Loss of *ARNT/HIF1*β Mediates Altered Gene Expression and Pancreatic-Islet Dysfunction in Human Type 2 Diabetes

Jenny E. Gunton,[1] Rohit N. Kulkarni,[1] SunHee Yim,[2]
Terumasa Okada,[1] Wayne J. Hawthorne,[3]
Yu-Hua Tseng,[1] Russell S. Roberson,[1]
Camillo Ricordi,[4] Philip J. O'Connell,[3]
Frank J. Gonzalez,[2] and C. Ronald Kahn[1,]*
[1]Joslin Diabetes Center and Harvard Medical School
1 Joslin Place
Boston, Massachusetts 02215
[2]Laboratory of Metabolism
National Cancer Institute
Bethesda, Maryland 20892
[3]National Pancreas Transplant Unit
Westmead Hospital
University of Sydney
Sydney 2145
Australia
[4]University of Miami Diabetes Research Institute
Miami, Florida 33136

Summary

β cell dysfunction is a central component of the pathogenesis of type 2 diabetes. Using oligonucleotide microarrays and real-time PCR of pancreatic islets isolated from humans with type 2 diabetes versus normal glucose-tolerant controls, we identified multiple changes in expression of genes known to be important in β cell function, including major decreases in expression of *HNF4*α, insulin receptor, *IRS2*, *Akt2*, and several glucose-metabolic-pathway genes. There was also a 90% decrease in expression of the transcription factor *ARNT*. Reducing ARNT levels in Min6 cells with small interfering RNA (siRNA) resulted in markedly impaired glucose-stimulated insulin release and changes in gene expression similar to those in human type 2 islets. Likewise, β cell-specific ARNT knockout mice exhibited abnormal glucose tolerance, impaired insulin secretion, and changes in islet gene expression that mimicked those in human diabetic islets. Together, these data suggest an important role for decreased ARNT and altered gene expression in the impaired islet function of human type 2 diabetes.

Introduction

Type 2 diabetes is an epidemic disease affecting almost 200 million people worldwide (Zimmet et al., 2001). The pathogenesis involves two core defects: insulin resistance and impaired β cell function (Martin et al., 1992; Weyer et al., 1999; DeFronzo et al., 1992). β cell dysfunction is a critical component of all forms of diabetes. In type 1 diabetes, this occurs as a result of autoimmune β cell destruction, whereas in type 2 diabetes, β cell mass may be normal or only slightly reduced early in the disease (Clark et al., 1988), although there are marked functional defects, including impaired first-phase insulin secretion, loss of pulsatility of insulin secretion, altered insulin/proinsulin ratio, and increased β cell apoptosis (Porte and Kahn, 2001; Del Prato et al., 2002; Gerich, 1998; Gumbiner et al., 1996; Butler et al., 2003). Many substantial advances toward the understanding of peripheral insulin resistance have been made in animal models and in humans (Bruning et al., 1997; Lauro et al., 1998; Nandi et al., 2004; Sreekumar et al., 2002; McCarthy and Froguel, 2002; Mauvais-Jarvis and Kahn, 2000; Petersen et al., 2004). However, the mechanisms underlying β cell failure in humans are less well understood, partly due to difficulty accessing the pancreas and the small contribution (<2%) of islets to the total pancreatic mass (Kloppel et al., 1985).

Important understanding of the genetic control of β cell function has come from maturity-onset diabetes of the young (MODY) (Bell and Polonsky, 2001; Froguel and Velho, 2001; Habener and Stoffers, 1998; Polonsky, 1995). In these relatively rare autosomal dominant forms of diabetes, mutations in one of six identified genes leads to impaired insulin secretion and early-onset diabetes. The MODY genes are five transcription factors (*HNF1*α, *HNF1*β, *HNF4*α, *PDX-1/IPF-1*, and *NeuroD1/Beta2*) and glucokinase.

In addition, the glucose-sensing and insulin-signaling pathways have been shown to play important roles in β cell function. These pathways include the facilitative glucose transporters, especially GLUT2, glucokinase (which phosphorylates glucose to G6P), and several enzymes of the glycolytic pathway that metabolize glucose, resulting in an increased ATP:ADP ratio, membrane depolarization, and insulin release. Although glucose uptake in β cells is insulin independent, studies in animal and in vitro models have indicated important roles for insulin signaling in maintenance of normal glucose-responsive insulin secretion. Thus, genetically modified mice lacking insulin receptors, *IRS2*, or the serine kinase *Akt2* exhibit marked alterations in glucose sensing, insulin secretion, and/or altered β cell growth (Kulkarni et al., 1999a; Withers et al., 1998; Cho et al., 2001).

In the present study, we sought to identify defects in islets isolated from humans with type 2 diabetes by examining gene-expression profiles using oligonucleotide microarrays and real-time polymerase chain reaction (PCR). These revealed decreased expression of several genes known to be associated with defective β cell function, including *HNF4*α and glucose-sensing and insulin-signaling genes. In addition, islets from humans with type 2 diabetes demonstrated marked downregulation of the aryl hydrocarbon receptor nuclear translocator (*ARNT*) (also called hypoxia-inducible factor 1 β or *HIF1*β).

ARNT is a member of the basic helix-loop-helix Per/AhR/ARNT/Sim (bHLH-PAS) family of transcription factors and is essential for normal embryonic development (Abbott and Buckalew, 2000; Kozak et al., 1997; Maltepe et al., 1997). All bHLH-PAS proteins function as obligate heterodimers, with ARNT acting as the general partner for other members of the family, including

*Correspondence: c.ronald.kahn@joslin.harvard.edu

HIF1α, HIF2α, HIF3α, and AhR. HIF proteins mediate signal transduction in response to conditions of hypoxia, and AhR responds to certain environmental toxins such as polycyclic aromatic hydrocarbons and the notorious dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (Kozak et al., 1997; Kewley et al., 2004).

We find that knockdown of ARNT in cultured Min6 cells using small interfering RNA (siRNA) and β cell-specific knockout of *ARNT* in mice produces both defects in glucose-stimulated insulin release and alterations in islet gene expression that mimic those in islets of humans with type 2 diabetes. Together, these different models suggest an important role for ARNT and altered gene expression in impaired β cell function and the pathogenesis of human type 2 diabetes.

Results

Microarray Analysis

cRNA prepared from islets of seven normal and five type 2 diabetic patients was hybridized to Affymetrix U133A and B microarrays. Demographic characteristics are shown in Table S1 in the Supplemental Data available with this article online. Of the ~44,928 genes and ESTs represented on these chips, 370 showed differences in expression at the $p < 0.01$ level (Table S2). Of these, 243 were upregulated in diabetic subjects relative to normal, and 137 were downregulated. All of the microarray data will be available on the website of the Diabetes Genome Anatomy Project (DGAP) at http://www.diabetesgenome.org.

None of the 13 genes that were recently reported to best differentiate acinar and islet contributions to mRNA (Cras-Meneur et al., 2004) differed, indicating equivalent islet purification in the two groups (Figure 1A). As expected, expression of insulin, glucagon, somatostatin, and pancreatic polypeptide mRNAs in human islets was high but did not differ significantly between groups (Figure 1B). Figure 1C shows array expression of a number of non-MODY transcription factors known to be associated with β cell development and function. None of these was significantly altered, although *Foxa2/HNF3*β and *Pax4* both showed trends to increased expression in diabetics ($p = 0.06$). Expression of neurogenin3 was low and did not differ between groups.

MAPPFinder 2.0 analysis of the microarray data identified the group of RNA-processing genes as significantly altered (Figure S1). No other group of genes was identified using this approach. Therefore, we focused our initial attention on families of genes that have previously been linked to β cell function.

Expression of MODY, Glucose-Sensing, and Insulin-Signaling Genes Was Altered in Human Islets

Three groups of genes that have been shown to affect glucose-stimulated insulin secretion are the MODY genes, glucose-uptake and -metabolism genes, and several insulin-signaling genes. Expression of genes from these groups was measured by both microarray and quantitative real-time PCR. For simplicity, the quantitative RT-PCR data are presented here, but as noted above, the array data are available at the DGAP website.

Of the six MODY genes, *HNF4*α was markedly and significantly decreased in human diabetic islets, with expression at 18% of control ($p = 0.002$) (Figure 2A). *HNF1*α showed a trend toward decreased expression ($p = 0.08$). Expression of other MODY genes did not differ significantly, although there was wide interindividual variability in *PDX-1* and glucokinase.

mRNAs for the glucose transporters *GLUT1*, *GLUT2*, and *GLUT3* (Figure 2B), as well as *GLUT9* and *GLUT10* (data not shown), were all clearly detectable in islets, but there were no significant differences in expression. Interestingly, in human islets, *GLUT1* was much more highly expressed than *GLUT2*. By RT-PCR, *GLUT1* expression in controls was 4.4 times that of *GLUT2* ($p = 0.037$). Markedly higher expression of *GLUT1* compared to *GLUT2* in human islets has been previously reported (De Vos et al., 1995) and is a potentially important difference between human and rodent islets.

In contrast to glucose-transporter expression, several downstream enzymes in the glycolytic pathway were significantly decreased in diabetic islets, including glucose-6-phosphate isomerase (*G6PI*), aldolase (*Aldo*), phosphofructokinase (*PFK*), and phosphoglucomutase (*PGM*) at 16%, 18%, 19%, and 11% of control ($p = 0.002$, $p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, respectively).

Among the insulin-signaling genes, insulin-receptor mRNA was significantly lower in subjects with diabetes (23% of control, $p = 0.027$) (Figure 2C). In addition, *IRS2* and *Akt2* mRNAs were significantly and markedly lower in the diabetic subjects (9%, $p < 0.0001$, and 16% of control, $p = 0.008$, respectively). On the other hand, expression of *SHIP2*, a phosphatidylinositol phosphatase that inhibits insulin signaling, was significantly increased in islets of the type 2 diabetics (81% increase, $p = 0.04$). These changes would be likely to lead to impaired insulin signaling in diabetic islets. There were no significant differences in expression of proinsulin, *IRS4*, phosphatidylinositol (PI) 3-kinase subunits, or *Akt1* between the two groups (data not shown).

In mice, the two alternately spliced isoforms of the insulin receptor have been suggested to play different roles in regulation of islet gene expression (Leibiger et al., 2001). Furthermore, some studies have suggested an altered ratio of expression of insulin-receptor isoforms in muscle of humans with type 2 diabetes (Norgren et al., 1993). Semiquantitative PCR using primers selected to amplify both A (minus exon 11) and B (plus exon 11) isoforms (Entingh et al., 2003) revealed that isoform A was the dominantly expressed form in human islets and confirmed the decrease in total insulin-receptor mRNA, but the ratio of isoforms did not differ significantly between the diabetics and controls (data not shown).

ARNT Expression Was Decreased in Islets from Humans with Type 2 Diabetes Mellitus

By microarray, the most significantly decreased gene in islets from people with type 2 diabetes was *ARNT*, 18% of control levels, $p = 0.000012$, Figure 2D. Real-time PCR confirmed a substantial and highly statistically significant decrease in ARNT, with expression at 10.2% of normal islets, $p < 0.0000001$, Figure 2E. Among the
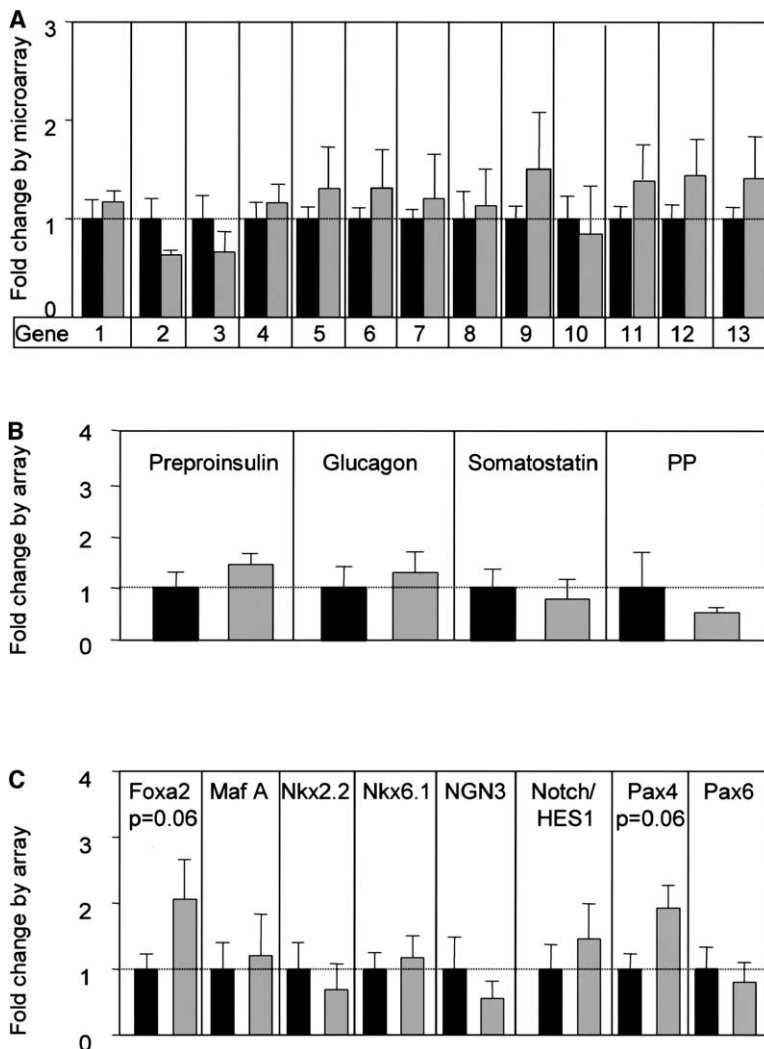
Figure 1. Expression of Islet Genes, Hormones, and Islet Transcription Factors Did Not Differ in Normal Glucose-Tolerant or Diabetic Humans

RNA was purified from pancreatic islets isolated from five people with type 2 diabetes and seven with normal glucose tolerance.

(A) By Affymetrix oligonucleotide microarray, the 13 genes reported to best differentiate between islet and acinar pancreatic tissue (Cras-Meneur et al., 2004) did not differ between diabetic and control subjects, suggesting equivalent islet purification. Gene 1 = carboxypeptidase A1; 2 = protease serine 2; 3 = protease serine 1; 4 = amylase α-2A; 5 = carboxypeptidase B1; 6 = elastase 3A; 7 = pancreatic colipase; 8 = carboxypeptidase A2; 9 = pancreatic lipase; 10 = carboxyl ester lipase; 11 = elastase 3B; 12 = chymotrypsinogen; 13 = phospholipase A2, group IB.

(B) Expression of pancreatic hormones did not differ significantly between the diabetic and normal glucose-tolerant groups. By array, expression of preproinsulin was 186,000 in the diabetic group. The median array expression was 1,500, indicating the expected very high level of preproinsulin expression in islets.

(C) Expression of a number of important islet transcription factors did not differ significantly in diabetic islets as determined by Affymetrix microarray analysis. There were trends toward increased expression of *Foxa2/ HNF3β* (p = 0.06) and *Pax4* (p = 0.06).

Bars show fold change in microarray results compared to control subjects, and error bars indicate ± 1 SEM. Black bars = control; gray bars = type 2 diabetes.

bHLH-PAS family members, *AhR*, *BMAL1*, *HIF1α*, and *HIF2α/EPAS1* were detected in human islets by microarray and real-time PCR. Of these, *AhR*, *BMAL1*, and *HIF1α* were also significantly decreased (Figure 2F).

Small Interfering RNA Impaired Glucose-Stimulated Insulin Release and mRNA Expression in Min6 Cells

To determine the role of ARNT in a glucose-responsive β cell-derived model, Min6 cells were treated with siRNA directed against *ARNT* or scrambled-siRNA control. As shown in Figure 3A, siRNA treatments decreased ARNT protein with varying effectiveness. This was paralleled by decreases in *ARNT* mRNA (data not shown). Scrambled siRNA (control, group 1) had no effect on ARNT protein levels. Among the six active siRNAs tested, there were two levels of effect: an intermediately effective group (group 2), which caused a moderate decrease in ARNT (approximately 40%) and the most effective siRNAs, which markedly reduced ARNT (>80% decrease, group 3). When each of these was assessed for effect on β cell function, both the moderately effective and most effective siRNAs pro-

duced marked reductions in glucose-stimulated insulin secretion from Min6 cells. Group 2 produced a decrease of approximately 50% in the stimulatable component of insulin release (p < 0.01 by ANOVA for repeated measures), whereas group 3 abolished glucose-stimulated insulin release (p < 0.001 by ANOVA) (Figure 3B).

Real-time PCR of RNA isolated from Min6 cells revealed that siRNA treatment also led to significant decreases in the expression of *HNF4α* (60% decrease, p = 0.012) and *HNF1α* (68% decrease, p < 0.0001) (Figure 3C); *G6PI* (35% decrease, p = 0.039), aldolase (50% decrease, p = 0.048), and *PFK* (59% decrease, p = 0.047) (Figure 3D); and insulin receptor (88% decrease, p = 0.044), *IRS2* (78% decrease, p < 0.001), and *Akt2* (97% decrease, p < 0.0001) (Figure 3E). Together, these marked changes in gene expression would be expected to impair β cell function, glucose metabolism, and glucose-responsive insulin secretion, which was seen in Figure 3B.

The changes in mRNA expression in the Min6 cells with reduced *ARNT* expression closely paralleled the changes observed in the human islets from people with
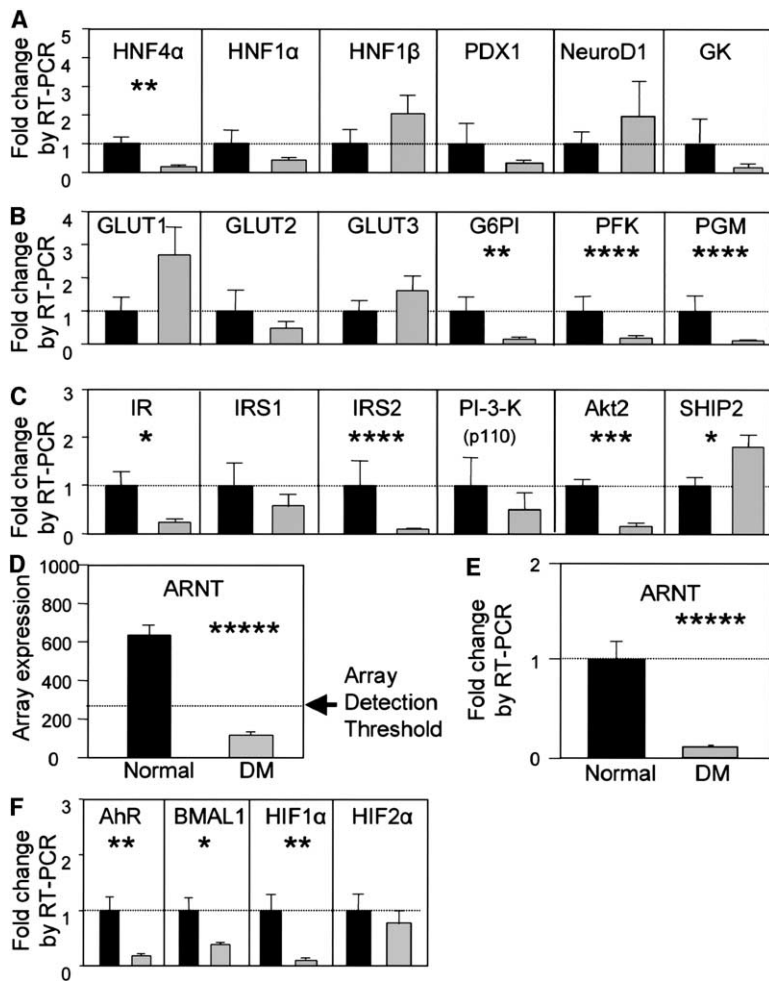
Figure 2. mRNA Expression Was Altered in Human Pancreatic Islets Isolated from Subjects with Type 2 Diabetes

(A) Real-time PCR was used to measure mRNA expression in human pancreatic islets, with correction for expression of the control gene TATA-box binding protein (*TBP*). Of the MODY genes, *HNF4α* was significantly decreased in diabetic islets. Glucokinase, *PDX-1*, and *HNF1α* were lower but did not reach significance due to wide interindividual variability.

(B) By real-time PCR, the expression levels of glucose transporters *GLUT1*, *GLUT2*, and *GLUT3* in human islets did not differ significantly. However, the expression of the enzymes involved in several successive steps in the glucose metabolic pathway was significantly decreased: glucose-6-phosphoisomerase (*G6PI*), phosphofructokinase (*PFK*), and phosphoglucomutase (*PGM*).

(C) Expression of insulin receptor, *IRS2*, and *Akt2* was significantly decreased in type 2 diabetes. Additionally, expression of the PIP3 phosphatase *SHIP2* was increased.

(D) By microarray, *ARNT* was detectable in islets isolated from 7 of 7 people with normal glucose tolerance but was below background levels in 5 of 5 subjects with type 2 diabetes.

(E) Decreased *ARNT* was confirmed by real-time PCR, with *ARNT* expression in diabetic subjects being 10.2% of that in controls ($p = 2.5 \times 10^{-9}$).

(F) Expression of other bHLH/PAS family members was decreased in diabetic human pancreatic islets, including aryl hydrocarbon receptor (*AhR*), *BMAL1*, and hypoxia-inducible factor 1 α (*HIF1α*).

Error bars are ± 1 SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; *****$p < 0.00001$. Black bars = normal glucose tolerance; gray bars = type 2 diabetic subjects.

type 2 diabetes. When the real-time PCR fold changes were plotted against each other, there was a strong and highly significant correlation between the change in type 2 islets and the change in siRNA-treated Min6 cells for genes with a putative ARNT binding site in the first 2 kb of their promoter regions ($r^2 = 0.69$ and $p = 0.006$, Figure 3F).

To examine which ARNT partner (or partners) was important in the effect upon β cell function, Min6 cells were treated with siRNAs directed against *HIF1α*, *HIF2α*, or *AhR*. Each of these was effective, achieving >65% decrease in expression (Figure 3G). At low glucose concentration, knockdown of the AhR led to a small but significant decrease in insulin release, whereas knockdown of HIF1α and HIF2α was without effect (Figure 3H). At 33 mM glucose, knockdown of HIF1α, HIF2α, and AhR each independently produced small but significant decreases in insulin release, but none of these achieved the same magnitude of decrease seen with ARNT knockdown ($p < 0.01$), suggesting that the effect of decreased ARNT may result from loss of function of multiple of these bHLH-PAS heterodimers in β cells.

Glucose Tolerance and Insulin Secretion in β-ARNT Mice

To determine the role of ARNT in vivo, mice with β cell-specific knockout of *ARNT* (β-ARNT) were created as described in Experimental Procedures. β-ARNT mice were born at the expected frequencies, were fertile, and did not differ in weight or length from their *flox*ed control littermates. However, on glucose-tolerance testing at 9–11 weeks of age, female β-ARNT mice showed significantly higher serum glucose values with 1 g/kg glucose load (Figure 4A) as well as with 2 g/kg of glucose (Figure 4B) (both $p < 0.01$ by ANOVA). Female β-ARNT mice had fasting insulin levels similar to those in control mice, but first-phase glucose-stimulated insulin release was totally abolished (Figure 4D, $p < 0.01$). Furthermore, female β-ARNT mice had no augmentation of insulin secretion following addition of arginine to the glucose stimulation, while controls displayed a clear augmentation (Figure 4F, $p < 0.01$). Male β-ARNT mice, on the other hand, had a milder phenotype with slightly, but significantly, higher glucose values compared to controls (Figure 4C) and a normal insulin-secretory response to glucose (Figure 4E). Male β-ARNT mice ex-
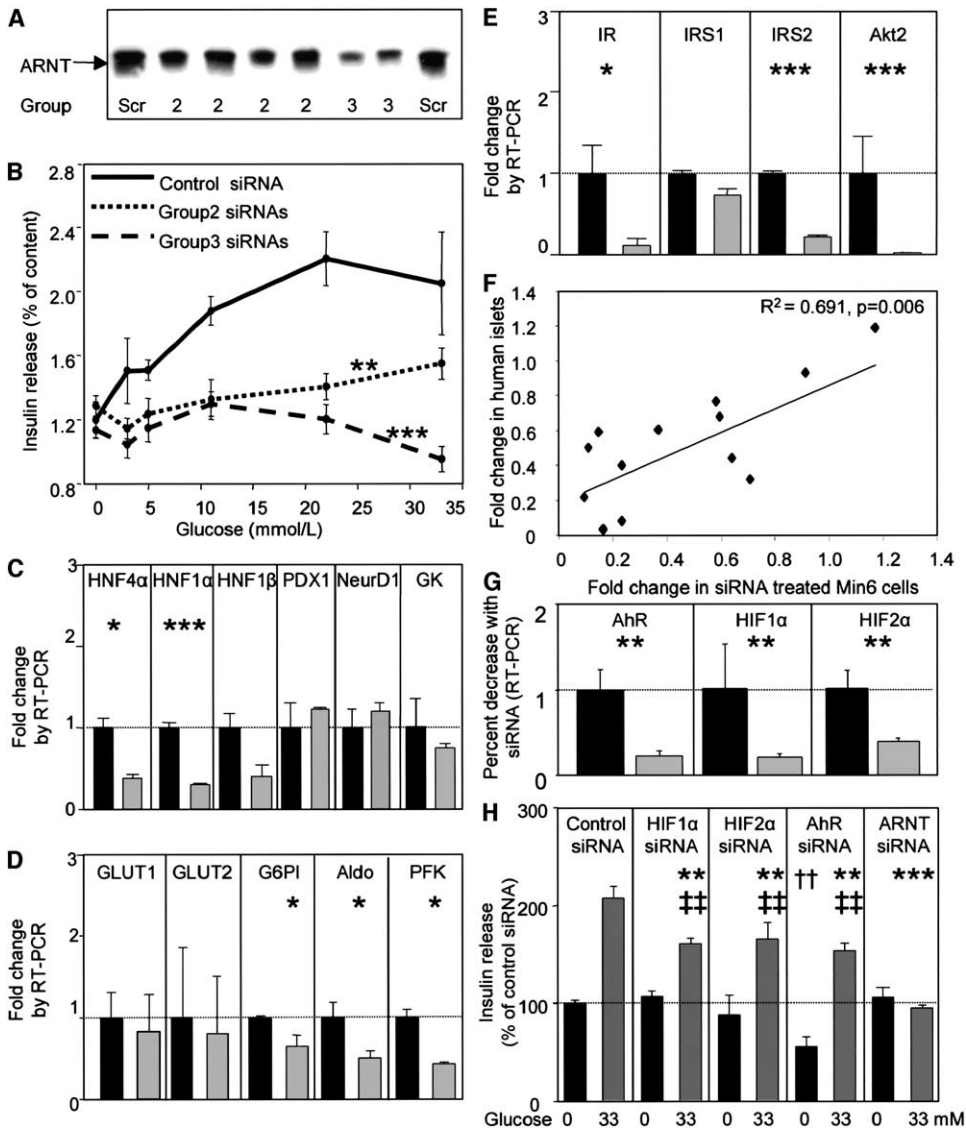
Figure 3. Decreasing *ARNT* In Vitro with Small Interfering RNA (siRNA) Impairs Glucose-Stimulated Insulin Release (GSIR) in Min6 Cells

(A) Western immunoblot of ARNT protein following treatment with either scrambled-siRNA controls (Scr) or six different anti-ARNT siRNAs for 48 hr. siRNAs were grouped as moderately effective (group 2) and highly effective (group 3).

(B) Cells treated with control siRNA showed normal glucose-stimulated insulin release. Compared to Scr controls, cells treated with group 2 siRNAs showed significant impairment of glucose-stimulated insulin release ($p < 0.01$), and cells treated with group 3 siRNAs had no response to glucose stimulation ($p < 0.001$).

(C–E) Min6 cells treated with anti-ARNT siRNA had altered gene expression, measured by real-time PCR.

(C) Among the MODY genes, decreased ARNT was associated with significantly decreased *HNF4α* and *HNF1α*.

(D) Expression of glucose-6-phosphoisomerase (*G6PI*), aldolase (*Aldo*), and phosphofructokinase (*PFK*) were significantly decreased following siRNA treatment.

(E) siRNA treatment led to decreased expression of insulin receptor (*IR*), insulin receptor substrate-2 (*IRS2*), and *Akt2*.

(F) For genes with a potential ARNT binding site in the promoter, the fold change in mRNA expression in type 2 human islets was correlated with the fold change in siRNA-treated Min6 cells, $r^2 = 0.69$.

(G) To examine the role of potential ARNT partners, Min6 cells were treated with RNAi directed against HIF1α, HIF2α, AhR, or ARNT for 48 hr. These caused >65% decreases in mRNA expression.

(H) Insulin release was measured after RNAi treatment. AhR RNAi decreased basal insulin secretion, and RNAi directed against HIF1α, HIF2α, and AhR significantly decreased insulin release at 33 mM glucose; however, none of these was as effective as ARNT knockdown.

Error bars are ± 1 SEM. *$p < 0.05$; **$p < 0.01$ versus control; ***$p < 0.001$; ††$p < 0.01$ versus control at 0 mM glucose; ‡‡$p < 0.01$ versus ARNT siRNA at 30 mM glucose. Black bars = scrambled siRNA; gray bars = active siRNA.
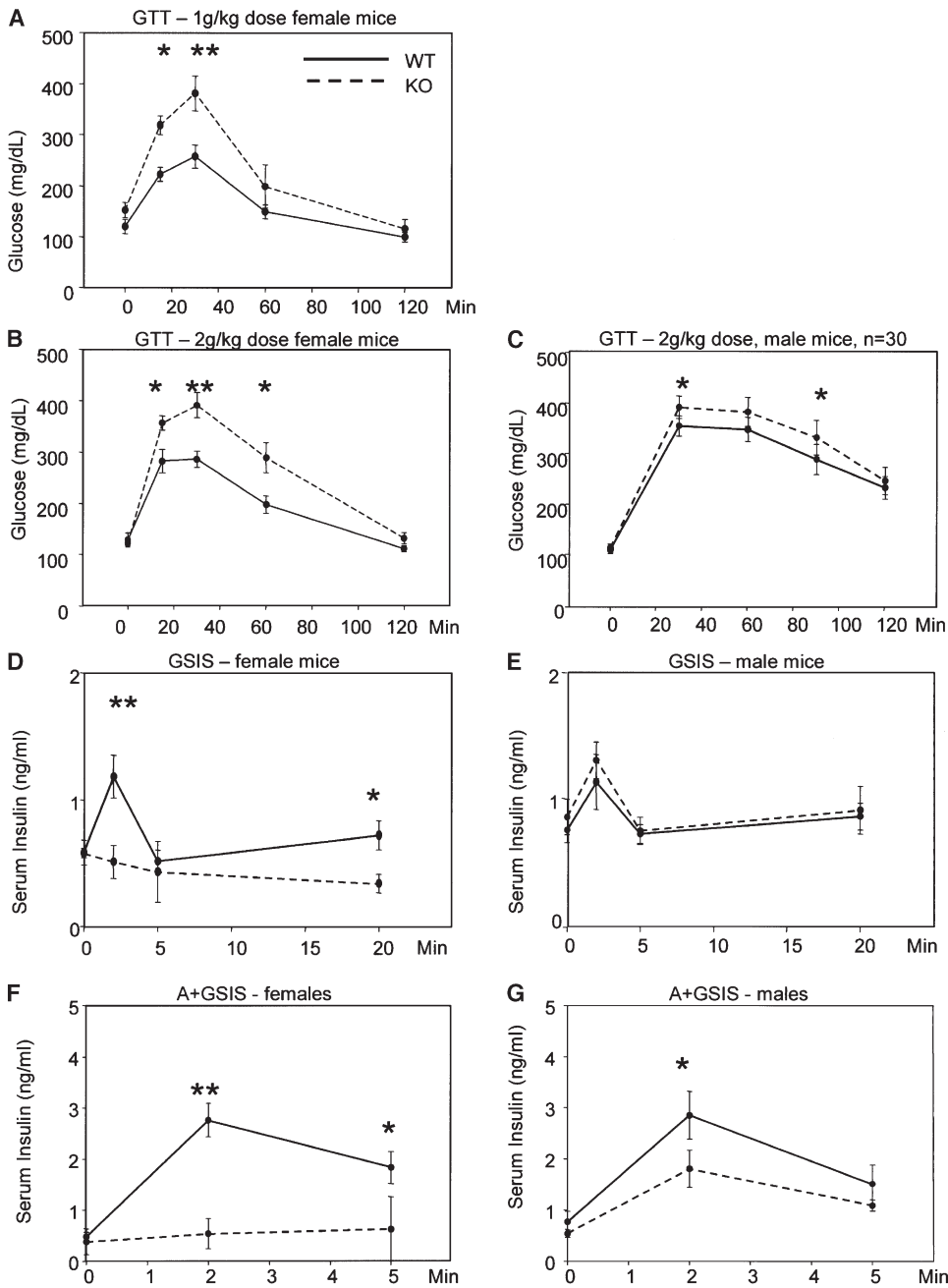
Figure 4. Glucose Tolerance and Insulin Secretion Were Abnormal in 9- to 11-Week-Old Mice with β Cell-Specific Deletion of ARNT (β-ARNT Mice)

(A) Intraperitoneal glucose-tolerance testing (GTT) with a 1 g/kg glucose load led to significantly greater glucose excursion in female β-ARNT mice.

(B) During GTT with 2 g/kg glucose load, female β-ARNT mice showed significantly higher glucose levels than *flox*ed control littermates (p < 0.01 by ANOVA for repeated measures).

(C) Male β-ARNT mice had slightly, but significantly, higher glucose levels than controls when large numbers of mice were compared.

(D) Glucose-stimulated insulin secretion (GSIS) was markedly abnormal in female β-ARNT mice, with total loss of first-phase insulin secretion (p < 0.01).

(E) Male mice had preserved GSIS.

(F) Arginine augmentation of GSIS was normal in *flox*ed control mice but was lacking in female β-ARNT mice (p < 0.01).

(G) Male β-ARNT mice showed mild impairment of arginine-augmented GSIS (p < 0.05).

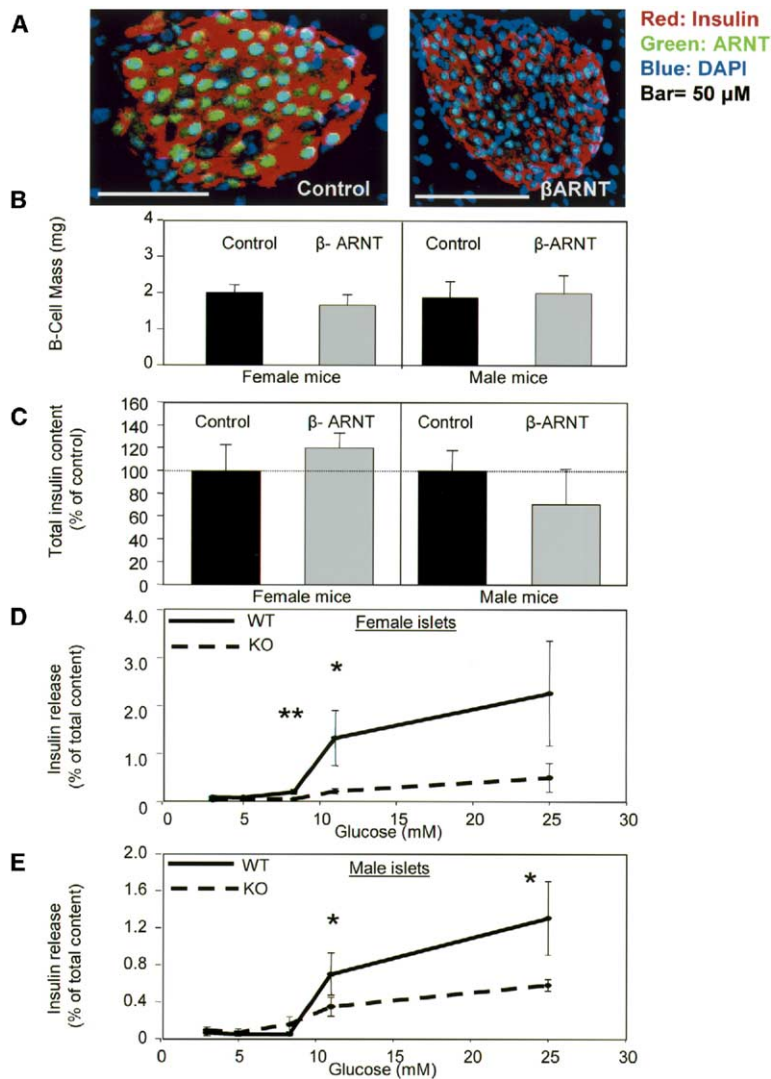Error bars are ± 1 SEM. *p < 0.05; **p < 0.01. Solid line = control mice; dashed line = β-ARNT mice.

Figure 5. Insulin Release Was Abnormal in Islets Isolated from β-ARNT Mice

Equal numbers of islets were selected for insulin-secretion and -content studies by investigators blinded to mouse genotype.

(A) Islets isolated from β-ARNT and control mice were used for immunohistochemistry to confirm efficacy of ARNT deletion.

(B) β cell mass was quantified by an observer blinded to mouse genotype and did not differ significantly between control and β-ARNT mice for either males or females.

(C) Total insulin content also did not differ between β-ARNT and control mice for either female or male mice.

(D) Insulin release was significantly lower from female β-ARNT islets at glucose concentrations of 8.3 and 11 mM and showed a trend toward lower secretion at 25 mM glucose.

(E) In male mice, insulin release was also significantly lower from β-ARNT islets than from *flox*ed control islets at glucose concentrations of 11 and 25 mM.

Error bars are ± 1 SEM. *p < 0.05; **p < 0.01.

hibit a 40% reduction in peak levels of insulin following combined arginine and glucose stimulation compared to controls (p < 0.05, Figure 4G).

Islets from β-ARNT Knockout Mice Display Defective Glucose-Stimulated Insulin Release and Altered mRNA Expression

To determine the basis of the defect in glucose-stimulated insulin secretion in the β-ARNT mice, islets from β-ARNT and control animals were examined. Isolated islets were stained with a cocktail for insulin, DAPI, and ARNT. As shown, ARNT deletion was highly effective in the knockout mice (Figure 5A). β cell mass was examined by an observer blinded to mouse genotype and did not differ between β-ARNT and control mice (Figure 5B). Islet isolation gave similar yields of 227 ± 19 and 236 ± 12 islets in β-ARNT and control mice, respectively. Total insulin content did not differ between β-ARNT and control mice for either female or male mice (Figure 5C). Using islets from either females or males, insulin release was low and equal at low glucose concentrations

(3 or 5 mM). However, at glucose concentrations of 8.3 and 11 mM, insulin release from female β-ARNT islets was significantly lower than in controls (p < 0.01 and p < 0.05, respectively) (Figure 5D). This trend continued at 25 mM glucose, but the difference did not achieve statistical significance at this high glucose level. In male β-ARNT mice, insulin release was also lower at both 11 and 25 mM glucose (Figure 5E).

RNA was isolated from the remaining islets and analyzed by real-time PCR. The results for male and female mice did not differ significantly, so they are presented together. As shown in Figure 6A, of the genes associated with MODY in humans, *HNF4α* was substantially and significantly decreased (92% decrease, p < 0.0001). Expression of glucose transporters *GLUT1* and *GLUT2* was not altered. However, *G6PI* and aldolase were significantly decreased (46%, p = 0.044 and 54%, p = 0.048, respectively) (Figure 6B). Insulin-receptor expression was decreased by 46% (p < 0.0001), and there was a 73% decrease in *Akt2* expression (p = 0.007) (Figure 6C). Again, the changes observed in mRNA expression in β-ARNT islets paralleled those seen in human
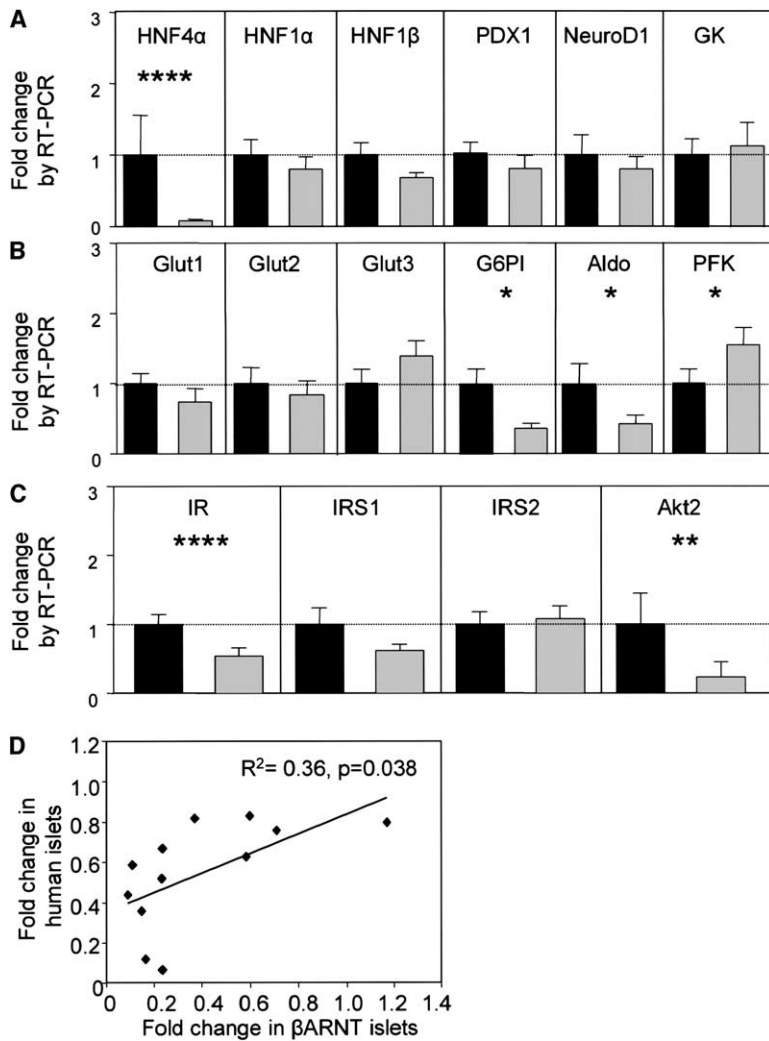
Figure 6. By Real-Time PCR, mRNA Expression in β-ARNT Mouse Islets Was Abnormal and Mimicked Those Changes Seen in Human Islets from Type 2 Diabetic Subjects and Anti-ARNT siRNA-Treated Min6 Cells

There were no significant differences in gene expression between males and females, so results are shown together.

(A) Of the MODY genes, *HNF4α* was highly significantly decreased in β-ARNT islets compared to *floxed* controls.

(B) Of the glucose-uptake and -metabolism genes, glucose-6-phospho-isomerase (*G6PI*) and aldolase (*Aldo*) were significantly decreased in islets from knockout mice. Phosphofructokinase (*PFK*) expression was significantly increased.

(C) Insulin-receptor and *Akt2* expression were significantly decreased in islets from β-ARNT mice.

(D) There was a significant correlation between the fold change in mRNA expression in type 2 human islets and the fold change in the islets of the β-ARNT mice ($r^2 = 0.36$; $p = 0.038$).

Error bars are ± 1 SEM. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$. Black bars = *floxed* control islets; gray bars = β-ARNT islets.

pancreatic islets and those in Min6 cells treated with siRNA to decrease ARNT. The fold change of gene expression in human type 2 islets plotted against fold change in βARNT mouse islets is shown in Figure 6D for genes with a putative ARNT binding site in the first 2 kb of their promoter regions and was significant ($r^2 = 0.36$, $p = 0.038$). Interestingly, for the mRNAs examined, the results for male and female mice did not differ significantly, suggesting that the ability of male mice to maintain near-normal glucose tolerance and insulin secretion depends on compensation by some other component of the insulin-secretion or insulin-action pathway.

Among potential ARNT partners, expression of mRNAs for *AhR*, *BMAL1*, *HIF1α*, and *HIF2α/EPAS1* were clearly detectable in mouse islets (data not shown).

Comparison to Other Mouse Models of Diabetes

RNA was isolated from islets of three other mouse models of islet dysfunction and diabetes: ob/ob mice, db/db mice, and β cell-specific insulin-receptor knockout mice (βIRKO), all at 10–12 weeks of age. The first two models have hyperglycemia (456–464 mg/dl versus 195–286 mg/dl), while βIRKO mice are normoglycemic (197 ± 18 mg/dl versus 187 ± 33 mg/dl in controls), as expected at this young age.

There was a trend toward decreased *ARNT* expression in βIRKO mice (74% of expression in IR-lox animals, $p = 0.07$) and no significant change in *ARNT* expression in db/db or ob/ob mice (Figure 7). These results suggest that the substantial decrease in ARNT in human islets is not likely to be mediated solely by diabetes, hyperglycemia, or insulin resistance.

Discussion

It is well known that humans with type 2 diabetes exhibit marked defects in β cell function, the most characteristic of which is impaired first-phase insulin secretion (Edlund, 2001; Del Prato et al., 2002; Gerich, 1998; Gumbiner et al., 1996; Butler et al., 2003). This defect is intrinsic, as demonstrated by recent studies using islets isolated from subjects with type 2 diabetes (Anello et al., 2005). Our data show that pancreatic islets isolated from patients with type 2 diabetes also exhibit multiple alterations in gene expression. The magnitude
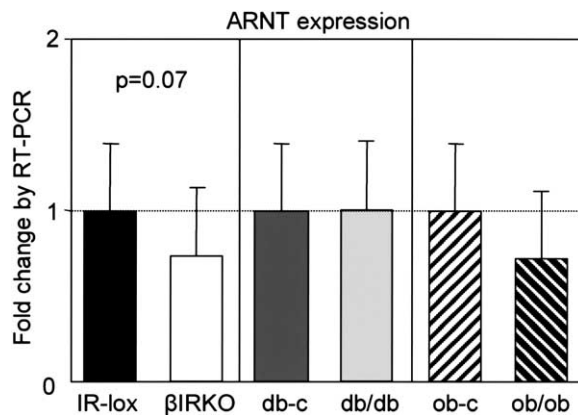
ARNT expression

p=0.07

Figure 7. *ARNT* Expression in Islets Isolated from Other Mouse Models of Diabetes and siRNA Treatment to Identify Potential ARNT Partners in Min6 Cells

Pancreatic islets were isolated from IR-lox mice and β cell-specific insulin-receptor knockout (βIRKO), ob control (ob-c), ob/ob, db control (db-c), and db/db mice aged 10–12 weeks, and RNA was isolated for real-time PCR. As shown, βIRKO mice showed a trend toward decreased *ARNT* expression (74% of control, p = 0.07), whereas ob/ob mice and db/db mice had no significant changes in *ARNT* expression. Error bars are ± 1 SEM.

of some of these changes is quite impressive and involves a number of genes demonstrated to affect β cell function, including *HNF4α*, the insulin receptor, *IRS2*, *Akt2*, and several enzymes of the glycolytic pathway. *HNF4α* is a MODY gene, and studies in knockout mice show that decreased levels of insulin receptor, *IRS2*, or *Akt2* can lead to altered glucose sensing and defective insulin secretion (Bell and Polonsky, 2001; Kulkarni et al., 1999a; Withers et al., 1998; Cho et al., 2001).

In an effort to identify an upstream regulator of these changes, we examined the mRNA expression of transcription factors. While most were unchanged, there was a substantial and highly significant decrease in expression of *ARNT*. In vitro studies in cultured β cells using siRNA to decrease ARNT resulted in severe impairment of glucose-stimulated insulin secretion and alterations in mRNA expression that largely paralleled those seen in human type 2 diabetic islets. Likewise, β cell-specific deletion of *ARNT* in mice led to significant abnormalities in glucose metabolism and insulin secretion and a number of changes in RNA expression in isolated islets, which also paralleled those seen in human pancreatic islets. These data suggest that changes in gene expression could lead to the altered insulin secretion in human type 2 diabetes and that ARNT is a likely upstream regulator of many of these changes.

ARNT is essential for the normal function of HIF1α, HIF2α, and the aryl hydrocarbon receptor (AhR). These heterodimeric complexes are required for cellular responses to hypoxia (HIF proteins) and environmental toxins (AhR), respectively (Kozak et al., 1997; Kewley et al., 2004). ARNT-containing dimers have been reported to regulate expression of many genes. With short DNA consensus binding sites for ARNT hetero- and homodimers, there are more than 13,000 putative ARNT binding sites in promoters in the human genome. Many of

these promoters have multiple potential binding sites. Thus, a substantial decrease in ARNT could result in many alterations in gene expression. Many of the genes found to be altered in association with decreased ARNT in this study have putative ARNT-dimer consensus binding sites in their promoters (including *HNF4α*, *HNF1α*, *Akt2*, *G6PI*, *PFK*, and aldolase), consistent with a direct role of ARNT-containing dimers in the regulation of their expression. Preliminary results using chromatin immunoprecipitation suggest a direct role of ARNT-HIF dimers in the regulation of expression of these glycolytic enzymes.

It has been previously reported that ARNT regulates transcription of a number of genes involved in the pathogenesis of diabetes. These include mRNAs coding for proteins involved in glucose metabolism and genes involved in vascular function and hypoxic response, including vascular endothelial growth factor (*VEGF*), plasminogen activator inhibitor 1 (*PAI1*), and erythropoietin (*EPO*) (Zelzer et al., 1998; Salceda et al., 1996; Forsythe et al., 1996; Okino et al., 1998). ARNT has also been shown to regulate aldolase in hepatoma and breast carcinoma cells (Zelzer et al., 1998; Salceda et al., 1996). The lack of decrease in *GLUT1* with decreased ARNT was surprising, in that HIF1α is known to regulate *GLUT1*. RNAi directed against HIF1α in Min6 cells resulted in a decrease in *GLUT1* expression (data not shown), suggesting that diminishing ARNT may lead to other compensatory changes that oppose the HIF1α effect.

One of the genes that appears to be regulated by ARNT is *HNF4α*, which was decreased in association with decreased ARNT in human islets, as well as in cells after RNAi knockdown and in knockout mice. While *HNF4α* is a MODY gene and decreased *HNF4α* is likely to have contributed to the phenotype by inducing secondary gene-expression changes, mice with β cell-specific deletion of *HNF4α* have mild abnormalities in β cell function (Gupta et al., 2005; F.J.G., unpublished data). This suggests that the changes in the β-ARNT mice are not mediated solely via HNF4α. This is not surprising since ARNT dimerizes with multiple transcription partners to alter the expression of target genes.

Dioxins are potent environmental toxins that signal through the ARNT-AhR heterodimer. Dioxin exposure is associated with an increased risk of type 2 diabetes in women (Fierens et al., 2003; Pesatori et al., 2003). The data in men are less clear, with some studies suggesting an increased risk of diabetes following dioxin exposure (Fierens et al., 2003; Kim et al., 2003) and some not (Pesatori et al., 2003; Michalek et al., 2003). This parallels our findings in that the phenotype of the β-ARNT mice was greater in females than in males. Whether the alteration in ARNT in human type 2 diabetes is the result of exposure to an environmental toxin will require further study.

The presence of sexual dimorphism is observed in almost all mouse models of diabetes. In most models of type 2 diabetes, including those generated by altering insulin signaling, there is a male predominance in the phenotype. In the NOD mouse model of type 1 diabetes in which there is autoimmune β cell destruction, there is a strong female predominance in the development of diabetes, even though both genders develop lympho-

cytic infiltration of the islets (Kikutani and Makino, 1992). The specific factors leading to the sexual dimorphism remain largely unexplained in virtually all of these models.

ARNT has multiple potential partners, and which of these is most important in β cells remains to be determined. By real-time PCR, mRNA expression of *AhR*, *BMAL1*, *HIF1α*, and *HIF2α* was clearly detectable in human islets, mouse islets, and Min6 cells. *ARNT2* expression has not been reported in pancreas. Mice with inactivating mutations in *AhR*, *BMAL1*, *HIF1α*, and *HIF2α/EPAS1* have been generated. A small proportion (23%) of female *AhR* null mice develop glucose intolerance at 7 months of age, suggesting that the ARNT-AhR dimer plays a role in normal β cell function (Thackaberry et al., 2003). However, the phenotype is less pronounced in the AhR mice, less penetrant, and occurs at an older age than in our mice, suggesting an additional pathogenic component in the β-ARNT mice. *BMAL1* null mice, on the other hand, have lower serum glucose and insulin levels, suggesting that inactivation of the ARNT-BMAL1 heterodimer is not responsible for the diabetic phenotype seen in our animal model (D. Kennaway, personal communication). Since these data are at present only available for male mice, it will be of interest to determine whether the female *BMAL1* null mice have a different phenotype, given the dimorphic phenotype that we have observed.

The *HIF1α* null mutation is embryonic lethal, and thus the glucose homeostasis of this model is unknown, but there are no reported major abnormalities in pancreatic development. Similarly, the *HIF2α* null mouse shows high embryonic/neonatal lethality, and glucose-homeostasis data have not been reported. HIF1α protein and HIF2α protein are undetectable in Min6 cells in the basal state but are clearly detected upon treatment with the hypoxic mimics such as desferrioxamine (DFO) or CoCl (data not shown). In preliminary coimmunoprecipitation studies in Min6 cells, we find that ARNT associates with AhR under normoxic conditions and, following DFO treatment, also associates with HIF1α and 2α. Since islets in vivo are in a slightly hypoxic environment (pO$_2$ 31–37 mm Hg [Carlsson et al., 1998]), there may be increased expression of HIF proteins in the pancreas in vivo that mimics the latter condition. Our preliminary studies using siRNA in Min6 cells suggest that the loss of HIF1α, HIF2α, and AhR individually produce only minor effects of glucose-stimulated insulin secretion, suggesting that the altered insulin secretion observed within islets and Min6 cells with decreased ARNT may be due to the combined effects of these transcription factors rather than one heterodimer in particular.

A possible driving force for additional changes in gene expression seen in human islets is the altered expression of insulin-signaling proteins. Mice with β cell-specific knockout of the insulin receptor or global knockout of *IRS2* or *Akt2* develop diabetes with early β cell failure (Kulkarni et al., 1999a; Withers et al., 1998; Cho et al., 2001). It is therefore very interesting that the mRNAs encoding these insulin-signaling proteins were significantly decreased in the type 2 diabetic subjects, suggesting an important role for alterations in this pathway in human diabetes. Furthermore, *SHIP2*, a member

of the inositol polyphosphate 5-phosphatase family, has been shown to be a negative regulator of insulin signaling and insulin sensitivity in vivo (Clement et al., 2001) and was increased in diabetic islets in the present study. Insulin signaling has been shown to stimulate the expression of preproinsulin, glucokinase, and other genes in the β cell, and, in the liver and pancreatic islet, acts upstream of HNF4α or PDX-1 by stimulating Akt-mediated phosphorylation of Foxa2 (HNF3β) (Kellerer et al., 2001; Wolfrum et al., 2003; Lee et al., 2002).

In summary, although much remains to be learned about the biology of human pancreatic islets and type 2 diabetes, our data suggest that changes in gene expression, and, in particular, changes in *ARNT*, are likely to be important in the β cell dysfunction in humans with type 2 diabetes. As an obligate partner of several transcription factors involved in the response to toxins and hypoxic stress, ARNT is at a potential site to integrate genetic and environmental insults. These findings also provide new insights into the pathogenesis of type 2 diabetes and a possible new target for treatment of this disease.

Experimental Procedures

Isolated islets were purified from five type 2 diabetic subjects and seven normoglycemic controls using the modified Ricordi method (Ricordi et al., 1988). Average age was 47 years in both groups. The mean duration of type 2 diabetes was 5.8 ± 2.1 years, and no subjects were insulin requiring. Mean HbA1c was 7.5% ± 0.5% in the diabetic subjects. There was no difference in islet yield, islet purity, or islet viability between groups. Islet purity was also compared by examining expression of 13 genes reported to best differentiate islets from acinar tissue (Cras-Meneur et al., 2004). None of these differed between the two groups, again suggesting equivalent purification (Figure 1A).

RNA was extracted from at least 1000 islet equivalents per subject, and no samples were pooled. cRNA was prepared separately for each subject (Yechoor et al., 2002) and hybridized to Affymetrix U133A and B microarrays (24 arrays in total). The number of subjects gave an 80% power to detect differences of >1.83 standard deviations. 1779 cDNA/ESTs represented on the arrays differed with p value of <0.05, and 370 differed with p values of <0.01 (the p < 0.01 genes are listed in Table S2). Complete microarray data sets are available on the Diabetes Genome Anatomy Project (DGAP) website (http://www.diabetesgenome.org).

Real-time PCR was performed in a two-step reaction using the Advantage RT-for-PCR kit (BD Biosciences, Palo Alto, California). The second step was performed in a fluorescent temperature cycler (ABI-Prism 7700 Sequence Detection System, Applied Biosystems) with LightCycler-RNA Master SYBR-Green-I (Roche, Mannheim, Germany) and specific primers for each of the genes (sequences available on request). Every plate included a control gene for every subject. Results were analyzed by Student's unpaired t test. Semiquantitative PCR of insulin-receptor isoforms was performed as per Entingh et al. (2003).

siRNA Treatment of Min6 Cells
Using Min6 cells, ARNT mRNA and protein were decreased by 48 hr of treatment with small interfering RNAs (Dharmacon), which were prepared and transfected using Lipofectamine 2000 (Invitrogen) according to the respective manufacturers' protocols to achieve final siRNA concentrations of 100 nM. siRNA sequences are available on request. Scrambled-sequence siRNA was used as a control in all experiments (Dharmacon). Following siRNA treatment, ARNT protein was assessed by Western immunoblot using a monoclonal anti-ARNT antibody (BD Biosciences). Glucose-stimulated insulin release was assessed in triplicate wells in three separate experiments. In separate experiments, treated cells were lysed and RNA isolated for real-time PCR. siRNAs directed against mu-

rine HIF1α, HIF2α, and AhR were purchased in "smartpool" form from Dharmacon and used according to manufacturer instructions. Each achieved >65% knockdown of the respective expression (shown in Figure 3G). Knockdown of AhR produced a small decrease in basal insulin release, and decreasing HIF1α, HIF2α, and AhR each led to decreased insulin release at 33 mM glucose (p < 0.01) (Figure 3H). However, the impairment in insulin secretion after ARNT RNAi was significantly greater than that seen with the other siRNAs (p < 0.01 for all).

Generation of β-ARNT Mice

Whole-body *ARNT* knockout mice die before embryonic day 10 (Abbott and Buckalew, 2000; Kozak et al., 1997; Maltepe et al., 1997). To study the effect of deletion of ARNT upon the development of diabetes, β cell ARNT knockout mice (β-ARNT) were generated using the Cre-lox recombination system. Two *lox*-p sites were inserted into the *ARNT* gene flanking exon 4, which contains the DNA binding domain, as previously described (Tomita et al., 2000). Homozygous *lox/lox* mice were interbred with mice expressing Cre recombinase under control of the rat insulin promoter (*RIP-Cre*), a kind gift from Dr. Mark Magnuson. This breeding resulted in mice with β cell deletion of ARNT (β-ARNT) and *flox*ed control littermates. β-ARNT mice were born at the expected frequencies, were fertile and did not differ in weight or length.

Glucose-Tolerance Tests and Insulin-Secretion Studies

Glucose-tolerance tests were performed after overnight fasting at 9–11 weeks of age, using either 1 or 2 g/kg of intraperitoneal (i.p.) glucose as indicated in the figure legend. GTTs were also performed in female mice using a smaller glucose load of 1 g/kg. Glucose-stimulated insulin secretion (GSIS) was assessed following i.p. injection of 3 g/kg of glucose (Kulkarni et al., 1999a). Arginine-stimulated augmentation of GSIS was studied following IP injection of 3 g/kg of glucose plus 0.3 mg/kg of arginine (Kulkarni et al., 1999a). Insulin was measured by ELISA (Crystal Chem, Chicago).

Islet Immunohistochemistry and Measurement of β Cell Mass

Islets isolated from β-ARNT and control mice were used for immunohistochemical staining for ARNT, insulin, and DAPI (Kulkarni et al., 1999b). β cell mass was quantified by an observer blinded to the genotype of the mouse according to the method in Kulkarni et al. (1999b).

Islet Isolation from β-ARNT Mice

Pancreatic islets were isolated from mice aged 10–12 weeks, as previously described (Kulkarni et al., 1999b). Islet isolation was performed blinded to genotype to avoid bias in islet selection for secretion studies. The total number of islets isolated from β-ARNT and control mice did not differ. Equal numbers of islets were subjected to stimulation of insulin secretion at glucose concentrations of 3.3, 5.5, 8.3, 11, and 25 mM. The remaining islets were reserved for RNA isolation and quantitative real-time PCR.

Islets from Other Mouse Models of Diabetes

Pancreatic islets were isolated from ob/ob mice, db/db mice, β cell-specific insulin-receptor knockout mice, and their appropriate controls (obc, dbc, and IR-lox mice) at 10–12 weeks of age. RNA was isolated and used for real-time PCR to examine gene expression of *ARNT*.

Supplemental Data

Supplemental Data include Supplemental References, two tables, and one figure and can be found with this article online at http://www.cell.com/cgi/content/full/122/3/337/DC1/.

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