Aryl Hydrocarbon Receptor Nuclear Translocator/Hypoxia-inducible Factor-1β Plays a Critical Role in Maintaining Glucose-stimulated Anaplerosis and Insulin Release from Pancreatic β-Cells*[S]

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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)[3] 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 K_{ATP} 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 "K_{ATP} 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 K_{ATP} channel-dependent and -independent pathways (7–11).

Important support for the K_{ATP} 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 K_{ATP} channels are held open by application of diazoxide and high K^+ (12, 13) or in islets obtained from rodents that lack functional K_{ATP} 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/AhR/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

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–4.

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[3] The abbreviations used are: GK, glucokinase; ARNT/HIF-1β, aryl hydrocarbon receptor nuclear translocator/hypoxia-inducible factor-1β; GSIS, glucose-stimulated insulin secretion; LC-CoA, long-chain acyl CoA; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; DIC, dicarboxylate

carrier; CIC, citrate carrier; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; PPP, pentose phosphate pathway; MEc, cytosolic malic enzyme; OGC, α-ketoglutarate carrier.

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 K_{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[TM] 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*: si*ARNT1*, nucleotide 389 (CCA UCU UAC GCA UGG CUG UUU CUC A), and si*ARNT2*, nucleotide 891 (GGA AGG AGA GCC UCA CUU UGU GGT A). A previously described siRNA sequence (5′-GAGACCCUAU-CCGUGAUUA-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 nucleo-fection, 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 (see 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% BisTris 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). γ-Tubulin was detected by immunoblotting with a mouse antibody against γ-tubulin (1:4000) (Sigma) 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-^{3}H]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-^{14}C]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 UniScint 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-d_{27} acid and vortexed followed by centrifugation. Supernatant was dried under nitrogen. Samples were then derivatized by the addition of methoxamine to protect carbonyls and incubated for 30 min at 50 °C followed by the addition of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide 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-d_{27} 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 1–2 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 Perifusion—Pseudoislets were perifused 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 in-line solution heater (Warner Instruments, Hamden, CT). The solution was gassed with 95% O_2, 5% CO_2 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 (si*ARNT1* and si*ARNT2* 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 si*ARNT1* resulted in a 78 ± 4% reduction of *ARNT/HIF-1β* mRNA as compared with the 56 ± 5% knockdown achieved by si*ARNT2* (Fig. 1*A*). 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 Suppression Impairs GSIS in 832/13 Cells—832/13 cells were either untreated or transfected with siControl, si*ARNT1*, or si*ARNT2* 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 si*ARNT1* and by 52 ± 17% for si*ARNT2* as compared with the siControl (Fig. 1*D*).

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. 2*A*). However, in the presence of 16.7 mM glucose the glycolytic flux was significantly reduced by 31 ± 6% in si*ARNT1*-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. 2*B*).

ARNT/HIF-1β Suppression Does Not Significantly Alter the Glucose-induced Changes in the ATP:ADP Ratio in 832/13 Cells—Fig. 2*A* shows that there was a reduction in glycolytic flux by 31% in si*ARNT1*-treated cells; however, glucose oxida-
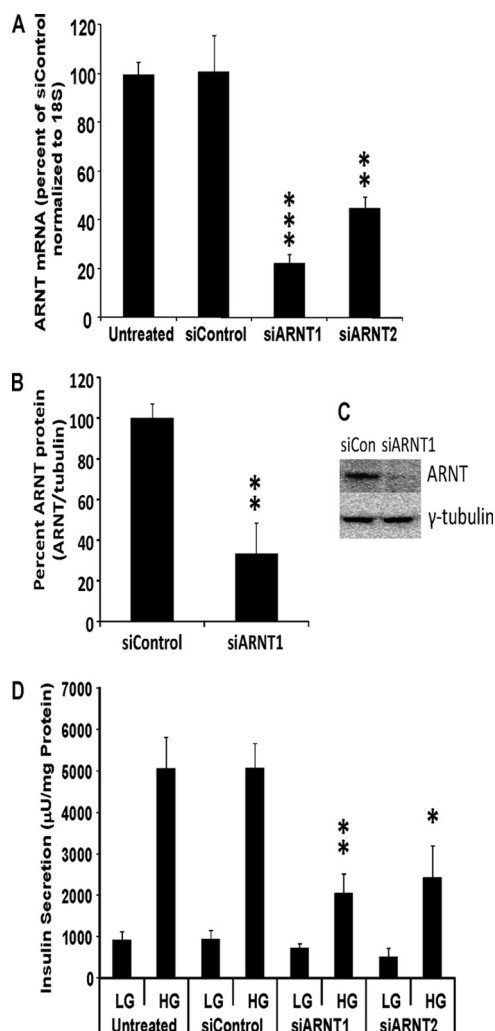
FIGURE 1. **Effects of si*ARNT1* and si*ARNT2* siRNA duplexes on gene expression, protein levels, and insulin secretion in 832/13 cells.** *A*, both si*ARNT1* and si*ARNT2* decrease *ARNT/HIF-1β* mRNA levels in 832/13 cells. *B* and *C*, immunoblot analysis of extracts from 832/13 cells treated with siControl (*siCon*) and si*ARNT1*. *D*, si*ARNT1* and si*ARNT2* 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*, ***, $p < 0.001$; **, $p < 0.01$l; siControl *versus* si*ARNT*. *D*, **, $p < 0.01$; *, $p < 0.05$; high glucose siControl *versus* high glucose siARNT.

tion was not affected suggesting that glucose-stimulated ATP levels may not be altered as well in si*ARNT1*-treated cells. We next investigated whether or not glucose-stimulated ATP production was altered in *ARNT/HIF-1β* knockdown cells. Although we did observe a slight reduction in both ATP levels and the ATP:ADP ratio following *ARNT/HIF-1β* knockdown, this was not statistically different from that observed in siControl-treated 832/13 cells (Fig. 3). This is consistent with the observation that glucose oxidation remained unaltered under these conditions.

GC/MS-based Metabolic Analysis of Glycolysis in 832/13 Cells with Reduced ARNT/HIF-1β Levels—To further elicit the key metabolic effects of *ARNT/HIF-1β* knockdown, we employed a metabolomics approach. Assessment of the metabolic profile of the glycolytic, pentose phosphate, TCA cycle, free fatty acid, and amino acid pathways in both siControl and si*ARNT1*-treated cells was performed using an Agilent
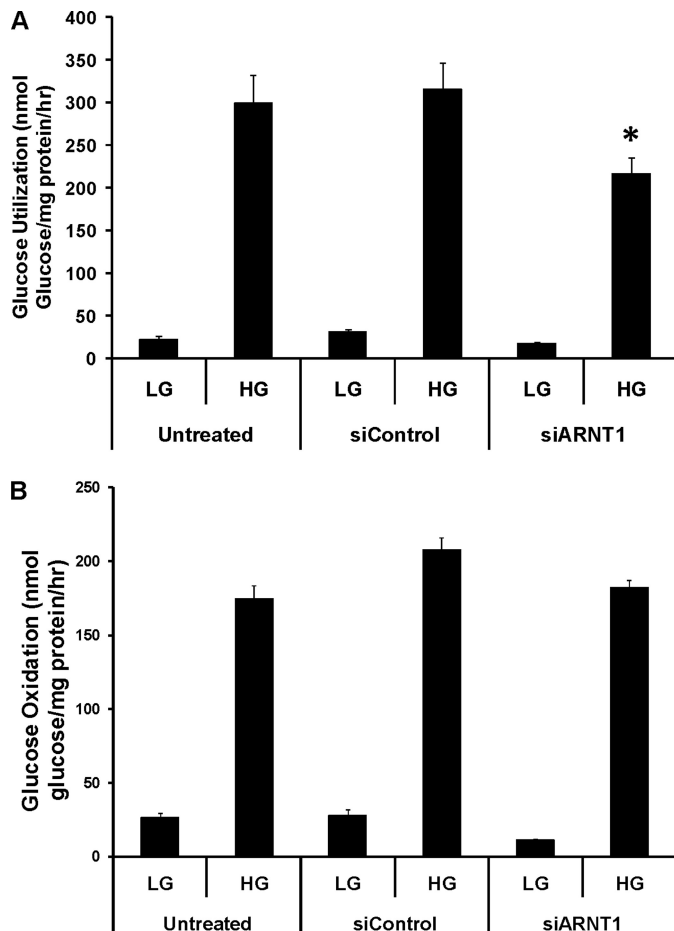
FIGURE 2. **Effects of si*ARNT1* on glucose utilization and glucose oxidation in 832/13 cells.** *A,* glucose utilization. *B,* glucose oxidation. *LG,* low glucose (2 mM); *HG,* high glucose (16.7 mM). *, $p < 0.05$ HG siControl *versus* HG si*ARNT1*. Data represent mean ± S.E. of 3–4 independent experiments.

FIGURE 3. **Effects of siRNA-mediated suppression of *ARNT/HIF-1β* 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.

GC/MS system. This comparative analysis revealed that most of the glycolytic (Fig. 4*A*) and TCA cycle intermediates (Fig. 4*B*) are markedly reduced by *ARNT/HIF-1β* knockdown. For glycolysis, the glucose-stimulated increase in glucose 6-phosphate levels was not affected by *ARNT/HIF-1β* knockdown, suggesting that glucokinase activity is preserved in si*ARNT1*-treated cells. However, other glycolytic intermediates, such as dihydroxyacetone phosphate, 3-phosphoglycerate, and pyruvate were all significantly reduced in si*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-1β* knockdown at 16.7 mM glucose. The amount of lactate was also significantly lower in si*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-1β Levels—The glucose-stimulated increase in TCA intermediates were all significantly lower in si*ARNT1*-treated cells as compared with siControl-treated cells (Fig. 4*B*), including citrate/isocitrate, α-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
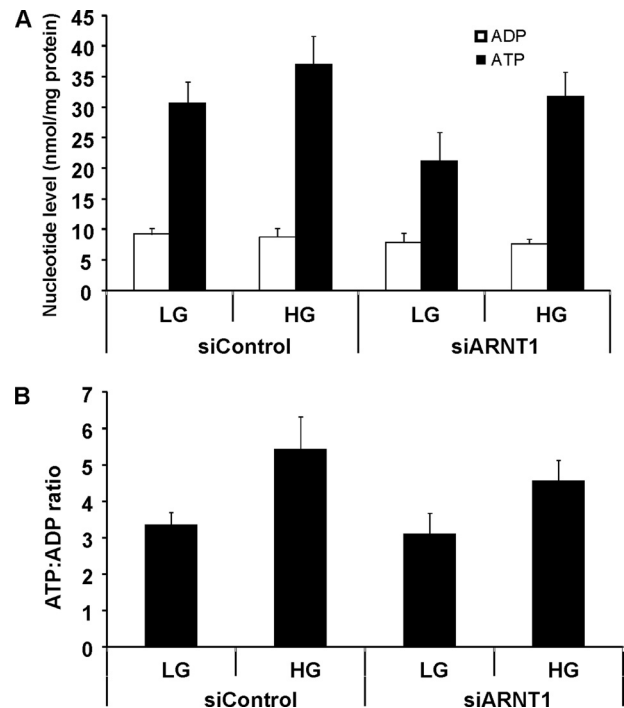
significantly reduced at low glucose concentrations. At high glucose levels, the reduction in TCA metabolite levels following *ARNT/HIF-1β* knockdown was most notable for α-ketoglutarate (90 ± 5%), followed by succinate (68 ± 2%), fumarate (67 ± 1%), and malate (65 ± 2%) and was less marked in case of citrate/isocitrate (49 ± 8%). Considering that glucose oxidation was not significantly affected under these conditions, the amounts of TCA metabolites seen in si*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-1β Levels—Although glucose utilization is negatively affected by *ARNT/HIF-1β* 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*ARNT1*-treated cells as compared with siControl-treated cells (supplemental Fig. 1). However, the final step in the oxidative phase of the PPP seemed to be negatively affected by *ARNT/HIF-1β* silencing as the glucose-induced rise in ribulose 5-phosphate levels was diminished in si*ARNT1*-treated cells, although not significantly. *ARNT/HIF-1β* 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*ARNT1*-
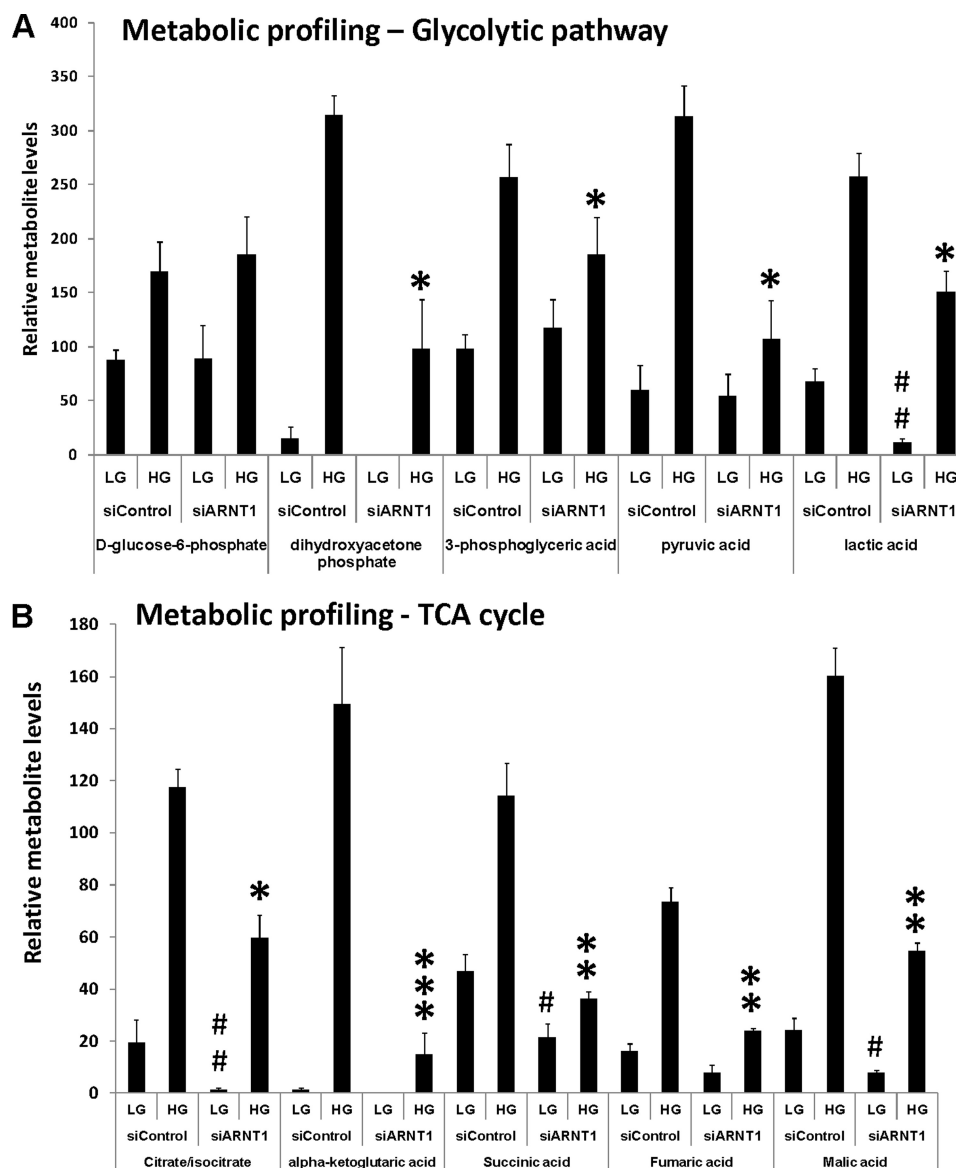
FIGURE 4. **Effects of siRNA-mediated suppression of *ARNT/HIF-1β* on glycolytic metabolite levels (*A*) and TCA cycle intermediates (*B*) in 832/13 cells.** Data are presented as a ratio of target metabolite corrected for an internal control (myristic acid-d_{27}) and represents mean ± S.E. of 3 independent 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* HI si*ARNT1*; #, $p < 0.05$; ##, $p < 0.01$, LG siControl *versus* LG si*ARNT1*.

treated cells exposed to elevated glucose concentrations. Also, the product of transaldolase, erythrose 4-phosphate, was also significantly lower in si*ARNT1*-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* 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. 5*A* 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 si*ARNT1*-treated cells as compared with siControl-treated cells. The levels of fatty acid species, including myristic acid (supplemental Fig. 2*A*), palmitic acid, palmitoleic acid, oleic acid, stearic acid (Fig. 5*A*), arachidic acid, and caprylic acid (also known as octanoic acid) (supplemental Fig. 2*B*) did

not increase when si*ARNT1*-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* also seems to extend into amino acid metabolism but to a lesser extent (Fig. 5*B* and supplemental Fig. 3). Only a few amino acids were affected in si*ARNT1*-treated cells as the levels of β-alanine, L-glutamate, L-threonine, and L-alanine were significantly lowered at high glucose concentrations (Fig. 5*B* and supplemental Fig. 3). The lower levels of L-alanine in si*ARNT1*-treated cells are interesting considering that pyruvate serves as a precursor for its pro-
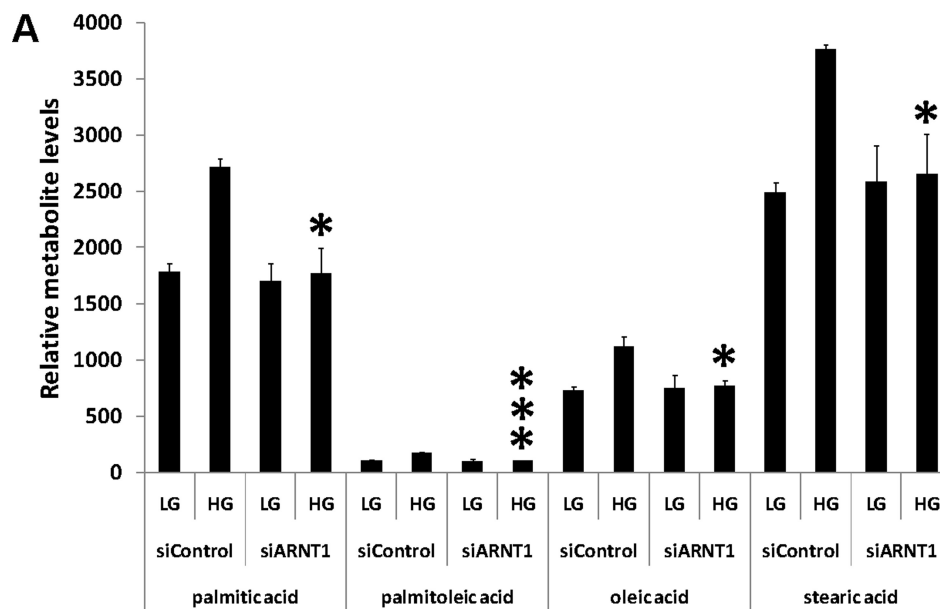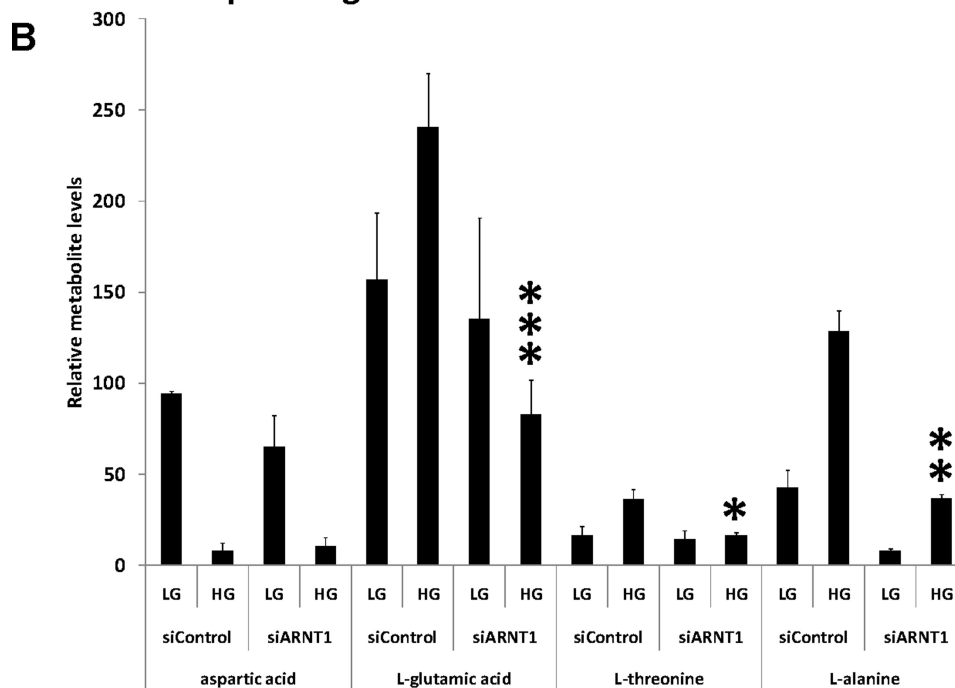
FIGURE 5. **Effects of siRNA-mediated suppression of *ARNT/HIF-1β* on selected free fatty acid (*A*) and amino acid (*B*) levels in 832/13 cells.** The data are presented as a ratio of target metabolites corrected for an internal control (myristic acid-d_{27}) and represents mean ± S.E. of 3 independent experiments. *LG*, low glucose (2 mM); *HG*, high glucose (16.7 mM). *, $p < 0.05$; **, $p < 0.01$, HG siControl *versus* HI si*ARNT1*; ***, $p < 0.001$, HG siControl *versus* HG si*ARNT1*.

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
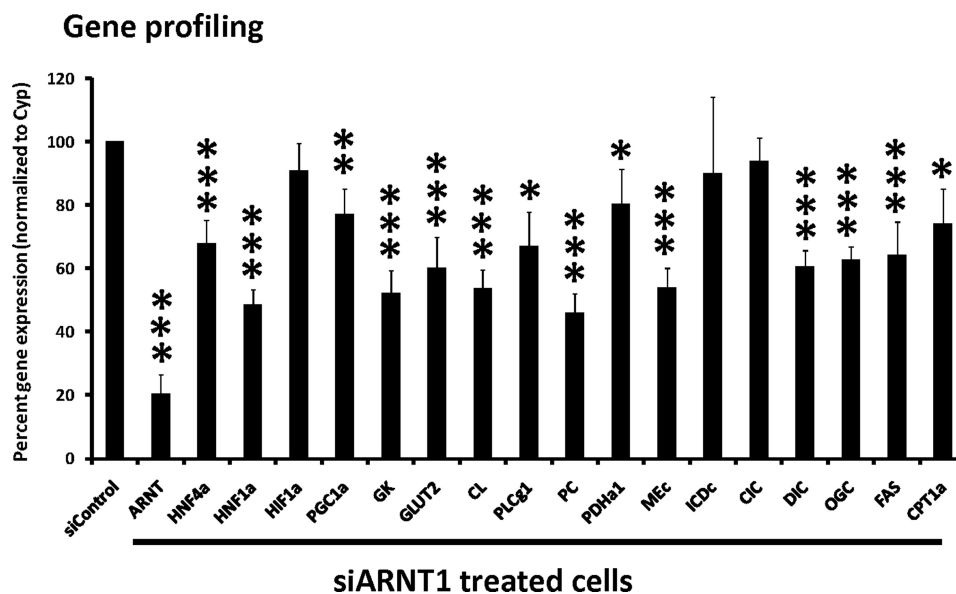
Gene profiling

FIGURE 6. **Effects of siRNA-mediated suppression of *ARNT/HIF-1β* on key genes involved in the metabolic regulation of β-cell function in 832/13 cells.** Gene expression is expressed as a percentage of the target gene from siControl-treated cells and corrected for by an internal control gene cyclophilin E (*Cyp*). There was no significant difference seen between treatment groups for cyclophilin E ($n = 5$). *HNF4a*, hepatocyte nuclear factor-4α; *HNF1a*, hepatocyte nuclear factor-1α; *HIF1a*, hypoxia-inducible factor 1α; *GK*, glucokinase; *GLUT2*, glucose transporter-2; *PLGγ1*, phospholipase γ-1; *PC*, pyruvate carboxylase; *PDHα1*, pyruvate dehydrogenase (α1 subunit); *MEc*, cytosolic malic enzyme; *ICDc*, cytosolic isocitrate dehydrogenase; *CIC*, citrate carrier; *DIC*, dicarboxylate carrier; *OGC*, α-ketoglutarate carrier; *FAS*, fatty-acid synthase; *CPT1a*, carnitine palmitoyltransferase 1α. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ siControl *versus* si*ANRT1*.

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 (*PDHα1*) and pyruvate carboxylase (*PC*) are significantly reduced in response to *ARNT/HIF1β* knockdown and are important genes involved in mitochondrial glucose metabolism (Fig. 6). *PDHα1* is important for the generation of NADH and ATP, and *PC* is important for anaplerotic entry of glucose. Export of mitochondrial metabolites to the cytosol occurs via dicarboxylate carrier (*DIC*; malate transporter), α-ketoglutarate carrier (*OGC*; α-ketoglutarate transporter), and citrate carrier (*CIC*; citrate transporter). Interestingly, both *DIC* and *OGC* are both significantly reduced after treating cells with si*ARNT1*. Reductions in *PC*, *DIC*, and *OGC* are in agreement with a reduction of anaplerosis 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 si*ARNT1*-treated cells. As certain free fatty acids were lower in si*ARNT1*-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 si*ARNT*-treated cells.

Islet Perifusion—Previous studies have suggested that β-cell-specific *ARNT/HIF1β* knock-out mice have defective insulin secretion; however, biphasic insulin secretion has not yet been assessed. Transfection of dispersed islets with
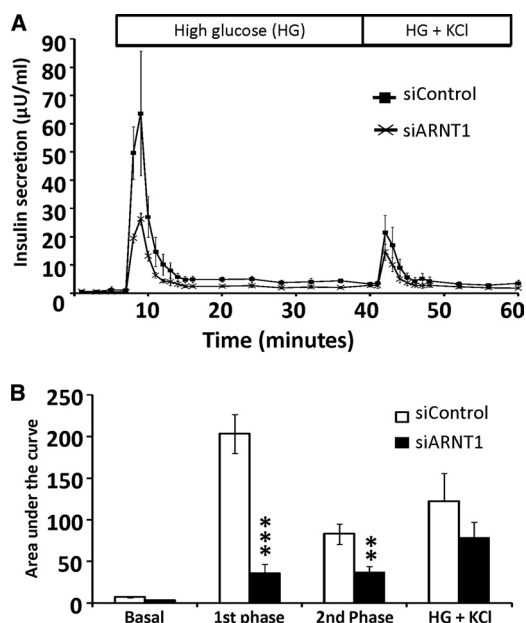

FIGURE 7. **Biphasic insulin secretion in pseudoislets 72 h after transfection with either siRNA control (*siControl*) or siRNA against *ARNT/HIF-1β* (si*ARNT1*).** *A,* pseudoislets were perifused from 1 to 8 min with 2.8 mM low glucose (*LG*) followed by 16.7 mM high glucose (time points from 9–40 min) and finally with 16.7 mM high glucose with 40 mM KCl (time points from 41–60 min) ($n = 5$). *B,* area under the curve of the insulin secretion responses in *A* (*basal*, 1–8 min; *1st phase*, 9–18 min; *2nd phase*, 19–40 min; *HG + KCl*, 41–60 min). **, $p < 0.01$; ***, $p < 0.001$ siControl *versus* si*ANRT1*.

siRNAs against *ARNT/HIF-1β* (si*ARNT1*) 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 si*ARNT1*-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 si*ARNT1*- and si*ARNT2*-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 other 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*).

Gunton *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 si*ARNT1*-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 si*ARNT1*-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-
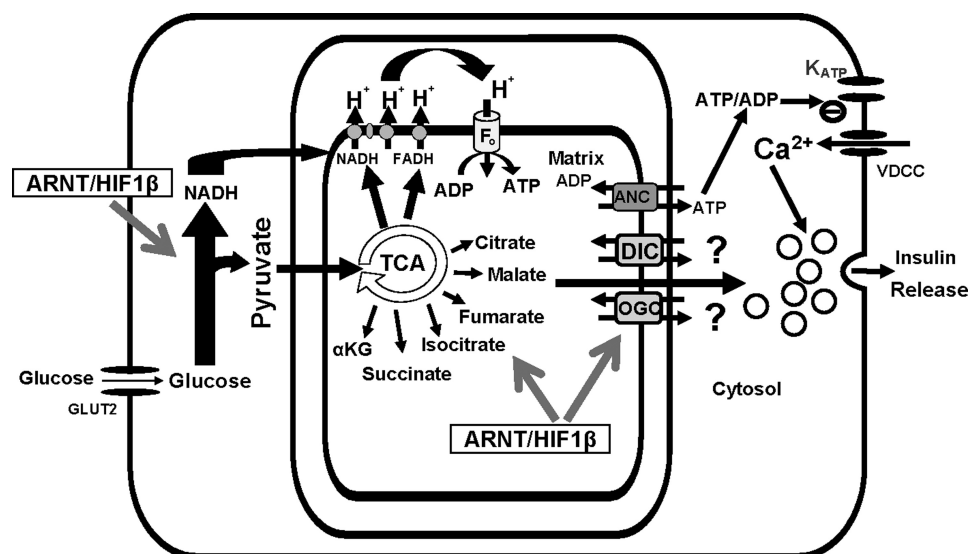
FIGURE 8. **Schematic showing the involvement of *ARNT/HIF-1β* in insulin release.** Glycolysis, DIC, OGC, and anaplerosis are significantly reduced in the absence of ARNT/HIF-1β in β-cells. In addition, ARNT/HIF-1β appears to also be involved in the regulation of glucose-induced fatty acid and glutamate metabolism (data not shown). Interestingly, ARNT/HIF-1β does not seem to play a significant role in the regulation ATP production in β-cells. *OGC,* α-ketoglutarate carrier; *DIC,* dicarboxylate carrier; *OGC,* α-ketoglutarate carrier; *Fo*, ATP synthase; *ANC,* adenine nucleotide carrier; *VDCC,* voltage-dependent calcium channels.

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 si*ARNT1*-treated cells than in siControl-treated cells exposed to 16.7 mM glucose. The 31% reduction in glycolytic flux in si*ARNT1*-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 si*ARNT1*-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 particu-

larly 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 si*ARNT1*-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 si*ARNT1*-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 K_{ATP} 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 si*ARNT1*-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).

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