Defective insulin secretion in response to glucose is an important component of the β cell dysfunction seen in type 2 diabetes. As mitochondrial oxidative phosphorylation plays a key role in glucose-stimulated insulin secretion (GSIS), oxygen-sensing pathways may modulate insulin release. The von Hippel–Lindau (VHL) protein controls the degradation of hypoxia-inducible factor (HIF) to coordinate cellular and organismal responses to altered oxygenation. To determine the role of this pathway in controlling glucose-stimulated insulin release from pancreatic β cells, we generated mice lacking Vhl in pancreatic β cells (Vhl KO mice) and mice lacking Vhl in the pancreas (PVhlKO mice). Both mouse strains developed glucose intolerance with impaired insulin secretion. Furthermore, deletion of Vhl in β cells or the pancreas altered expression of genes involved in β cell function, including those involved in glucose transport and glycolysis, and isolated βVhlKO islets displayed impaired glucose uptake and defective glucose metabolism. The abnormal glucose homeostasis was dependent on upregulation of Hif1α expression, and deletion of Hif1α in Vhl-deficient β cells restored GSIS. Consistent with this, expression of activated Hif1α in a mouse β cell line impaired GSIS. These data suggest that VHL/HIF oxygen-sensing mechanisms play a critical role in glucose homeostasis and that activation of this pathway in response to decreased islet oxygenation may contribute to β cell dysfunction.

Introduction
Blood glucose levels are normally tightly controlled by the regulation of insulin release from the pancreatic β cells. Glucose-stimulated insulin secretion (GSIS) is a complex metabolic process involving the uptake and phosphorylation of glucose via GLUT2 transporters and glucokinase (Gck), respectively, metabolism of glucose-6-phosphate via the glycolytic pathway, and subsequent activation of mitochondrial metabolism to produce coupling factors such as ATP (1). A rise in the cytoplasmatic ATP/ADP ratio leads to closure of KATP channels, depolarization of the plasma membrane, opening of voltage-sensitive Ca2+ channels, and activation of Ca2+-dependent exocytotic mechanisms, resulting in insulin secretion (1). This metabolic sensing mechanism requires molecular oxygen for the quantitative generation of ATP from glucose. Understanding the complex physiology of this mechanism may give insights into both the pathogenesis and treatment of the β cell dysfunction seen in type 2 diabetes.

Hypoxia-inducible factor (HIF) is a transcription control complex containing a constitutive β subunit and regulatory α subunit, which acts as a master regulator of the responses to altered cellular and tissue oxygen concentration (2). In the presence of oxygen, HIF-α subunits are hydroxylated, enabling capture by the von Hippel–Lindau (VHL) tumor suppressor gene product, which is the substrate recognition component of an ubiquitin E3 ligase complex (3, 4). At low oxygen concentrations, HIF-α is stabilized and active. In the absence of VHL, HIF is constitutively active. Key processes regulated by HIF include erythropoiesis, angiogenesis, and cellular energy metabolism, thereby adapting the organism, tissue, and cell to hypoxia (4). HIF is responsive within the range of oxygen tensions encountered in normal tissues and is increasingly recognized as an important physiological regulator rather than a simple stress response mechanism, playing roles, for example, in innate immunity (5), neutrophil survival (6), muscle performance (7), and skin oxygen sensing (8).

HIF upregulates expression of the high-affinity glucose transporter GLUT1 and glycolytic enzymes and decreases mitochondrial oxygen consumption in a range of cell types (4). Since glucose uptake, glycolysis, and mitochondrial respiration are key steps in β cell glucose sensing, activation of the HIF pathway has the potential to provide a major input modulating GSIS. This could potentially be important in a wide range of disease states in which oxygen delivery is altered, including obstructive sleep apnea and acute and chronic respiratory disease, or when islet oxygenation is directly compromised, such as

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Nonstandard abbreviations used: ABRT, aryl hydrocarbon receptor nuclear translocator; BHIF1αKO, mice, lacking HIF1α in pancreatic β cells; BHIF1αKO mice, mice lacking Vhl and HIF1α in pancreatic β cells; BHIF1αKO mice, mice lacking Vhl in pancreatic β cells; E-cadherin; FAD, flavin adenine dinucleotide; Gck, glucokinase; GSIS, glucose-stimulated insulin secretion; 2-NBDG, 2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxy-D-glucose; Pdk1, pyruvate dehydrogenase kinase 1; RIPCre mice, mice expressing Cre under control of the rat insulin II promoter; VHL, von Hippel–Lindau.

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Deletion of the von Hippel–Lindau gene in pancreatic β cells impairs glucose homeostasis in mice


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in islet transplantation. VHL disease is associated with pancreatic tumors believed to be of endocrine origin, also indicating a potential role for this pathway in islet endocrine cell growth and function. Furthermore, small-molecule HIF activators are currently under evaluation for the treatment of anemia, and understanding the potential effects of pharmacological manipulation of this pathway on pancreatic islet function is also of clinical interest.

Therefore, to determine the effect of activating HIF, we investigated the effect of deleting the Vhl gene, specifically in β cells or the pancreas in mice. After we initiated these studies, it was reported that islets of patients with type 2 diabetes show reduced expression of the HIF-α dimerization component aryl hydrocarbon receptor nuclear translocator/HIF1b (ARNT/HIF1B) and that knockdown of Arnt/Hif1b in β cells or mice impaired GSIS (9). In light of these studies, and assuming a monotonic relationship between GSIS and HIF activation, it was reasonable to predict that HIF activation would have the opposite effect of deletion of Arnt/Hif1b and therefore enhance GSIS. Our genetic experiments showed that Hif activation in mice results in profound disruption of β cell function and, in contrast, that β cell deletion of Hif1a does not impair glucose homeostasis.

**Results**

Deletion of Vhl in β cells or the pancreas impairs glucose homeostasis in mice. Initially, we crossed mice expressing Cre under control of the rat insulin II promoter (RIPCre mice; ref. 10) and mice with a floxed allele of Vhl (11) to generate mice lacking Vhl in pancreatic β cells or mice impaired GSIS (9). In light of these studies, and assuming a monotonic relationship between GSIS and HIF activation, it was reasonable to predict that HIF activation would have the opposite effect of deletion of Arnt/Hif1b and therefore enhance GSIS. Our genetic experiments showed that Hif activation in mice results in profound disruption of β cell function and, in contrast, that β cell deletion of Hif1a does not impair glucose homeostasis.

**Figure 1**

Deletion of Vhl in β cells or the pancreas impairs glucose homeostasis in mice. (A) Recombination of the Vhl allele was assayed by PCR in islets, cerebral cortex (cortex), and hypothalami (hyp) from WT and KO mice for VhlKO and PVhlKO mice. Positive and negative PCR controls for deletion were performed using DNA from Vhl-null cells, Vhl control (cont) cells, WT cells, and without addition of DNA. Arrows indicate the deleted allele. (B and C) Hif-1α staining using chromogenic detection (left and middle panels) in islets from control, VhlKO, and PVhlKO mice and combined chromogenic/immunofluorescence staining (right panels) co-localizing Hif-1α and insulin in islets from VhlKO and PVhlKO mice. (D and E) Fed blood glucose levels in 12-week-old female control, VhlKO, and PVhlKO mice. n = 8. (F and G) Blood glucose after an intraperitoneal injection (2 g/kg body weight) of glucose in 12-week-old female control, VhlKO, and PVhlKO mice. n = 8 for null alleles; n = 24 for control animals. (H and I) Fed plasma insulin levels in 12-week-old female control, VhlKO, and PVhlKO mice. n = 8. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control.
β cells (βVhlKO mice) and in a small population of hypothalamic neurons (12). βVhlKO mice were viable and born with expected Mendelian ratios (data not shown). The Vhl allele was deleted in islets and hypothalami from βVhlKO mice (Figure 1A), and Hif-1α stabilization and expression was induced in more than 95% of β cells of βVhlKO mice, consistent with Vhl deletion (Figure 1B and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI26934DS1). However, βVhlKO mice were proportionate dwarfs with reduced pituitary growth hormone (GH) levels (Supplemental Figure 1, B and C), while relative food intake and fat mass were normal in these animals (Supplemental Figure 1, D and E). These findings suggest that Vhl acts in RipCre hypothalamic neurons to regulate Gh secretion. This endocrine phenotype may have had an impact on subsequent studies of glucose homeostasis in βVhlKO mice. Therefore, to test the possibility that potential β cell phenotypes in βVhlKO mice resulted wholly or partly from hypothalamic deletion of Vhl, we used mice expressing Cre under control of the mouse pancreatic and duodenal homeobox 1 promoter (PdxCre mice) to generate PVhlKO mice, which lacked Vhl only in the pancreas (13, 14). PVhlKO mice were born with the expected Mendelian frequency and, in contrast to βVhlKO mice, displayed normal body weight (Supplemental Figure 1F). Recombination of the Vhl allele was observed in PVhlKO islets but not in hypothalami (Figure 1A). Stabilization and expression of Hif-1α was induced in the pancreas of PVhlKO mice (Figure 1C) with greater than 70% of β cells expressing Hif-1α and a 70% reduction in Vhl mRNA (Supplemental Figure 1, G and H). We did not detect Hif-2α in the pancreas.
of βVhlKO or PVhlKO mice, and pancreatic weight was normal in PVhlKO mice with no clinical evidence of exocrine dysfunction in these animals (data not shown).

Generation of βVhlKO and PVhlKO mice permitted examination of the role of Vhl in glucose homeostasis in vivo. Twelve-week-old female βVhlKO and PVhlKO mice displayed significantly elevated fed glucose levels and impaired glucose tolerance (Figure 1, D–G). Glucose intolerance was also seen in 12-week-old male βVhlKO and PVhlKO mice (Supplemental Figure 2, A and B). Fed insulin levels were reduced in both βVhlKO and PVhlKO mice (Figure 1, H and I), fasting insulin levels in PVhlKO mice were similar to those seen in control animals, and βVhlKO mice displayed a mild fasting hypoinsulinemia compared with control mice (Supplemental Figure 2, C and D). Insulin tolerance tests in PVhlKO mice demonstrated no impairment of insulin sensitivity (data not shown). The similarity of the glucose homeostasis phenotypes in both βVhlKO and PVhlKO mice indicated that this was not due to the hypothalamic deletion of Vhl seen in the βVhlKO animals.

Deletion of Vhl in β cells or in the pancreas does not alter β cell mass, proliferation, or survival or cause pancreatic tumors. The VHL/HIF pathway has been implicated in both growth factor–mediated cell proliferation and cell survival. Therefore, we undertook islet morphometric analysis in βVhlKO and PVhlKO mice to exclude reduction of β cell mass as the cause for the impaired glucose homeostasis. Absolute β cell mass was reduced in βVhlKO animals, but when expressed as a percentage of body mass was equivalent to that in control mice (Supplemental Figure 3, A and B). β cell area in βVhlKO mice was equivalent to that seen in control animals (Supplemental Figure 3C). Likewise, we detected no significant difference in β cell mass (either expressed as an absolute value or as a function of body weight) or β cell area in PVhlKO mice compared with control animals (Supplemental Figure 3, D–F). Consistent with these findings, there were no alterations in β cell apoptosis or proliferation rates in both strains (Supplemental Figure 3, G and H). In both mouse lines, organization of α and β cells was preserved, but in βVhlKO mice occasional α cells were scattered among the β cells (Supplemental Figure 4, A and B). Taken together, these findings imply that the in vivo defects in glucose handling were not the result of ablation of β cells. We did detect alteration in the vascularization of the pancreas in βVhlKO and PVhlKO mice by staining for Cd31, although expression of this vascular marker as determined by RT-PCR was not increased in βVhlKO and PVhlKO islets compared with those of controls (Supplemental Figure 4, C–E). Interestingly, however, and consistent with the normal cell proliferation and survival parameters, we did not detect tumors or cyst formation in the pancreases of βVhlKO or PVhlKO mice up to 12 months old (data not shown), suggesting that these pathologies arise from cell types other than those in which we deleted Vhl (i.e., β cells or Pdx1-expressing cells) or involve additional events such as expression of Hif-2α (15).

Deletion of Vhl impairs GSIS in vivo and in vitro. The absence of changes in β cell mass in the islets of βVhlKO or PVhlKO mice suggested that functional defects in β cells underlie the alterations in
Figure 4
Abnormal expression of glucose-sensing apparatus and glycolytic genes in βVhlKO and PVhlKO mice. (A and B) Expression of Glut1, Glut2, and Gck mRNA in βVhlKO and PVhlKO islets relative to control islets. n = 5. (C) Glut1 immunostaining in control, βVhlKO, and PVhlKO islets. Representative images are shown. Scale bars: 150 μm. (D and E) Glut2 (green) and insulin (red) staining in control, βVhlKO, and PVhlKO islets. Representative images are shown. Scale bars: 100 μm. (F) Western blotting for Hif-1α, Glut1, Glut2, and α-tubulin (loading control) in islets isolated from PVhlKO mice. Glut1, Glut2, and α-tubulin were resolved on a 10% gel, and blots were probed and stripped for each of the 3 antibodies. Hif-1α was analyzed on a separate 6% gel with an equal amount of the same cell lysate loaded. Representative blots are shown and are typical of 2 independent experiments. (G) Expression of Glut1 and Glut2 mRNA in Min6 cells transfected with activated HIF1α mutant relative to empty vector–transfected control cells. n = 3. (H and I) Expression of Gapdh, Aldola, Pfk, and Pdk1 mRNA in isolated βVhlKO and PVhlKO islets relative to control islets. n = 5. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control.
glucose homeostasis. Consistent with this idea, in vivo GSIS was severely blunted in PVhlKO mice (Figure 2A). To define the nature of the defects in GSIS seen with the deletion of Vhl, we undertook further in vitro analysis of β cell function using isolated islets and β cells. In perfusion studies, insulin release was equivalent in isolated control and βVhlKO islets under basal conditions (2 mmol/l glucose; Figure 2B). As the perfusate glucose concentration was increased to 20 mmol/l, peak and sustained GSIS were markedly impaired in βVhlKO islets (Figure 2B). When the same islets were challenged with 20 mmol/l glucose and 500 nmol/l phorbol ester to induce potentiated insulin release, βVhlKO islets had a response that reached 60% of control islets, suggesting significant preservation of the response to a non-glucose potentiator of GSIS (Figure 2C). In static incubation experiments, GSIS was also markedly impaired in PVhlKO islets (Figure 2D). To further probe the site of the defective GSIS we used the non-glucose secretagogues α-ketoisocaproic acid (which is exclusively metabolized in mitochondria) and potassium chloride (which depolarizes cell membranes independently of glucose metabolism). Stimulated insulin secretion in βVhlKO islets was normal with both of these agents, while GSIS was again impaired, suggesting that the defect is proximal to mitochondrial metabolism (Figure 2E).

Increased expression of constitutively activated HIF-1α in β cells impairs GSIS. We next examined whether the defects in GSIS could result from increased activation of HIF-1α due to the hypothalamic deletion. Elevated expression of HIF-1α was achieved in Min6 cells transduced with a retrovirus expressing a constitutively active HIF-1α (Figure 3A). Examination of these cells revealed increased impaired insulin release in response to glucose, indicating that activation of HIF-1α perturbs GSIS (Figure 3B).

Rescue of defective glucose homeostasis in βVhlKO mice by concomitant deletion of Hif1α and lack of glucose homeostasis phenotype in βHif1aKO mice. To test genetically in vivo the role of Hif1α in mediating the perturbation in glucose homeostasis following Vhl deletion, we generated mice lacking both Vhl and Hif1α in pancreatic β cells (βVhlHif1αKO mice) using a mouse with a floxed allele of Hif1α. These mice had normal fasted and fed glucose levels (Supplemental Figure 4, F and G) and normal glucose tolerance at 4–6 months of age (Figure 3C). Furthermore the dwarf phenotype seen in βVhlKO mice was reversed in these animals (Supplemental Figure 4H).

The generation of βHif1aKO mice, which lack Hif1α in RIPCre-expressing cells as part of the genetic crosses used to breed βVhHif1aKO mice, also permitted us to analyze the impact of reduced Hif-1α activity upon glucose homeostasis. These animals, which had normal body weight (Supplemental Figure 4I), displayed normal glucose handling on glucose tolerance testing at both 12 weeks and 4–6 months of age (Figure 3D and Supplemental Figure 4J). Studies in isolated islets from these mice revealed a slight but significant increase in insulin release under basal (2 mmol/l) glucose conditions, but no difference in GSIS compared with control mice (Figure 3E). Furthermore, when we examined GSIS in islets from βVhHif1aKO mice, this was slightly enhanced compared with that seen in control animals (Figure 3E). Together, these findings suggest that Hif1α may have a mild restraining effect upon β cell function and that the abnormal GSIS seen in βVhlKO mice is dependent upon upregulation of Hif1α.

These results indicate that appropriate expression of Hif1α is required for normal β cell function, and for GSIS in particular. The rescue of the phenotype of βVhlKO mice by concomitant deletion of Hif1α, the defective GSIS in cells expressing constitutively active HIF-1α, and the concordant phenotype seen in βVhlKO mice suggest that this abnormality is not the result of other features that may be present in the βVhlKO mice due to the hypothalamic deletion.

Alteration in expression of key components of the glucose-sensing apparatus and glycolytic pathway in islets lacking Vhl. The profound defects in GSIS in the absence of abnormalities in β cell mass and the preserved insulin release in response to α-ketoisocaproic acid suggested that β cells lacking Vhl had abnormalities in glucose-sensing or in the proximal elements of glucose metabolism. We therefore examined expression of components of the glucose-sensing and glycolytic apparatus, which are known targets of the VHL/HIF pathway. In both βVhlKO and PVhlKO islets, there was a significant reduction of Glut2 mRNA expression and a concomitant induction in Glut1 mRNA expression with parallel changes in Glut2 and Glut1 production in pancreatic sections and in isolated islets (Figure 4, A–F). In particular, there was a striking loss of cell membrane Glut2 immunofluorescence and protein production in βVhlKO and PVhlKO islets (Figure 4, D–F). Expression of Gck mRNA was also reduced in islets of both genotypes (Figure 4, A and B). Glut1 expression was increased and Glut2 expression was reduced in Min6 cells expressing constitutively active HIF-1α or exposed to either hypoxia or the HIF activator dimethylfumarate (16) (Figure 4G and Supplemental Figure 5, A and B), supporting the conclusion that the observed expression changes in βVhlKO and PVhlKO islets are mediated by increased Hif1α activity.

Analysis of the expression of a panel of glycolytic genes revealed that expression of pyruvate dehydrogenase kinase 1 (Pdk1), phosphofructokinase (Pfk), and Gapdh was increased in islets isolated from βVhlKO and PVhlKO mice, while aldolase expression was reduced (Figure 4, H and I). Increased Pdk1 expression inhibits pyruvate dehydrogenase activity and restricts the entry of pyruvate to mitochondrial oxidative pathways, while reduced aldolase expression further contributes to reducing flux through glycolysis.

We next examined the expression of key β cell transcription factors. In both βVhlKO and PVhlKO islets, there was a significant reduction in the expression of Nkx6.1, a homeodomain transcription factor that regulates GSIS in β cells (17) (mRNA expression as a percentage of control: βVhlKO, 51.5% ± 11.5% vs. control, 100.0% ± 10.1%; P < 0.01, n = 6; PVhlKO, 56.6% ± 3.4% vs. control, 100.0% ± 13.8%; P < 0.05, n = 5). In contrast, the expression of hepatic...
tocyte nuclear factor 1α (Hnf1α), Hnfβb, Hnf4α, and NeuroD was unaltered (data not shown). The glycoprotein E-cadherin (Ecad) has been implicated in β cell function (18), and we have recently demonstrated that the VHL/HIF pathway suppresses its expression in renal cancer cells (19). In both BVhlKO and PVhlKO islets, expression of Ecad was reduced (mRNA expression as a percentage of control: BVhlKO, 46.6% ± 5.3% vs. control, 100% ± 10%, P < 0.01, n = 6; PVhlKO, 39.7% ± 2.0% vs. control, 100% ± 10.7%, P < 0.01, n = 5).

Islets lacking Vhl have impaired glucose uptake and metabolism. The net effect of the expression changes in glucose-sensing and glycolytic genes might be expected to reduce coupling of glucose uptake and glycolysis to mitochondrial ATP production and insulin secretion. Therefore, we undertook further functional analysis in islets isolated from BVhlKO and PVhlKO mice, initially studying glucose uptake by isolated β cells. Control β cells incubated in 2.5 mmol/l glucose rapidly took up 2-[N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino]-2-deoxy-D-glucose (2-NBDG), a fluorescent D-glucose derivative (20) (Figure 5A). In contrast, both total steady-state fluorescence and the rate of 2-NBDG uptake were significantly reduced in BVhlKO and PVhlKO islets (Figure 5, A–C, and data not shown).

Next we monitored the increase in metabolic flux as glucose is metabolized by β cells, by measuring cellular NADH and flavin adenine dinucleotide (FAD)⁺ levels using autofluorescence (21). In response to increasing glucose concentrations, control β cells exhibited a robust increase in NADH and a concomitant fall in FAD⁺ fluorescence (Figure 5, D and F). In contrast, these responses were essentially absent in BVhlKO and PVhlKO β cells, indicating altered mitochondrial metabolism in response to glucose (Figure 5, E and G). To probe the site of this defect, we monitored Ca²⁺ fluxes in Fura-2AM-loaded β cells from BVhlKO and PVhlKO mice in response to glucose, methyl-succinate (which enters the mitochondrial respiratory chain at complex II) and the Kₒ₁₈₆ channel blocker tolbutamide. Glucose-stimulated Ca²⁺ mobilization was markedly attenuated in BVhlKO and PVhlKO β cells compared with controls (Figure 5, H and I). In contrast, responses to methyl-succinate and tobutamide were preserved in BVhlKO and PVhlKO β cells (Figure 5, J and K), which, in combination with the normal secretory response to α-ketoglutaric acid (Figure 2E), places the defect in GSIS between the glucose uptake machinery and mitochondrial metabolism. Taken together with the abnormalities in the proximal components of the glucose-sensing apparatus, these findings demonstrate that the VHL/HIF pathway is critical in regulating mammalian pancreatic β cell function.

**Discussion**

Pancreatic β cells have high rates of aerobic metabolism compared with other cell types, and islet oxygenation is markedly higher than in the exocrine pancreas or other tissues (22). In the current studies, we investigated whether inactivation of Vhl in β cells and concomitant Hif activation alters glucose sensing. Although available transgenic lines are effective at β cell deletion, they are not completely selective (12, 13). We therefore used 2 inactivation strategies, both of which inactivated Vhl in β cells. The concordant defects in GSIS that we observed in BVhlKO and PVhlKO mice, combined with our analyses of isolated islets, imply that loss of Vhl in the β cell results in a profound defect in GSIS.

The most extensively studied function of VHL is its role in the destruction of HIF-1α and HIF-2α in the presence of oxygen (3). VHL also interacts with a number of other proteins and influences diverse intracellular pathways (reviewed in ref. 23). Proteins that have been reported to interact with VHL in other cell types, and which could alter glucose uptake and GSIS in β cells, include PCK-λ/ι and 8 (24, 25) and Sp1 (26). To determine whether the effects of Vhl loss of function were mediated by Hif-1α, we therefore generated BVhlHif1αKO mice. Our finding of normal glucose homeostasis and body weight in BVhlHif1αKO mice suggests that the alteration in both these parameters caused by deletion of Vhl is mediated via activation of Hif-1α. This experiment does not formally exclude the possibility that hypothalamic deletion of Hif1α in BVhlHif1αKO mice contributes to the correction of glucose homeostasis. Nevertheless, our data on β cells expressing a constitutively activated HIF-1α show that HIF-1α activation is sufficient to have major effects on GSIS and gene expression. Together with the data from PVhlKO mice, we conclude that the β cell phenotypes most likely arise from increased β cell Hif-1α activation and consequent engagement of the Hif-1α-dependent gene expression program.

The VHL/HIF pathway has many downstream targets, and therefore abnormal β cell function in BVhlKO and PVhlKO mice is likely to be due to a combination of effects. We demonstrate marked abnormalities in GSIS in Vhl-deficient islets but no alterations in either β cell proliferation or survival. The molecular mechanisms underlying this defect include reduced Glut2 expression, which has been shown to be sufficient to perturb glucose homeostasis in mice and humans (27, 28), and attenuated Gek expression, which alone (10, 29) or in combination with additional β cell defects (30) causes β cell dysfunction. Our findings also suggest that increased Glut1 expression compromises appropriate glucose handling by β cells and does not compensate for the marked reduction in Glut2 expression. The Km for D-glucose of rodent Glut2 is 17 mmol/l, allowing a wide range of physiological blood glucose levels to be sensed by the β cell. GLUT1, however, has been shown to have a Km for D-glucose of 2.3–2.6 mmol/l in humans and to confer a human β cell Vₘₖₐₜ of 3 mmol/l/min, compared with a rodent β cell Vₘₖₐₜ of 32 mmol/l/min for glucose uptake via Glut2. This suggests that BVhlKO and PVhlKO islets relying on Glut1 to supply glucose into the β cell sense little difference between 2 and 20 mmol/l glucose, since Glut1 facilitates glucose transport at near maximum capacity at these concentrations. The absolute levels of glucose uptake depend on the absolute Glut1 expression levels, but our data show the increased Glut1 expression does not normalize glucose uptake in an appropriate manner to restore GSIS in Vhl-null islets.

We also demonstrate alterations in the expression of glycolytic genes that have an additional negative impact upon GSIS. Increased Pdk1 expression inhibits pyruvate dehydrogenase activity and would restrict the entry of pyruvate to mitochondrial oxidative pathways, while reduced aldolase expression would be anticipated to reduce flux through glycolysis as well. Our studies using non-glucose secretagogues together with metabolic and imaging studies place the defects in GSIS proximal to the mitochondria. The combination of markedly reduced glucose uptake and impaired glycolytic flux would prevent the burst of mitochondrial oxidative phosphorylation required for quantitative coupling of glucose uptake to insulin release when glucose levels rise. Taken together, this combination provides a sufficient explanation for the abnormalities in BVhlKO and PVhlKO mice.

Recently it was reported that mice with β cell–specific deletion of the Hif-1α dimerization component Arnt/Hif1b show impaired glucose homeostasis (9). In contrast, our genetic manipulation of deleting Vhl in insulin-producing cells led to increased β cell Hif activity. While it might have been predicted that these 2 distinct perturbations of the Hif pathways would have opposite effects
upon glucose homeostasis, a number of explanations could underlie the similar phenotypes resulting from deletion of Vhl or Arnt in mouse β cells. Firstly, in addition to HIF substrate, ARNT dimerizes with the aryl hydrocarbon receptor, and loss of the AHR/ARNT response may influence β cell function. Indeed, siRNA for Abra was reported to decrease GSIS in the Min6 β cell line (9). Secondly, Arnt will have been deleted not only in β cells, but also in the hypothalamus, which could contribute to the reported abnormalities in glucose homeostasis seen in mice generated using the RIPC Cre animal (9). Third, glucose intolerance has been demonstrated in the pure RIPC Cre strain on certain genetic backgrounds, and our own use of PdxCre animals (14) provides evidence that the effect we observed was not due to the RIPC Cre transgene. We also demonstrate normal glucose tolerance in the RIPC Cre strain in our hands. Our studies of mice lacking Hif1a in insulin-producing cells give further insights into the role of this pathway in β cells and suggest that Hif1α may have a mild restraining role on β cell function under basal conditions. There are additional considerations in evaluating the potential pathological relevance of our findings compared with those of Gunton et al. (9), which suggested that decreased HIF could contribute to human type 2 diabetes. First, type 2 diabetes is clearly multifactorial, and it is plausible that perturbations of HIF in either direction could contribute to its pathophysiology, although our studies with Hif1a deletion in β cells suggest that this may not be the case. Second, HIF activation by tissue hypoxia is a well-understood pathophysiological construct, whereas the circumstances under which HIF would be suppressed – other than by genetic manipulation – are unclear. In this context it is also important to appreciate that HIF is activated, at least in the kidney, in response to very minor alterations in oxygen delivery that are within the physiological range. Finally, our results indicate that although engagement of the HIF-1α–dependent transcriptional program would be anticipated to protect β cells from hypoxia, the consequence is perturbation of the intimate relationship between oxidative phosphorylation and GSIS, highlighting the complexity of the role of the VHL/HIF pathway in β cell function.

Our findings are likely to have clinical relevance independent of the putative role of the VHL/HIF pathway in human type 2 diabetes. Tissue oxygen delivery is reduced in many settings, including sepsis syndrome, acute and chronic pulmonary conditions, and obstructive sleep apnea. Indeed, sepsis and obstructive sleep apnea are associated with impaired glucose metabolism, and acute hypoxia causes glucose intolerance in humans (31). Our findings suggest that HIF activation in the β cell in these settings would impair GSIS. However, understanding the overall effects of the VHL/HIF pathway and hypoxia on metabolic networks in these human illnesses represents a complex challenge in systems biology, as it is unclear precisely which tissues may contribute to abnormal glucose handling in the setting of hypoxia in humans. However, a particular clinical scenario in which islet hypoxia may be relevant is islet transplantation. This potentially attractive therapy for diabetes is hampered by hypoxic graft failure, and it has been suggested that impaired graft function may occur in part through activation of the HIF pathway (32). Our studies show that this is likely and provide mechanisms by which this would occur. Our genetic experiments also suggest that pharmacological manipulation of the HIF system, which is under investigation as a therapeutic strategy in cancer, anemia, cardiac ischemia and failure (33), and inflammatory conditions such as glomerulonephritis (34), may have profound effects on β cell function unless this treatment is targeted to specific organs. Vhl is a tumor suppressor gene, and pancreatic cysts and tumors are a feature of human VHL disease (35). However, the βVhKO and PVhKO mice in our studies did not develop these types of tumors, although they did show some increase in vascularization. One explanation would be that the human tumors and cysts do not arise from β cells or other cells expressing Pdx1, which would reinforce the cell and tissue specificity of the tumor suppressor action of Vhl. An alternative possibility is that further events are required for the development of cysts and tumors in the pancreas, which typically present in the third and fourth decades in humans. An important question has been the extent of redundancy in the VHL/HIF system, and whether differential redundancy of HIF components between cell types might account for this striking tissue specificity of tumors in VHL disease (36). Our results may help to clarify this by showing that in β cells Vhl loss is sufficient for Hif-1α stabilization and activation, does not result in detectable Hif-2α activation, and does not produce an increase in β cell mass. The lack of Hif-2α activation is likely to reflect cell-type specificity in Hif2a mRNA expression and is consistent with a previous study in which acute hypoxia did not activate Hif-2α in rat islets (37). Interestingly in kidney cells, the site of malignant tumors in VHL patients, acquisition of Hif2a expression appears to be crucial in progression and tumor growth (15). Finally, it is becoming increasingly clear that the VHL/HIF pathway also has important physiological roles that may not necessarily be manifest as disease processes, such as involvement in innate immunity (5), in muscle performance (7), in neutrophil survival decisions (6), in stem cell differentiation and homing (38–40), and in the role of skin oxygen sensing (8).

In summary, our studies show that VHL/HIF oxygen-sensing mechanisms play a critical role in glucose homeostasis, and activation of this pathway causes β cell dysfunction.

Methods

Mice and animal care. Floxed Vhl allele (Vhlfl/fl, The Jackson Laboratory) mice were crossed with RIPC Cre mice (The Jackson Laboratory) and PdxCre mice (14) to generate compound heterozygote mice for both Cre strains. Double heterozygote mice were crossed with Vhlfl/fl mice to obtain WT, Vhlfl/fl, Cre, and Cre Vhlfl/fl mice for each Cre strain. Mice lacking Vhl in RIPC Cre-expressing cells were designated βVhKO mice, and those lacking Vhl in PdxCre-expressing cells were designated PVhKO mice. Hif1afl/fl mice (41) were intercrossed with RIPC Cre and compound heterozygote mice mated with Hif1afl/fl mice to obtain mice lacking Hif1α in insulin-producing cells, designated βHif1aKO mice. βHif1aKO mice were intercrossed with RIPC Cre Vhlfl/fl mice and compound heterozygote mice for all alleles were subsequently crossed to generate mice lacking both Vhl and Hif1α in insulin-producing cells, designated βVhβHif1aKO mice. Mice were maintained on a 12-hour light/12-hour dark cycle with free access to water and standard mouse chow (4% fat, RM1; Special Diet Services) and housed in specific pathogen–free barrier facilities. Mice handling and all in vivo studies were performed in accordance with the United Kingdom Home Office Animal Procedures Act of 1986 and with approval of the University College London Animal Ethics Committee. All mice were studied on a mixed 129S/C57BL/6 background with appropriate littermate controls. WT, Cre transgenic, and Vhlfl/fl mice were phenotypically indistinguishable with no differences in glucose tolerance between WT, Cre transgenic, and Vhlfl/fl mice for both βVhKO and PVhKO strains (Supplemental Figure 5, C and D). Balanced numbers of mice of these genotypes were therefore used as controls. All phenotypes described for the mice in Results were present in both male and female animals. Genotyping of the mice was performed by PCR amplification of tail DNA as previously described (11, 12, 14). Cre-mediated excision of Vhl was detected
by PCR on genomic DNA isolated from pancreatic islets and hypothalami. DNA (150 ng) was assayed on the Opticon 2 system (MJ Research) using the primers mVHLPCR5 (5′-CAAATCGCATGCTGTTACCCAC-3′) and mVHLPCR8 (5′-CTGACCTCAGTGGTCTGTCAGC-3′).

**Physiological measurements.** Body and tissue weights were determined using a Sartorius BP610 balance. Blood samples were collected from mice via tail vein bleeds or from cardiac puncture on terminally anesthetized mice. Blood glucose was measured using an Ascensia Elite Glucometer (Bayer Corp.). Islet insulin levels were determined using an ultrasensitive rat insulin ELISA (Crystalzech Inc.) using a mouse standard or with a mouse ELISA (Linco Research). Glucose tolerance tests and GSIS tests were performed on mice after a 16-hour fast as previously described (14). Animals were injected intraperitoneally with 2 g/kg or 3 g/kg D-glucose, and blood glucose or insulin levels, respectively, were determined.

**Immunohistochemistry and morphometric analysis.** Immunohistochemistry for insulin and glucagon and morphometric analysis were performed using methods described previously (12, 14). Antibodies used were as follows: mouse anti-insulin antibody (clone K36aC10; Sigma-Aldrich), rabbit anti-glucagon antibody (Abcam), chicken anti-mouse IgG–Alexa Fluor 488 conjugate, and anti-rabbit IgG–Alexa Fluor 594 (Invitrogen). For immunostaining of Hif-1α (rabbit polyclonal; Novus Inc.), Glut1 (rabbit polyclonal; Alpha Diagnostics Ltd.), Cd31 (rabbit polyclonal; Santa Cruz Biotechnology Inc.), and Glut2 (rabbit polyclonal, gift from B. Thorens, University of Lausanne, Switzerland) in paraffin-embedded sections, antigen retrieval with DAKO target retrieval was performed, and for visualization either a DAKO CSA kit or DAKO Envision kit or anti-rabbit Alexa Fluor 594–conjugated secondary antibodies were used according to the manufacturers’ instructions. For Hif-1α and insulin double-staining, paraffin-embedded sections were first immunostained and visualized for Hif-1α (rabbit polyclonal, Alpha Diagnostics) using the DAKO CSA kit. After visualization, sections were immunostained with mouse anti-insulin antibody (clone K36aC10; Sigma-Aldrich) and Alexa Fluor 488–conjugated chicken anti-mouse IgG. Mounting solution containing DAPI ( Vectashield DAPI, Vector Laboratories) was used to identify nuclei. For detection of apoptosis, rabbit anti-active caspase-3 antibody (BD Biosciences – Pharmingen) was used, and to detect proliferation, rabbit anti-Ki67 antibody (Abcam) was used, both as previously described (14). Sections were imaged via light, fluorescence, or confocal microscopy, and images were captured as previously described (12, 14).

**Western blotting.** Urea-SDS lysis buffer was used to prepare protein lysates, and protein concentrations were measured using BCA ( Pierce Biotechnology). Lysates were run on SDS/PAGE gels and then transferred to Immobilon-P PVDF membranes (Millipore). Membranes were blocked in 5% milk/1% BSA for 45 minutes and primary antibodies applied for 2 hours at room temperature. Membranes were then washed and incubated with an HRP-linked secondary antibody (DAKO) for 1 hour at room temperature. Following further washes, the membrane was developed with ECL plus (Amersham Biosciences). Primary antibodies used were Hif-1α, Glut1, and Glut2 as described above, and α-tubulin (Sigma-Aldrich).

**Islet and Min6 cell experiments.** Mice were sacrificed by cervical dislocation, and the common bile duct was cannulated and its duodenal end occluded by clamping. Liberase solution (2 ml at 0.25 mg/ml in HBSS) was injected into the duct to dissect the pancreas. The pancreas was excised, incubated at 37°C for 15 minutes, and mechanically disrupted in 10 ml of HBSS (supplemented with 1% BSA). Cellular components were collected by centrifugation (201 g for 1 minute), washed, and resuspended in 10 ml of HBSS. Islets were hand-picked under a microscope and washed once in HBSS. Prior to DNA or protein extraction, islets were collected by centrifugation at 5,724 g for 2 minutes and stored at −80°C. For insulin secretion studies, isolated islets were cultured overnight in DMEM with 11 mmol/l glucose. Medium was replaced with KRHB (Kreb’s-Ringer buffer containing 2 mmol/l D-glucose and 10 mmol/1HEPES) 1 hour prior to study. Dynamic insulin release was then assessed using a multichannel perfusion system at 37°C in a temperature-controlled environment, as previously described (42). Insulin was measured in perfusate samples by radioimmunoassay, as previously described (42). Static insulin release was assessed using batch cultures of 5 islets in 150 μl KRHB plus additional D-glucose, 25 mmol/1 KCl, or 10 mmol/l L-ß-ktetraisocaproic acid (Sigma-Aldrich) for 1 hour at 37°C. Insulin release was measured by ELISA. Min6 cells were cultured as previously described (42). To activate Hif-1α, cells were incubated in 1% oxygen or 0.5 mmol/l dimethylglyoxal for 16 hours. For examination of gene expression in Min6 cells, retroviral transduction with active HIF-1α (P042A and P564A) was performed as previously described (19). GSIS from infected Min6 cells was measured by static culture in a 96-well plate (3 × 104 cells per well). Following overnight culture, media was replaced with KRHB for 1 hour before static secretion assays were performed (as described above).

**Gene expression studies.** Isolated islets or Min6 cells were harvested and RNA extracted immediately with RNAse reagent (Biogenesis) or RNeasy kits (Qiagen) according to the manufacturers’ instructions. Total RNA (0.5–2 μg) was reverse transcribed using a first-strand cDNA synthesis kit (Roche). Quantitative PCR was carried out with first-strand cDNA and commercial TaqMan Assays (Applied Biosystems) on the Opticon 2 System (MJ Research Inc.). Expression levels of Glut1, Glut2, Gck, Ecad, Nkx6.1, NeuroD1, Hnf3β, Hnf1α, Hnf4α, Ipk, Aldolase, Gapdh, Pdk1, Vhl, and Cld1 were normalized to hypoxanthine-guanine phosphoribosyl transferase (Hprt), and data were analyzed using the 2−ΔΔCt method (12). Details of the primers used are presented in Supplemental Methods.

**Imaging studies in isolated islets.** Islets isolated as described above were dissociated into single cells by incubation in trypsin at 21°C for 2.5 min with gentle agitation. Cells were washed once before being allowed to attach to poly-Llysine–treated glass coverslips. Cells were incubated for 48 hours in DMEM culture medium with 1-glutamine supplemented with 1% heat-inactivated FBS and 5.5 mmol/l D-glucose. The medium was changed to HBSS supplemented with 20 mmol/l HEPES and 2 mmol/l D-glucose 1 hour prior to imaging. Cells were imaged on a 37°C heated stage on a Zeiss LSM 510 confocal microscope. β cells were stained following analysis, using dithizone for identification (43). Uptake of the fluorescent glucose analogue 2-NBDG (Invitrogen) was measured by addition of 18 mmol/l 2-NBDG in the presence of 2.5 mmol/l D-glucose during continuous imaging with an excitation wavelength of 458 nm and collection of light emitted at greater than 505 nm (20). Reduced NADH and oxidized flavoprotein (FAD)′ autofluorescence in response to the addition of D-glucose in 9 mmol/l increments were captured as previously described (21, 44). The mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenylla (FCCP) was used to artificially evoke maximally oxidized states of the NADH/flavoproteins to establish the dynamic range of the signals. Ca2+ imaging using Fura-2AM was performed as previously described (21, 44). Measurements were obtained by averaging the signal collected in a region of interest drawn across the maximum area of the β cell. Mono-methyl succinate (Fluka) and tolbutamide (Sigma-Aldrich) were used to stimulate Ca2+ influx via mitochondrial metabolism and pharmacological KATP channel closure respectively.

**Statistics.** Data are presented as mean ± SEM, unless otherwise indicated. All statistics were performed using Minitab (version 13) and GraphPad ( Prism 4) software. Paired and unpaired t tests and 1- and 2-way ANOVA were performed, using the post-hoc Newman-Keuls test where appropriate. P < 0.05 was regarded as statistically significant.

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