determined in the LDA by real time quantitative reverse transcription-PCR. The fold changes were subtracted from $2^{ΔΔC_t}$ real time quantitative reverse transcription-PCR values from four independent experiments.

| Symbol                  | Gene description                              | GenBank\textsuperscript{TM} accession number | TaqMan assay | $C_{\Delta C_t}$ 832/13 cells | $C_{\Delta C_t}$ 832/2 cells | Differential fold changes (832/13 cells/832/2 cells) | $P$  |
|-------------------------|------------------------------------------------|---------------------------------------------|--------------|-------------------------------|-------------------------------|-------------------------------------------------|------|
| Glycolysis-related genes|                                                |                                             |              |                               |                               |                                                 |      |
| Gck                     | Glucokinase                                    | NM_012565                                  | Rn00561265_m1| 26.41                         | 29.03                         | 5.56                             | <0.001|
| HK1                     | Hexokinase                                     | NM_012734                                  | Rn00562436_m1| Not detected                  | Not detected                  | 29.81                           |      |
| Ldha                    | Lactate dehydrogenase A                        | NM_017025                                  | Rn00820751_g1| 25.00                         | 25.28                         | 0.81                             |      |
| Me1                     | Malic enzyme 1                                 | NM_012600                                  | Rn00561502_m1| 24.59                         | 25.97                         | 1.33                             | <0.05|
| Pklr                    | Pyruvate kinase, liver, and red blood cells    | NM_012624                                  | Rn00561764_m1| 25.00                         | 26.35                         | 2.35                             | <0.001|
| Pfkm                    | Phosphofructokinase                            | NM_053291                                  | Rn00581848_m1| 24.78                         | 25.18                         | 0.42                             | <0.01|
| Tril                    | Triosephosphate isomerase 1                    | NM_022922                                  | Rn00821155_g1| 23.03                         | 21.79                         | 0.42                             |      |
| Mitochondria-related genes|                                              |                                             |              |                               |                               |                                                 |      |
| Acoc2                   | Acetate-CoA carboxylase α                      | NM_016987                                  | Rn005672936_g1| 26.95                         | 25.55                         | 1.10                             | <0.05|
| Ca                      | Citrate synthase                               | NM_012376                                  | Rn00562472_m1| 23.44                         | 23.92                         | 0.56                             | <0.05|
| Gpd2                    | Glycerol-3-phosphate dehydrogenase 2, mitochondrial | NM_031151                                 | Rn00577832_m1| 24.83                         | 24.53                         | 0.77                             | <0.05|
| Mo1                     | Malate dehydrogenase, mitochondrial            | NM_012776                                  | Rn00561264_m1| 27.17                         | 27.17                         | 0.00                             |      |
| Pc                      | Pyruvate carboxylase                            | NM_012985                                  | Rn00562434_m1| 23.59                         | 24.09                         | 1.34                             | <0.001|
| ATP5e                   | ATP synthase, H ÷ transporting mitochondrial F1 complex, e subunit | NM_019383                                  | Rn00581986_g1| 23.05                         | 23.96                         | 0.54                             | <0.001|
| Cox4i1                  | Cytochrome c oxidase subunit IV isoform 1      | NM_017202                                  | Rn00578590_m1| 25.99                         | 26.22                         | 0.59                             | <0.05|
| Ndufa5                  | NADH dehydrogenase 1 α subcomplex 5           | NM_012985                                  | Rn00566528_m1| 24.39                         | 30.93                         | 0.25                             | <0.001|
| Nqo1                    | NADH dehydrogenase quinone 1                  | NM_017000                                  | Rn00820691_g1| 25.99                         | 27.75                         | 0.81                             | <0.05|
| Miscellaneous            |                                               |                                             |              |                               |                               |                                                 |      |
| Acaca                   | Acetoc-CoA carboxylase α                       | NM_016987                                  | Rn00672936_g1| 26.95                         | 25.55                         | 1.10                             | <0.05|
| Aclcy                   | ATP citrate lyase                              | NM_016987                                  | Rn00656411_m1| 22.27                         | 23.93                         | 3.03                             | <0.001|
| Cpt1α                   | Carnitine palmitoyltransferase 1, liver        | NM_031559                                  | Rn00580702_m1| 26.56                         | 26.00                         | 0.65                             | <0.05|
| Fasn                    | Fatty acid synthase                            | NM_017332                                  | Rn00569117_m1| 25.39                         | 26.16                         | 1.00                             | <0.05|
| Glucl1                  | Glutamate dehydrogenase                       | NM_012570                                  | Rn00561306_m1| 24.43                         | 25.20                         | 1.60                             | <0.05|
| Mlcyd                   | Malonyl-CoA dehydrogenase                      | NM_053477                                  | Rn00585056_m1| 28.18                         | 29.47                         | 2.00                             | <0.01|
| Pdk2                    | Pyruvate dehydrogenase kinase 2               | NM_030872                                  | Rn00578427_m1| 27.85                         | 27.04                         | 0.55                             | <0.01|

normally involves tightly coupled metabolic fluxes between the tricarboxylic acid cycle and glycolysis, subserved by the malate-aspartate and glycerolphosphate shuttles. These shuttles provide NAD$^+$ in the cytosol to maintain glycolytic flux and NADH in the mitochondrion to be used by the electron transport chain for oxidative phosphorylation. Indeed, these shuttles have been shown to be critical for β-cell function (24). Another alternative is altered pyruvate processing. Normally, in glucose-responsive pancreatic β-cells, pyruvate from glycolysis is further processed through two regulated metabolic pathways: PC that requires ATP and provides oxaloacetate for the tricarboxylic acid cycle and pyruvate dehydrogenase that feeds acetyl-CoA into the tricarboxylic acid cycle (13). Pyruvate can be reduced to lactate by LDH-A, particularly under hypoxic conditions (25) or when mitochondrial metabolism is compromised, with the result that NADH is oxidized to NAD$^+$; however, this is normally not observed in glucose-responsive β-cells. As in MIN6-m14 cells (18), the high glycolytic rate in 832/2 cells was indeed accompanied by the production of lactate. This finding was corroborated by the detection of a high LDH-A expression level in 832/2 cells. The enzyme may thus serve the role of providing additional cytosolic NAD$^+$ for the reaction catalyzed by glycolaldehyde-3-phosphate dehydrogenase, substituting for the mitochondrial shuttles, hereby supporting the high glycolytic rate. In contrast, LDH-A expression was not found in glucose-responsive 832/13 cells, which lacked detectable production of lactate.

In the mitochondrion, oxidation of pyruvate generates NADH, which provides electrons for the electron transport chain that in turn creates a proton gradient over the inner mitochondrial membrane. This proton gradient is finally dissipated by ATP synthase for the synthesis of ATP from ADP (26, 27). This process is of paramount importance in the pancreatic β-cell, because a rise in the ATP:ADP ratio triggers GSIS. However, it requires that O$_2$ serves as an electron acceptor in the mitochondrial matrix. Thus, the activity of the electron transport chain and oxidative phosphorylation can be monitored by O$_2$ consumption. Using the Seahorse XF24, O$_2$ consumption rates can be monitored in real time. Such analysis showed that respiration in 832/13 cells responded to a rise in ambient glucose; this control was lacking in the glucose-unresponsive 832/2 line. We augmented these studies with experiments on permeabilized cells, where mitochondrial ATP production upon the addition of glutamate and succinate was monitored. This confirmed that 832/13 cells exhibit a significantly greater capacity to produce ATP in mitochondria in response to met-
The notion that mitochondrial metabolism is favored in 832/13 cells was further supported by the fact that many of the genes for mitochondrial enzymes and proteins were more highly expressed in the glucose-responsive cell line. Moreover, measurements of Complex I, III, and IV activities in the electron transport chain of 832/13 cells showed significant increases compared with glucose-nonresponsive 832/2 cells. This was further supported by increased protein expression of selected constituents of these complexes. Notably, 832/13 and 832/2 clones contain equal amounts of mitochondrial DNA/cell (data not shown).

The observed phenomena in the 832/2 line are reminiscent of the Warburg effect (28): cancer cells preferentially employ glycolytic metabolism, even in the presence of O2. Clearly, such ability confers a survival advantage, particularly because cancer cells are likely to exist under anaerobic conditions during tumor progression. Both clonal cell lines in the study originate from an insulinoma, and therefore it is not surprising that some subclones may exhibit a more de-differentiated, cancer-like phenotype. The cancer cell characteristics evolve at the expense of the more highly differentiated capacities, e.g. glucose-regulated insulin secretion. The Warburg-like effect in 832/2 cells can also be understood in light of LDH-A expression, which is required for a high glycolytic rate when mitochondrial metabolism and its associated shuttles re-oxidizing NADH in the cytosol are compromised.

In pancreatic β-cells the diversion of metabolites from mitochondrial metabolism by LDH-A expression, e.g. in response to increased HIF-1α, has been associated with dampened GSIS despite a simultaneously increased glycolytic activity (29). Previous investigations made by Ishihara et al. (30) revealed that overexpression of LDH-A in the clonal INS-1 cells and isolated rat islets does not affect GSIS but resulted in stimulation of insulin secretion by low lactate concentrations with a concomitant increase in its oxidation. However, Zhao and Rutter (19) showed that overexpression of LDH-A in clonal MIN6 cells reduces GSIS.

To summarize, by combining a number of biochemical, functional, and transcriptomic analyses, we have further defined how two related clonal β-cell lines can exhibit such different capacities to respond to glucose with appropriate insulin secretion. The fundamental difference is that the glucose-unrespon-

![Graphs and Data](supplemental_Fig_S1)
Metabolism and Insulin Secretion in β-Cells

cells rely on glycolytic metabolism, whereas the glucose-responsive cells exhibit robust coupling of the glucose stimulus and mitochondrial metabolism. In this respect, the ability to produce ATP in response to accelerated metabolism of glucose appears to be critical. Loss of this capacity would also lead to impaired insulin secretion in individuals predisposed to develop Type 2 diabetes.

As a first step to extrapolate the observed metabolic alterations relevant for metabolic control of insulin secretion in 832 cells to human cells, we have performed a global expression analysis of islets from human donors. We selected the 46 genes assayed by the LDA in clonal cells and plotted the corresponding mRNA levels found in the microarray analysis of the human islets against HbA1c levels. A pattern of regulation of these genes in human islets emerged that was similar to that found in clonal β-cells. In general, higher long term plasma glucose levels in donors correlated with up-regulation of genes involved in glycolysis, whereas genes involved in mitochondrial metabolism were down-regulated. Admittedly, this does not prove a causal relationship involved in mitochondrial metabolism were down-regulated. Similarly, changes in HbA1c levels. Furthermore, samples from donors with discrete changes in glucose homeostasis are reflected by mitochondria metabolism, which is required for robust insulin secretion. The 832 cells to humancells, we have performed a global expression analysis of islets from human donors. We selected the 46 genes assayed by the LDA in clonal cells and plotted the corresponding mRNA levels found in the microarray analysis of islets from human donors. We selected the 46 genes assayed by the LDA in clonal cells and plotted the corresponding mRNA levels found in the microarray analysis of the human islets against HbA1c levels. A pattern of regulation of these genes in human islets emerged that was similar to that found in clonal β-cells. In general, higher long term plasma glucose levels in donors correlated with up-regulation of genes involved in glycolysis, whereas genes involved in mitochondrial metabolism were down-regulated. Admittedly, this does not prove a causal relationship involved in mitochondrial metabolism were down-regulated. Similarly, changes in HbA1c levels. Furthermore, samples from donors with discrete changes in glucose homeostasis are reflected by

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