The metabolic pathways that are involved in regulating insulin secretion from pancreatic β-cells are still incompletely understood. One potential regulator of the metabolic phenotype of β-cells is the transcription factor aryl hydrocarbon receptor nuclear translocator (ARNT)/hypoxia-inducible factor (HIF)-1β. ARNT/HIF-1β levels are profoundly reduced in islets obtained from type 2 diabetic patients. However, no study to date has investigated key pathways involved in regulating insulin release in β-cells that lack ARNT/HIF-1β. In this study, we confirm that siRNA-mediated knockdown of ARNT/HIF-1β inhibits glucose-stimulated insulin secretion. We next investigated the metabolic consequence of the loss of ARNT/HIF-1β knockdown. We demonstrate that β-cells with reduced ARNT/HIF-1β expression levels exhibit a 31% reduction in glycolytic flux without significant changes in glucose oxidation or the ATP:ADP ratio. Metabolic profiling of β-cells treated with siRNAs against the ARNT/HIF-1β gene revealed that glycolysis, anaplerosis, and glucose-induced fatty acid production were down-regulated, and all are key events involved in glucose-stimulated insulin secretion. In addition, both first and second phase insulin secretion in islets were significantly reduced after ARNT/HIF-1β knockdown. Together, our data suggest an important role for ARNT/HIF-1β in anaplerosis, and it may play a critical role in maintaining normal secretion competence of β-cells.

The ability of the pancreatic β-cell to maintain glucose homeostasis critically depends on the existence of a functional glucose sensor that operates within the physiologic range of glucose concentrations (1, 2). The glucose-phosphorylating enzyme glucokinase (GK) has been identified as the rate-limiting step in cytosolic glucose metabolism, allowing the β-cell to adapt the rate of insulin release in accordance to changes in the circulating glucose levels (2, 3). Downstream of GK, glucose metabolism leads to an elevation of the ATP:ADP ratio to a point where it promotes closure of KATP channels, resulting in the depolarization of the β-cell plasma membrane and opening of voltage-gated calcium channels, allowing calcium to enter the cytosol and promote insulin exocytosis (4–6). This so-called “KATP channel-dependent pathway” appears to be particularly important for the first acute phase of insulin release, whereas the second and more sustained phase of insulin secretion requires both KATP channel-dependent and -independent pathways (7–11).

Important support for the KATP channel-independent pathway of glucose-stimulated insulin release (GSIS) comes from studies showing that glucose can still elicit a significant increase in insulin secretion in conditions where KATP channels are held open by application of diazoxide and high K+ (12, 13) or in islets obtained from rodents that lack functional KATP channels (7, 8, 11, 14–18). These studies suggest that mitochondrial glucose metabolism generates other signals besides changes in the ATP:ADP ratio that are important for stimulus-secretion coupling in pancreatic β-cells (11, 19–23). Several molecules, including GTP, glutamate, malonyl-CoA/LC-CoA, α-ketoglutarate, and NADPH, have been proposed as candidate coupling factors in GSIS (11, 24–31).

ARNT/HIF-1β is a member of the basic helix-loop-helix/PER/ARNT/Sim family of transcription factors and is considered to be an obligate heterodimerization partner for other members of this family, such as HIF-1α, HIF-2α, HIF-3α, and AhR (32). In addition, ARNT/HIF-1β has been shown to homodimerize and regulate the transcription of genes that typically contain the palindromic E-box (CACGTG) signature in their promoter sequence (33, 34). Gene expression profiling of diabetic human islets revealed that ARNT/HIF-1β and its target genes are markedly reduced (35). The importance of ARNT/HIF-1β in GSIS was evidenced by the diminished glucose competence in islets obtained from β-cell-specific ARNT/HIF-1β knock-out mice as well as in Min6 cells where
the transcription factor was effectively silenced by siRNA technology (35).

In this study, our aim was to obtain a metabolic footprint of β-cells with low ARNT/HIF-1β levels and identify the metabolic pathways that are affected by the transcription factor. We demonstrate that impairment in GSIS become eminent when ARNT/HIF-1β is silenced in our INS-1-derived 832/13 cells. Our novel findings that ARNT/HIF-1β plays a role in regulating biphasic insulin secretion and anaplerosis as well as other key metabolic pathways suggest that the mechanism of ARNT/HIF-1β-regulated insulin release appears to be independent of ATP production and likely involves the altered K₅,ATP-independent pathway of insulin release.

EXPERIMENTAL PROCEDURES

Cell Lines and Insulin Secretion Assay—The cell line 832/13 derived from INS-1 rat insulinoma cells (36) was a kind gift from C. B. Newgard. Insulin secretion assay was performed as described previously (30, 36, 37).

siRNA Duplex Construction—Two siRNA duplexes were constructed against ARNT/HIF-1β (GenBank™ accession number NM_012780). Relative to the start codon, the 5′ end of the siRNA target sequence corresponded to the following nucleotide in ARNT/HIF-1β: siARNT1, nucleotide 389 (CCA UCU UAC GCA UGG CUG UUU CUC A), and siARNT2, nucleotide 891 (GGA AGG AGA GCC UCA CUU UGU GGT A). A previously described siRNA sequence (5′-GAGACCCCUAUCCGUGAUAU-3′) with no known gene homology was used as a control (siControl) (30, 37, 38). siRNA duplexes were introduced into 832/13 cells at ~50% confluence by nucleofection, using Lipofectamine RNAiMax according to the manufacturer’s instructions (Invitrogen). Experiments were performed 72 h after duplex transfection.

Real Time PCR Analysis of mRNA Expression—RNA isolation (Bio-Rad Aurum RNA mini kit), reverse transcription (iScript cDNA synthesis kit; Bio-Rad), and real time PCR analysis were performed on cell extracts of 832/13 cells or islets to determine the mRNA levels of target genes (supplemental Table 1 for target gene list and primer sequences). Gene expression levels were corrected by 18 S or by cyclophilin E (Cyp) expression. Fluorescent probes were purchased from Applied Biosystems, Inc. (ABI, Foster City, CA), and real time PCR primer sets were from Integrated DNA Technologies (IDT, San Diego) (see supplemental Table 1). Real time PCR reagents were from ABI or Bio-Rad (SSO Fast EvaGreen real time PCR supermix).

Western Blot—Cellular proteins were extracted with cell lysis buffer (Cell Signaling) containing phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (10 μg/ml), aprotinin (10 μg/ml), and pepstatin (5 μg/ml). Extracts (30 μg) were resolved on 10% Bis-Tris SDS-polyacrylamide gels and electrotransferred to nitrocellulose membranes (Invitrogen). ARNT/HIF-1β was detected with a monoclonal antibody against ARNT/HIF-1β (1:1500) (BD Biosciences) followed by horseradish peroxidase-conjugated anti-mouse antibody (1:20,000) (Sigma). Protein bands were detected with the Pierce ECL Western blotting substrate kit (Thermo Scientific).

ATP and ADP Measurements—Cellular ATP and ADP content was determined as described previously (30, 39).

Glucose Utilization—832/13 cells were preincubated with low glucose (2 mM) for 2 h. After the preincubation period, 832/13 cells were cultured in the presence of [5-3H]glucose (0.08 Ci/mol) as a tracer. Samples were processed for measurement of glucose utilization as described previously (30, 37).

Glucose Oxidation—832/13 cells were preincubated with low glucose (2 mM) for 2 h. After the preincubation, cells were cultured with radiolabeled [U-14C]glucose (0.5 Ci/mol of glucose) at low glucose (2 mM) or high glucose (16.7 mM) concentrations for 2 h. Following 2 h of incubation with radiolabeled glucose, samples were loaded into a trap system containing 1 N NaOH loaded into adjacent wells. The trap system was closed, and then the wells with media were injected with 70% perchloric acid. The trap was incubated on a shaker at 125 rpm for 90 min. NaOH was transferred to scintillation vials containing UniSorb BD scintillation fluid (BD Biosciences), mixed, and then counted (30, 37).

Metabolite Profiling by Gas Chromatography/Mass Spectrometry (GC/MS)—832/13 cells were treated with siARNT1 or siControl for 72 h. On the day of the experiment, cells were first incubated for 2 h at low glucose (2.8 mM) followed by incubating the cells with either low (2.8 mM) or high glucose (16.7 mM) for 2 h. Cells were subsequently washed in ice-cold PBS and lysed in 100 μl of ice-cold methanol containing internal standard myristic-d₂₃ acid and vortexed followed by centrifugation. Supernatant was dried under nitrogen. Samples were then derivatized by the addition of methoxamine to protect carbons and incubated for 30 min at 50 °C followed by the addition of N-methyl-N-trimethylsilyl trifluoroacetamide with 1% trimethylchlorosilane to protect the acidic groups and incubated for 30 min at 50 °C. One μl of the derivatized samples was injected in splitless mode onto an Agilent 7890A GC gas chromatograph equipped with a 30-m DB-5-ms column (Agilent). Each sample was run four times. Detection was performed with Agilent 5975C MSD. The chromatograms and mass spectra were processed by AMDIS software. Peak areas were normalized by the internal control myristic-d₂₃ acid.

Islet Isolation and Insulin Secretion—Islets were harvested from adult male Sprague-Dawley rats weighing ~250–300 g and cultured as described previously (30, 37).

Transfection of Primary Pancreatic β-Cells—300 islets were dispersed into single cells as described previously (39). Briefly, islets were placed in a sterile Eppendorf tube and washed three times with PBS. Islets were then incubated in 500 μl of 0.05% trypsin/EDTA containing 2 units/ml DNase I for ~5–10 min (until dispersed) followed by the addition of ice-cold growth medium (w/o antibiotics) to stop the reaction. The dispersed cells were then washed twice with ice-cold growth medium, and the cell pellet was resuspended in Opti-MEM. For β-cell transfection, cells were suspended in Opti-MEM containing Lipofectamine RNAiMAX (Invitrogen) and target siRNA or control siRNA (60 pmol). Dispersed β-cells were then reaggregated into pseudoislets by gently swirling the
cells every 30 min for 6 h. 72 h post-transfection, pseudoislets were used for perifusion.

Pseudoislets Perfusion—Pseudoislets were perfused in a similar fashion as seen for islets as we have previously described (37). 25 pseudoislets were layered between cotton in a Swinnex 13 chamber containing a nylon filter (Millipore, Burlington, MA). The chamber was pre-perifused with KRBH-BSA buffer containing 2.8 mM glucose at a flow rate of 1 ml/min using a Gilson Minipuls 3 pump (France) for 1 h. The temperature was maintained at 37 °C using an eight line inline solution heater (Warner Instruments, Hamden, CT). The solution was gassed with 95% O2, 5% CO2 to achieve a pH of 7.4 and maintained at 37 °C. Insulin assay was measured as described previously (30, 37, 39).

Statistics—Statistical significance was assessed by Student’s t test or analysis of variance post hoc Tukey’s test. All data were expressed as means ± S.E.

RESULTS

siRNA-mediated Knockdown of ARNT/HIF-1β in 832/13 Cells—832/13 cells were either transfected with two siRNA duplexes targeting different regions of the ARNT/HIF-1β gene transcript (siARNT1 and siARNT2 see under “Experimental Procedures” for details) or a control, nonspecific siRNA sequence (siControl) that has previously been characterized (30, 37, 38). 72 h after transfection, cells were harvested to measure ARNT/HIF-1β RNA and protein expression levels by real time PCR and Western blot, respectively. Treatment of 832/13 cells with siARNT1 resulted in a 78 ± 4% reduction of ARNT/HIF-1β mRNA as compared with the 56 ± 5% knockdown achieved by siARNT2 (Fig. 1A). ARNT/HIF-1β protein levels in 832/13 cells treated with siARNT1 were reduced by 67 ± 15% (Fig. 1, B and C).

ARNT/HIF-1β SuppressionImpairs GSIS in 832/13 Cells—832/13 cells were either untreated or transfected with siControl, siARNT1, or siARNT2 duplexes. After 72 h, an insulin secretion assay was performed first by preincubating cells for 2 h at low glucose (2 mM) followed by stimulating the cells with either low glucose (2 mM) or high glucose (16.7 mM). Insulin release at high glucose was significantly inhibited by 60 ± 10% for siARNT1 and by 52 ± 17% for siARNT2 as compared with the siControl (Fig. 1D).

ARNT/HIF-1β Suppression Lowers Glucose Utilization, without Affecting Glucose Oxidation in 832/13 Cells—Glucose utilization and oxidation were assessed to evaluate changes in glucose metabolism following ARNT/HIF-1β knockdown. Differences in glucose utilization were not statistically different at 2 mM glucose (Fig. 2A). However, in the presence of 16.7 mM glucose the glycolytic flux was significantly reduced by 31 ± 6% in siARNT1-treated cells as compared with siControl-treated cells. Although the glycolytic flux was negatively affected by ARNT/HIF-1β knockdown at 16.7 mM glucose, we could not detect significant changes in glucose oxidation at both 2 and 16.7 mM glucose (Fig. 2B).

ARNT/HIF-1β Suppression Does Not Significantly Alter the Glucose-induced Changes in the ATP:ADP Ratio in 832/13 Cells—Fig. 2A shows that there was a reduction in glycolytic flux by 31% in siARNT1-treated cells; however, glucose oxida-

![Figure 1](image-url)

**FIGURE 1.** Effects of siARNT1 and siARNT2 siRNA duplexes on gene expression, protein levels, and insulin secretion in 832/13 cells. A, both siARNT1 and siARNT2 decrease ARNT/HIF-1β mRNA levels in 832/13 cells. B and C, immunoblot analysis of extracts from 832/13 cells treated with siControl (siCon) and siARNT1. D, siARNT1 and siARNT2 inhibit glucose-stimulated insulin secretion in 832/13 cells. Results represent mean ± S.E. of 3–6 independent experiments. LG, low glucose (2 mM); HG, high glucose (16.7 mM). A and B, ***, p < 0.001; **, p < 0.01; *, p < 0.05; siControl versus siARNT, D, **, p < 0.01; *, p < 0.05; high glucose siControl versus high glucose siARNT.
GC/MS system. This comparative analysis revealed that most of the glycolytic (Fig. 4A) and TCA cycle intermediates (Fig. 4B) are markedly reduced by ARNT/HIF-1 \( \text{H9252} \) knockdown. For glycolysis, the glucose-stimulated increase in glucose 6-phosphate levels was not affected by ARNT/HIF-1 \( \text{H9252} \) knockdown, suggesting that glucokinase activity is preserved in si\text{ARNT1}-treated cells. However, other glycolytic intermediates, such as dihydroxyacetone phosphate, 3-phosphoglycerate, and pyruvate were all significantly reduced in si\text{ARNT1}-treated cells that were exposed to 16.7 mM glucose. These findings are in agreement with the observation that glycolytic flux is markedly reduced by ARNT/HIF-1 \( \text{H9252} \) knockdown at 16.7 mM glucose. The amount of lactate was also significantly lower in si\text{ARNT1}-treated cells both at low and high glucose concentrations. GC/MS-based Metabolic Analysis of the TCA Cycle in 832/13 Cells with Reduced ARNT/HIF-1 \( \text{H9252} \) Levels—The glucose-stimulated increase in TCA intermediates were all significantly lower in si\text{ARNT1}-treated cells as compared with si\text{Control}-treated cells (Fig. 4B), including citrate/isocitrate, \( \alpha \)-ketoglutarate, succinate, fumarate, and malate. The identities of citrate and isocitrate are indistinguishable, and therefore these metabolites have been grouped. In addition, this marked difference in the amount of TCA metabolites was also significantly reduced at low glucose concentrations. At high glucose levels, the reduction in TCA metabolite levels following ARNT/HIF-1 \( \text{H9252} \) knockdown was most notable for \( \alpha \)-ketoglutarate (90 ± 5%), followed by succinate (68 ± 2%), fumarate (67 ± 1%), and malate (65 ± 2%) and was less marked in case of citrate/isocitrate (49 ± 8%). Considering that glucose oxidation was not significantly affected under these conditions, the amounts of TCA metabolites seen in si\text{ARNT1}-treated cells are likely sufficient for maintaining glucose oxidation. The reduced amounts of malate, citrate, and isocitrate are of direct interest in the regulation of GSIS because these metabolites act as intermediates in the pyruvate cycling pathways, which have been suggested to play a key role in GSIS (11, 21, 30, 38, 40) and serve as a source of glucose carbon for lipogenesis (26, 41).

GC/MS-based Metabolic Analysis of the Pentose Phosphate Pathway (PPP) in 832/13 Cells with Reduced ARNT/HIF-1 \( \text{H9252} \) Levels—Although glucose utilization is negatively affected by ARNT/HIF-1 \( \text{H9252} \) knockdown at high glucose concentrations, the amount of glycolytic substrate entering the PPP under these conditions seems to be preserved as 6-phosphogluconate levels were similar in si\text{ARNT1}-treated cells as compared with si\text{Control}-treated cells (supplemental Fig. 1). However, the final step in the oxidative phase of the PPP under these conditions seems to be preserved as 6-phosphogluconate levels were similar in si\text{ARNT1}-treated cells as compared with si\text{Control}-treated cells (supplemental Fig. 1). However, the final step in the oxidative phase of the PPP seemed to be negatively affected by ARNT/HIF-1 \( \text{H9252} \) silencing as the glucose-induced rise in ribulose 5-phosphate levels was diminished in si\text{ARNT1}-treated cells, although not significantly. ARNT/HIF-1 \( \text{H9252} \) knockdown had its greatest impact on the non-oxidative arm of the PPP at the step catalyzed by ribulose-5-phosphate isomerase as ribose 5-phosphate levels were significantly reduced in si\text{ARNT1}-

![Graph A: Glucose Utilization and Oxidation](image1)

**FIGURE 2.** Effects of si\text{ARNT1} on glucose utilization and glucose oxidation in 832/13 cells. A, glucose utilization. B, glucose oxidation. LG, low glucose (2 mM); HG, high glucose (16.7 mM). *, p < 0.05 HG si\text{Control} versus HG si\text{ARNT1}. Data represent mean ± S.E. of 3–4 independent experiments.

![Graph B: ADP and ATP Ratio](image2)

**FIGURE 3.** Effects of siRNA-mediated suppression of ARNT/HIF-1 \( \beta \) on ADP and ATP (A) and ATP:ADP (B) ratio in 832/13 cells. LG, low glucose (2 mM); HG, high glucose (16.7 mM). Data represent mean ± S.E. of 6 independent experiments.
treated cells exposed to elevated glucose concentrations. Also, the product of transaldolase, erythrose 4-phosphate, was also significantly lower in siARNT1-treated cells. Overall, the glycolytic flow through the later phases of the PPP seems to be negatively affected by the low levels of ARNT/HIF-1β/H9252 in 832/13 cells.

**GC/MS-based Metabolic Analysis of Free Fatty Acid Levels in 832/13 Cells with Reduced ARNT/HIF-1β Levels**—As depicted in Fig. 5A and supplemental Fig. 2, the rise in fatty acid species that normally occurs when glucose concentrations are raised from basal to stimulatory concentrations is completely absent in siARNT1-treated cells as compared with siControl-treated cells. The levels of fatty acid species, including myristic acid (supplemental Fig. 2A), palmitic acid, palmitoleic acid, oleic acid, stearic acid (Fig. 5A), arachidic acid, and caprylic acid (also known as octanoic acid) (supplemental Fig. 2B) did not increase when siARNT1-treated cells were exposed to high glucose concentrations. These findings indicate that the mitochondrial export of citrate is diminished under these conditions. This is particularly interesting as the glucose-induced increments in LC-CoA species have been proposed to act as a coupling factor in GSIS (26, 41).

**GC/MS-based Metabolic Analysis of Amino Acid Levels in 832/13 Cells with Reduced ARNT/HIF-1β Levels**—Finally, the involvement of ARNT/HIF-1β/H9252 also seems to extend into amino acid metabolism but to a lesser extent (Fig. 5B and supplemental Fig. 3). Only a few amino acids were affected in siARNT1-treated cells as the levels of β-alanine, l-glutamate, l-threonine, and l-alanine were significantly lowered at high glucose concentrations (Fig. 5B and supplemental Fig. 3). The lower levels of l-alanine in siARNT1-treated cells are interesting considering that pyruvate serves as a precursor for its pro-

*FIGURE 4. Effects of siRNA-mediated suppression of ARNT/HIF-1β on glycolytic metabolite levels (A) and TCA cycle intermediates (B) in 832/13 cells. Data are presented as a ratio of target metabolite corrected for an internal control (myristic acid-d27) and represents mean ± S.E. of 3 independent experiments. LG, low glucose (2 mM); HG, high glucose (16.7 mM). *, p < 0.05; HG siControl versus HG siARNT1; **, p < 0.01; ***, p < 0.01, HG siControl versus HG siARNT1; #, p < 0.05; ##, p < 0.01, LG siControl versus LG siARN1.*
duction by alanine transaminase. The diminished glucose-stimulated production of L-glutamate, on the other hand, is interesting as it represents another potential candidate coupling factor that is negatively affected by ARNT/HIF-1β knockdown (23, 28, 43). Other potentially interesting metabolites include sorbitol, which is lower but not significantly, and creatinine, which is significantly lower at high glucose (supplemental Fig. 4).

**Gene Expression Profiling**—We next assessed several target genes related to diabetes, glucose metabolism, and anaplerosis (Fig. 6). The related ARNT/HIF1β gene, hypoxia-inducible factor 1α (HIF1α), was not significantly affected by ARNT/HIF1β knockdown. Diabetes-associated genes hepatocyte nuclear factor 1α (HNF1α), HNF4α, and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) were all significantly reduced in response to

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**FIGURE 5.** Effects of siRNA-mediated suppression of ARNT/HIF-1β on selected free fatty acid (A) and amino acid (B) levels in 832/13 cells. The data are presented as a ratio of target metabolites corrected for an internal control (myristic acid-d14) and represents mean ± S.E. of 3 independent experiments. LG, low glucose (2 mM); HG, high glucose (16.7 mM). *, p < 0.05; **, p < 0.01, HG siControl versus H1 siARNT1; †††, p < 0.001, HG siControl versus HG siARNT1.
siRNA-mediated reduction in ARNT/HIF1β gene as compared with control-treated cells. In addition, three important genes involved in glucose entry and glucose metabolism, glucose transporter 1 (GLUT2), glucokinase (GK), and ATP-citrate lyase (CL), are also significantly reduced.

Pyruvate dehydrogenase α1 (PDHa1) and pyruvate carboxylase (PC) are significantly reduced in response to ARNT/HIF1β knockdown and are important genes involved in mitochondrial glucose metabolism (Fig. 6). PDHa1 is important for the generation of NADH and ATP, and PC is important for anaerobic entry of glucose. Export of mitochondrial metabolites to the cytosol occurs via dicarboxylate carrier (DIC), α-ketoglutarate carrier (OGC), and citrate carrier (CIC). Interestingly, both DIC and OGC are both significantly reduced after treating cells with siARNT1. Reductions in PC, DIC, and OGC are in agreement with a reduction of anaerobic metabolism seen with the metabolomics results. An important proposed pathway involved in insulin secretion is pyruvate cycling, and two key enzymes in the proposed pyruvate cycling pathways are cytosolic NADP⁺-dependent malic enzyme (MEc) and cytosolic NADP⁺-dependent cytosolic isocitrate dehydrogenase (ICDc) (11). Only MEc and not ICDc were significantly reduced in siARNT1-treated cells. As certain free fatty acids were lower in siARNT1-treated cells, we also assessed key fatty acid metabolism enzymes fatty-acid synthase (FAS) and carnitine palmitoyltransferase 1α (CPT1α). Both FAS and CPT1α were significantly lower in siARNT1-treated cells.

Islet Perifusion—Previous studies have suggested that β-cell-specific ARNT/HIF1β knock-out mice have defective insulin secretion; however, biphase insulin secretion has not yet been assessed. Transfection of dispersed islets with siRNAs against ARNT/HIF-1β (siARNT1) followed by perifusion 72 h after transfection resulted in a significant reduction in both first and second phase insulin secretion (Fig. 7). These results support the concept that ARNT/HIF-1β is involved in both phases of insulin secretion.
DISCUSSION

Expression profiling of type 2 diabetic human islets showed that ARNT/HIF-1β was reduced by 90% (35). It was also demonstrated that ARNT/HIF-1β may control the expression levels of other candidate genes for type 2 diabetes, such as HNF4α, the insulin receptor, IRS-2, and Akt2, all of which play an important role in glucose homeostasis (44–47). In this study, we assessed the metabolic profile of pancreatic β-cells with low ARNT/HIF-1β expression levels to provide more insight into the metabolic pathways that are affected by ARNT/HIF-1β knockdown and to identify the underlying mechanisms that impair GSIS under these conditions.

Based on the available literature, we hypothesized that ARNT/HIF-1β would be required for maintaining glycolytic flux and normal GSIS. Although we confirmed that GSIS was dramatically reduced, we found that glucose utilization was only decreased by 31%, and glucose oxidation and glucose-stimulated ATP production were not significantly altered. To gain more insight into the metabolic consequence of the loss of ARNT/HIF-1β, we employed a metabolomics approach to assess a wide range of cellular metabolites. As expected, glycolytic intermediates were significantly reduced in siARNT1-treated cells; however, the most dramatic reductions were seen for TCA intermediates suggesting that anaplerosis is an important target of ARNT/HIF-1β. These studies also demonstrate that the role of ARNT/HIF-1β in regulating insulin release in response to glucose is independent of altered glucose oxidation and ATP production and likely involves altered anaplerosis.

Anaplerosis has been suggested to play an important role in insulin secretion. In β-cells, when glucose is abundant, the mitochondrial entry of pyruvate into the TCA cycle occurs via PDH and PC in almost equal proportions, giving rise to the existence of two separate pyruvate pools, with one feeding acetyl-CoA into the TCA cycle for oxidation (PDH pathway) and the other leading to a net accumulation of TCA intermediates (PC pathway) (21, 48–51). In the PDH pathway, the increase in mitochondrial glucose metabolism and acetyl-CoA will promote the transfer of reducing equivalents into the respiratory chain, resulting in mitochondrial membrane hyperpolarization and increased ATP production. The net accumulation of TCA intermediates in the PC pathway can play an important role in other proposed pathways involved in regulating insulin release, including pyruvate cycling, GTP, glutamate, α-ketoglutarate, and LC-CoA hypothesis (11, 24–31). Our results support the idea that ARNT/HIF-1β plays a role in regulating anaplerosis as both anaplerotic related genes (PC, DIC, and OGC) and TCA intermediates are both reduced in response to lower ARNT/HIF-1β expression levels. We also found that anaplerosis was lower at low glucose, and this was associated with a nonsignificant reduction in basal insulin secretion in siARNT1- and siARNT2-treated cells. This suggests that anaplerosis may play a role in basal insulin secretion as well as high glucose-stimulated insulin secretion.

Pyruvate cycling has been proposed to be a novel pathway involved in regulating insulin secretion by numerous groups (11, 30, 37, 38, 40, 52–56). Increased anaplerotic input is critical for proper functioning of all pyruvate cycling pathways. Pyruvate cycling has been suggested to generate two potentially important signaling molecules for insulin release, α-ketoglutarate and NADPH. NADPH can be produced via one of three pyruvate cycling pathways, the pyruvate/malate pathway, the pyruvate/citrate pathway, or the pyruvate/isocitrate pathway. A key NADPH-producing enzyme for both the pyruvate malate and pyruvate citrate pathways is the MEc (11, 52, 53). The key enzyme for the pyruvate isocitrate pathway is the cytosolic isocitrate dehydrogenase (38). Although strong evidence for a role of the pyruvate-isocitrate pathways exists (11, 30, 37, 38, 40, 52, 53), others have shown a role for the other two pyruvate cycling pathways (54–56). Which pyruvate cycling pathway(s) that is critical for insulin release is unknown. Our results support a role for ARNT/HIF-1β in pyruvate cycling as important genes in the pathway are downregulated (PC and MEc).

Guntert et al. (35) showed that the suppression or complete lack of ARNT/HIF-1β leads to abnormalities in insulin release in ARNT/HIF-1β-silenced Min6 cells and in primary islets obtained from β-cell-specific ARNT knock-out mice. Here, we confirm their findings as siRNA-mediated knockdown of ARNT/HIF-1β in INS-1-derived 832/13 cells also reduced GSIS by 60% when compared with control cells. We also found that both first and second phase insulin secretion was down-regulated in perifused pseudoislets. To identify the underlying mechanisms that cause this loss in glucose responsiveness, we initially measured glucose utilization and glucose oxidation. At 2 mM glucose, ARNT/HIF-1β knockdown did not significantly affect glucose utilization or glucose oxidation, which is in agreement with the observation that pyruvate levels did not deviate from control under these conditions. However, when exposed to 16.7 mM glucose, the glycolytic flux was clearly suppressed by ~31% in siARNT1-treated cells, and this reduction in glycolysis was not accompanied by a drop in the glucose oxidation. These findings are in agreement with an earlier report showing that the expression levels of several glycolytic enzymes are lowered upon ARNT/HIF-1β knockdown (35). Our data showing a lack of an effect on the oxidative entry of pyruvate into the TCA cycle is surprising considering that glycolytic flux was markedly reduced in ARNT/HIF-1β silenced β-cells.

Others have established that either suppression or complete loss of ARNT/HIF-1β in β-cells does not significantly affect the expression levels of the glycolytic pacemaker, glucokinase, or the glucose transporters GLUT1 and GLUT2 (35); however, our results suggest that there may be an effect on GLUT2 and GK. Although in our experiments GLUT2 and GK expressions are lower, the metabolomics results support the idea that overall glucose entry and glucokinase activity are not affected by ARNT/HIF-1β knockdown as glucose 6-phosphate levels were similar in siARNT1-treated cells exposed to the high glucose concentration. Therefore, it seems more likely that steps downstream of glucokinase have attained rate-limiting properties for glycolysis in β-cells where ARNT/HIF-1β function is impaired. One possible explanation is that low expression levels of other glycolytic enzymes, such as glu-
cose-6-phosphate isomerase, phosphofructokinase, and aldolase, become limiting for the substrate flow-through glycolysis (35). In agreement with this notion, we observed that the dihydroxyacetone phosphate and pyruvate levels were significantly lower in siARNT1-treated cells than in siControl-treated cells exposed to 16.7 mM glucose. The 31% reduction in glycolytic flux in siARNT1-treated cells was not reflected in a lack of an increase in glucose oxidation and the ATP:ADP ratio in response to glucose stimulation.

The novel finding that reducing ARNT/HIF-1β levels leads to a profound reduction in PC, DIC, and OGC expression levels and a reduction in TCA metabolites, even though glucose oxidation and ATP production were unaltered, is an unexpected result. These collective changes in metabolite levels demonstrate that the oxidative entry of pyruvate is preserved when siARNT1-treated cells are placed under a stimulatory glucose regime and such is likely to occur at the expense of the anaplerotic input into the TCA cycle.

Several arguments can be brought forward to support the idea that both anaplerotic input and pyruvate cycling activity are negatively affected by ARNT/HIF-1β knockdown. First, the reduction in glycolytic flux by ~31% is disproportionate to the 65% lower pyruvate levels detected in ARNT/HIF-1β-silenced 832/13 cells suggesting that anaplerotic and pyruvate cycling pathways are sacrificed at the expenses of maintaining mitochondrial oxidation.

Second, the glycolytic end product pyruvate and the TCA intermediates malate, citrate, and isocitrate, which collectively play a flux-determining role in the pyruvate cycling activity via the pyruvate-malate, pyruvate-citrate, and pyruvate-isocitrate shuttle, were significantly reduced by 50–70%. We also found that a key pyruvate cycling gene MEC was significantly reduced. As such, these pyruvate cycling metabolites control the amount of cytosolic NADPH being produced at stimulatory glucose concentrations, and this becomes particularly interesting from the perspective that NADPH has been proposed to act as a metabolic coupling factor (11, 29, 30, 38).

A third argument to support the idea that ARNT/HIF-1β knockdown negatively affects the glucose-induced anaplerotic input into the TCA cycle follows from the observation that there was a reduction in the expression of both FAS and CPT1α, and the glucose-induced rise in fatty acid production is virtually absent in siARNT1-treated cells. These observations suggest that the mitochondrial export of citrate via the citrate/isocitrate carrier (CIC), which provides an essential source of glucose carbon for lipogenesis (30), is severely diminished in ARNT/HIF-1β silenced β-cells. The abrogated glucose-stimulated fatty acid production in siARNT1-treated cells is relevant as the glucose-stimulated production of malonyl-CoA and LC-CoA has been suggested to serve as a metabolic coupling factor in GSIS (25, 26). The importance of mitochondrial export of substrate via CIC has been established as pharmacologic inhibition of the mitochondrial carrier protein in 832/13 cells, and isolated rat islets cause profound impairments in GSIS (30, 56). Our metabolic data also show that β-cells with low ARNT/HIF-1β levels have lost the ability to generate L-glutamate when glucose levels are raised from basal to stimulatory levels. This may represent another facet in the stimulus-secretion coupling that is affected by a profound reduction in ARNT/HIF-1β as L-glutamate has been proposed to act as a metabolic signal in the KATP channel-independent pathway that controls GSIS (42).

Our data support the concept that reducing ARNT/HIF-1β expression inhibits insulin secretion by lowering glucose-stimulated anaplerosis without affecting glucose oxidation and ATP production. ARNT/HIF-1β may lower insulin secretion by decreasing a novel anaplerotic dependent pathway involved in insulin secretion called pyruvate cycling. However, the link between ARNT/HIF-1β and pyruvate cycling has not yet been established.
In summary, we performed an extensive metabolic survey of β-cells with reduced ARNT/HIF-1β levels to identify the metabolic pathways that are affected by the transcription factor. We provide proof that ARNT/HIF-1β plays an important role in β-cell glucose metabolism as siRNA-mediated knockdown of the transcription factor causes a significant reduction in glycolytic flux, biphasic insulin secretion, and TCA substrate levels. Considering that glucose oxidation or glucose-induced ATP production was not significantly affected by ARNT/HIF-1β knockdown, the observed shift in the metabolic profile seems to be consistent with a marked reduction in anaplerosis. The idea that low levels of ARNT/HIF-1β negatively affects the anaplerotic input into the TCA cycle is furthermore supported by the observation that glucose-induced fatty acid and glutamate production are completely absent in siARNT1-treated 832/13 cells. Taken together, we provide evidence that ARNT/HIF-1β is absolutely required to keep pancreatic β-cells in a glucose-responsive state by ensuring sufficient substrate flow through the K_{ATP}−independent pathways that regulate GSIS (Fig. 8).

REFERENCES

1. Matschinsky, F. M. (1996) Diabetes 45, 223–241
2. Matschinsky, F. M. (2009) Nat. Rev. Drug Discov. 8, 399–416
3. German, M. S. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1781–1785
4. Prentki, M., and Matschinsky, F. M. (1987) Physiol. Rev. 67, 1185–1248
5. Ashcroft, F. M., and Rorsman, P. (1989) Prog. Biophys. Mol. Biol. 54, 87–143
6. MacDonald, P. E., Joseph, J. W., and Rorsman, P. (2005) Philos. Trans. R. Soc. Lond. B Biol. Sci. 360, 2211–2225
7. Henquin, J. C. (2009) IUBMB Life 61, 482–488
8. Buse, J. D., and Sherwin, R. S. (1999) Diabetes 48, 1725–1733
9. Buse, J. D., and Sherwin, R. S. (2000) Diabetes 49, 1139–1147
10. Gunawardana, S. C., Liu, Y. J., MacDonald, M. J., Stoker, S. W., Boonsaen, T., Jitrapakdee, S., Kendrick, M. A., Wallace, J. C., and MacDonald, M. J. (2008) J. Biol. Chem. 283, 28048–28059
11. Stoffers, D. A., and Stoffel, M. (2006) Annu. Rev. Physiol. 68, 459–486
12. Gembal, M., Gilon, P., and Henquin, J. C. (1992) J. Clin. Invest. 89, 1288–1295
13. Sato, Y., Aizawa, T., Komatsu, M., Okada, N., and Yamada, T. (1992) Diabetes 41, 438–443
14. Nenquin, M., Szollosi, A., Aguilar-Bryan, L., Bryan, J., and Henquin, J. C. (2004) J. Biol. Chem. 279, 32316–32324
15. Szollosi, A., Nenquin, M., and Henquin, J. C. (2010) Br. J. Pharmacol. 159, 669–677
16. Rafter, M. A., Nenquin, M., Miku, T., Seino, S., and Henquin, J. C. (2009) Endocrinology 150, 33–45
17. Quoix, N., Cheng-Xue, S., Martatt, L., Zeinoun, Z., Guiot, Y., Beaufils, M. C., Henquin, J. C., and Gilon, P. (2009) Diabetes 58, 412–421
18. Henquin, J. C. (2009) Diabetologia 52, 739–751
19. Hasan, N. M., Longacre, M. J., Stoker, S. W., Boonsaen, T., Jitrapadke, S., Kendrick, M. A., Wallace, J. C., and MacDonald, M. J. (2008) J. Biol. Chem. 283, 28048–28059
20. MacDonald, M. J., Stoker, S. W., and Hasan, N. M. (2008) Mol. Cell. Biochem. 313, 195–202
21. Lu, D., Mulder, H., Zhao, P., Burgess, S. C., Jensen, M. V., Kamzolova, S., Newgard, C. B., and Sherry, A. D. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 2708–2713
22. MacDonald, M. J., Fahien, L. A., Brown, L. J., Hasan, N. M., Buss, J. D., and Hendrick, M. A. (2005) Am. J. Physiol. Endocrinol. Metab. 288, E1–E15
23. Maechler, P., Antinoozi, P. A., and Wollheim, C. B. (2000) IUBMB Life 50, 27–31
24. Meredith, M., Rabaglia, M. E., and Metz, S. A. (1995) J. Clin. Invest. 96, 811–821
25. Corkey, B. E., Glennon, M. C., Chen, K. S., Deeney, J. T., Matschinsky, F. M., and Prentki, M. (1989) J. Biol. Chem. 264, 21608–21612
26. Prentki, M., Vischer, S., Glennon, M. C., Regazzi, R., Deeney, J. T., and Corkey, B. E. (1992) J. Biol. Chem. 267, 5802–5810
27. Newgard, C. B., Lu, D., Jensen, M. V., Schissler, J., Boucher, A., Burgess, S., and Sherry, A. D. (2002) Diabetes 51, S389–S393
28. Broca, C., Brennan, L., Petiti, P., Newsholme, P., and Maechler, P. (2003) FEBS Lett. 545, 167–172
29. Ivarsson, R., Quintens, R., Dejonghe, S., Tsukamoto, K., in, ’t Veld, P., Renstrom, E., and Schuit, F. C. (2005) Diabetes 54, 2132–2142
30. Joseph, J. W., Jensen, M. V., Ilkayeva, O., Palmieri, F., Alarcon, C., Rhodes, C. J., and Newgard, C. B. (2006) J. Biol. Chem. 281, 35624–35632
31. Kibbe, R. G., Pongratz, R. L., Romanelli, A. J., Wollheim, C. B., Cline, G. W., and Shulman, G. I. (2007) Cell Metab. 5, 253–264
32. Furness, S. G., Lees, M. J., and Whitelaw, M. L. (2007) FEBS Lett. 581, 3616–3625
33. Antonsson, C., Arulampalam, V., Whitelaw, M. L., Pettersson, S., and Poellinger, L. (1995) J. Biol. Chem. 270, 13968–13972
34. Arpiainen, S., Lämä, S., Pelkonen, O., Yim, S. H., Gonzalez, F. J., and Hakkkola, J. (2007) J. Mol. Biol. 369, 640–652
35. Gunton, J. E., Kulikarni, R. N., Yim, S., Okada, T., Hawthorne, W. J., Tseng, Y. H., Roberson, R. S., Ricordi, C. O., Connell, P. J., Gonzalez, F. J., and Kahne, C. R. (2005) Cell 122, 337–349
36. Hohmeier, H. E., Mulder, H., Chen, G., Henkel-Rieger, R., Prentki, M., and Newgard, C. B. (2000) Diabetes 49, 424–430
37. Joseph, J. W., Odegaard, M. L., Ronnebaum, S. M., Burgess, S. C., Muehlabauer, J., Sherry, A. D., and Newgard, C. B. (2007) J. Biol. Chem. 282, 31592–31600
38. Ronnebaum, S. M., Ilkayeva, O., Burgess, S. C., Joseph, J. W., Lu, D., Stevens, R. D., Becker, T. C., Sherry, A. D., Newgard, C. B., and Jensen, M. V. (2006) J. Biol. Chem. 281, 30593–30602
39. Joseph, J. W., Koshkin, V., Saleh, M. C., Sivitz, W. I., Zhang, C. Y., Lowell, B. B., Chan, C. B., and Wheeler, M. B. (2004) J. Biol. Chem. 279, 51049–51056
40. Joseph, J. W., Ilkayeva, O., Burgess, S. C., Sato, Y., Aizawa, T., Komatsu, M., Okada, N., and Yamada, T. (1992) Diabetes 41, 438–443
52. Ronnebaum, S. M., Jensen, M. V., Hohmeier, H. E., Burgess, S. C., Zhou, Y. P., Qian, S., MacNeil, D., Howard, A., Thornberry, N., Ilkayeva, O., Lu, D., Sherry, A. D., and Newgard, C. B. (2008) J. Biol. Chem. 283, 28909–28917
53. Brown, L. J., Longacre, M. J., Hasan, N. M., Kendrick, M. A., Stoker, S. W., and Macdonald, M. J. (2009) J. Biol. Chem. 284, 35359–35367
54. Xu, J., Han, J., Long, Y. S., Lock, J., Weir, G. C., Epstein, P. N., and Liu, Y. Q. (2008) Diabetologia 51, 2281–2289
55. Pongratz, R. L., Kibbey, R. G., Shulman, G. I., and Cline, G. W. (2007) J. Biol. Chem. 282, 200–207
56. Guay, C., Madiraju, S. R., Aumais, A., Joly, E., and Prentki, M. (2007) J. Biol. Chem. 282, 35657–35665