A Role for von Hippel-Lindau Protein in Pancreatic β-Cell Function

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OBJECTIVE—The *Vhlh* gene codes for the von Hippel-Lindau protein (VHL), a tumor suppressor that is a key player in the cellular response to oxygen sensing. In humans, a germline mutation in the *VHL* gene leads to the von Hippel-Lindau disease, a familial syndrome characterized by benign and malignant tumors of the kidney, central nervous system, and pancreas.

RESEARCH DESIGN AND METHODS—We use *Cre-lox* recombination to eliminate *Vhlh* in adult mouse pancreatic β-cells. Morphology of mutant islets is assessed by immunofluorescence analysis. To determine the functional state of $Vhlh^{-/-}$ islets, insulin secretion is measured in vivo and in vitro, and quantitative PCR is used to identify changes in gene expression.

RESULTS—Loss of VHL in β-cells leads to a severe glucose-intolerant phenotype in adult animals. Although VHL is not required for β-cell specification and development, it is critical for β-cell function. Insulin production is normal in β-cells lacking VHL; however, insulin secretion in the presence of high concentrations of glucose is impaired. Furthermore, the loss of VHL leads to dysregulation of glycolytic enzymes, pointing to a perturbation of the intracellular energy homeostasis.

CONCLUSIONS—We show that loss of VHL in β-cells leads to defects in glucose homeostasis, indicating an important and previously unappreciated role for VHL in β-cell function. We believe that the β-cell–specific *Vhlh*-deficient mice might be a useful tool as a "genetic hypoxia" model, to unravel the possible link between hypoxia signaling and impairment of β-cell function. *Diabetes* **58:433–441, 2009**

Oxygen homeostasis is essential to cellular function, and low O_2 pressure (hypoxia) has a profound impact on cell metabolism and physiological processes. The von Hippel-Lindau tumor suppressor protein (VHL) is a key player in the cellular response to oxygen sensing. Hypoxia inducible factors (HIFs) are global regulators of oxygen homeostasis, allowing cellular adaptation to oxygen deprivation by transcriptionally modulating genes involved in cellular energy metabolism, angiogenesis, apoptosis, and proliferation, among other biological processes (rev. in 1–5).

During normoxia, prolyl hydroxylases hydroxylate specific residues on HIF-α, a modification that is oxygen dependent (6,7). VHL specifically recognizes these modified residues, targeting HIF-α for ubiquitination via E3 ubiquitin ligase and subsequent proteasomal degradation (8,9). Under hypoxic conditions, HIF-α is stabilized and forms a complex with the β-subunit of a transcriptional complex (HIF-1β, also called aryl hydrocarbon receptor nuclear translocator, or ARNT) that then translocates to the nucleus to modulate expression of downstream targets, including genes involved in oxygen uptake and glucose metabolism. In the absence of VHL, HIF-α–dependent genes are inappropriately upregulated in spite of normal oxygen levels.

In humans, a germline mutation in the *VHL* gene leads to the development of the von Hippel-Lindau disease, a rare familial syndrome characterized by benign and malignant tumors of several organs, including the kidney, central nervous system, and pancreas (10). In mice, *Vhlh* (the murine homolog of *VHL*) inactivation results in midgestation lethality (11). The use of conditional alleles has thus proven a powerful approach to investigate the role of this protein in specific organ development and function (12). Conditional inactivation of *Vhlh* has been successfully achieved in several organs including kidney, liver, and bone (13–15). These studies have demonstrated that VHL plays a fundamental role in survival, proliferation, and differentiation of many cell types. Specific inactivation of *Vhlh* in kidney cells results in the development of blood-filled cavities that are reminiscent of the hemangioblastomas typically seen in human patients (13). Specific depletion of *Vhlh* in the liver leads to steatosis within the liver, presumably due to alterations in the glycolytic machinery within the organ (14,16).

Another hallmark of human VHL patients is the occurrence of cysts and tumors, including neuro-endocrine tumors, in the pancreas (17,18). To elucidate the role of VHL in pancreatic β-cells, we have eliminated the gene via *Cre-lox* technology. Specific inactivation of *Vhlh* in β-cells results in glucose intolerance in mice. This defect appears to be a consequence of a significant impairment in glucose-stimulated insulin secretion in β-cells that lack VHL. Further analysis has revealed a profound change in the expression pattern of genes coding for glycolytic enzymes that regulate the metabolic state of the β-cells. Thus, our data reveal a previously unappreciated role for VHL in maintaining a functional state in pancreatic β-cells.

RESEARCH DESIGN AND METHODS

Transgenic mouse handling. Mice used in these studies were maintained in the barrier facility according to protocols approved by the Committee on Animal Research at the University of California, San Francisco. *Ins-Cre* and *Pdx-1-Cre*^{ER} (tamoxifen-inducible) mice were obtained from Drs. Pedro Herrera's and Doug Melton's laboratories, and the *Vhlh*$^{loxP/loxP}$ mice have been described previously (14,19,20).

To activate *Cre*^{ER} in pancreatic β-cells, tamoxifen (TAM; Sigma-Aldrich, St.

Louis, MO) dissolved in corn oil (10 mg/ml) was administered intraperitoneally at 1 mg · mouse^{-1} · day^{-1} for 5 continual days.

For DNA genotyping, islet DNA was collected in 1 ml AT-Extraction solution (0.067N ammonium hydroxide, 0.2% Triton X-100) and sonicated for 10 s in ice water. Genomic DNA was concentrated by ethanol precipitation and used for PCR as previously described (19–21).

Histology and immunofluorescence analysis. For paraffin sections, isolated pancreata from adult mice were fixed in 4% (wt/vol) paraformaldehyde in PBS for 4 h to overnight at 4°C. For frozen sections, adult tissue was fixed in 4% (wt/vol) paraformaldehyde for 2 h at room temperature and incubated overnight in 30% (wt/vol) sucrose in PBS. The following day, tissues were frozen in optical cutting temperature cryoembedding media (OCT) after two washes in PBS and stored at −80°C. Hematoxylin/eosin staining and immunofluorescence analyses were performed as described previously (22). The following primary antibodies were used: guinea pig anti-insulin, 1:300; rabbit anti-glucagon, 1:300 (Linco Research, St. Charles, MO); rabbit anti-Glut2, 1:500 (Chemicon, Temecula, CA); mouse anti-Pax6, 1:25 (Developmental Studies Hybridoma Bank, Iowa City, IA); rabbit anti-somatostatin, 1:200 (Dako, Carpentaria, CA); rat anti-CD31, 1:100 (Pharmingen, San Diego, CA); and rabbit anti-Nkx6.1, 1:1,000 (23). Primary antibodies were detected with FITC-conjugated (1:200) and Cy3-conjugated (1:600) secondary antibodies (Jackson ImmunoResearch Laboratory, West Grove, PA).

Bright field images were acquired using a Zeiss Axio Imager D1 microscope. Fluorescence was visualized and photographed with a Zeiss Axiphoto2 plus microscope. Unless otherwise noted, all photomicrographs shown are representative of at least three independent samples of the indicated genotype.

Quantitative PCR. RNA isolation, cDNA preparation, and qPCR were performed as described previously (24). RNA expression of target genes was normalized based on comparison to cyclophilin expression. Primer sequences are available on request.

Intraperitoneal glucose tolerance test and acute insulin secretory response in vivo. After a 16- to 18-h fast, mice were weighed and fasting blood glucose level was measured using the Lifescan Glucometer. Mice were injected intraperitoneally with a 1 mol/l glucose solution at 10 μl per gram body weight. Blood glucose levels were then measured every 30 min for 2 h after injection. For in vivo insulin measurement, blood was collected from the tail vein before and 30 min after glucose injection. Serum containing protease inhibitors (Roche, Indianapolis, IN) was stored at −80°C. Insulin concentration was calculated using the Insulin EIA kit (ALPCO) as per the manufacturer's instructions.

Islet isolation and in vitro insulin secretion. The Islet Production Facility Core at the Diabetes Center at University of California–San Francisco (UCSF) isolated islets from adult mice. Glucose-stimulated insulin secretion in isolated islets was performed as previously described (25). To quantify the secretory response in the presence of K$^+$, 40 mmol/l KCl was added to the high glucose solution before incubation with the islets. For total insulin content of islets, insulin was extracted overnight at 4°C with acid/ethanol (1.5% HCl/75% ethanol) (300 μl). After centrifugation at 1,000g for 3 min, the supernatant was collected and frozen at −20°C for insulin determination as described above.

Insulin tolerance test. After a 16- to 18-h fast, mice were weighed and blood glucose was measured. Mice were injected intraperitoneally with a dose of 1 unit/kg of body weight. Blood glucose levels were measured every 30 min for 2 h after injection.

Islet lactate secretion assay. Islets isolated from control or *Pdx-1–Cre^{ER}*; *Vhlh$^{LoxP/LoxP}$* animals were incubated overnight in 200 μl growth medium with 16.7 mmol/l glucose. After the incubation, 20 μl supernatant was used in duplicate to measure lactate using a Lactate Assay Kit (Eton Biosciences, San Diego, CA). The islets were processed for RNA isolation using the RNeasy Mini Kit (Qiagen Sciences, MD). The amount of lactate was normalized to total RNA.

Islet area quantification. Paraffin-embedded tissue was sectioned 100 μm apart to exclude overlapping islets. Immunohistochemistry was performed using anti-insulin antibody and counterstaining with hematoxylin. Using Adobe Photoshop, the area of insulin-positive islets and total pancreatic tissue for every section was measured. β-Cell mass was calculated as a product of total pancreatic weight and β-cell area.

RESULTS

VHL inactivation in adult β-cells leads to glucose intolerance in mice. Homozygous deletion of *Vhlh* (the murine homolog of VHL) results in embryonic lethality due to defects in placental vasculogenesis, thus precluding the analysis of adult tissues in VHL-deficient mice (11). To investigate the role of VHL in adult β-cell function, we

specifically eliminated the *Vhlh* gene in β-cells via *Cre/loxP* recombination. The conditional allele of *Vhlh* (*Vhlh$^{LoxP/LoxP}$*) provides the capability to knockout the VHL protein in a tissue-specific manner. *Cre*-mediated excision of the floxed allele deletes the promoter and the first exon of the gene, resulting in a null allele. *Vhlh$^{loxP/loxP}$* mice have been used previously to successfully analyze the role of VHL in other organs such as liver and kidney (13,14). To specifically inactivate VHL function in β-cells, *Vhlh$^{loxP/loxP}$* mice were crossed with a transgenic mouse line that expresses the Cre recombinase under the control of the pancreatic and duodenal homeobox gene 1 (*Pdx-1*) promoter (*Pdx-1-Cre^{ER}*) (19). Although *Pdx-1* is broadly expressed during development, it becomes restricted to insulin producing β-cells in animals around 4–6 weeks of age. The *Pdx-1-Cre^{ER}* transgenic mouse line carries an altered form of the Cre recombinase that is fused to the estrogen receptor, rendering the protein inactive in the cytoplasm in the absence of the tamoxifen ligand. Double mutant transgenic mice carrying *Pdx-1-Cre^{ER}* and *Vhlh$^{LoxP/LoxP}$* allow for appropriate expression of *Vhlh* in the absence of tamoxifen treatment, thereby ensuring normal development. In *Pdx-1-Cre^{ER}*;*Vhlh$^{LoxP/LoxP}$* adults, exposure to tamoxifen leads to nuclear translocation of the Cre recombinase, allowing irreversible inactivation of *Vhlh* at a desired time.

As anticipated, *Pdx-1-Cre^{ER}*;*Vhlh$^{LoxP/LoxP}$* mutant mice were born in the expected Mendelian ratio and reached adulthood without any sign of compromised health. Administering tamoxifen to 8- to 10-week-old mice induced Cre expression in adult islets. Blood glucose concentration was monitored weekly after tamoxifen treatment. *Pdx-1-Cre^{ER}*;*Vhlh$^{LoxP/LoxP}$* mice treated with tamoxifen displayed normal fed and fasting blood glucose levels. To more vigorously test β-cell function, the response of these mice to a glucose challenge was assessed. In the absence of *Cre* expression, all mice (including *Pdx-1-Cre^{ER}*; *Vhlh$^{LoxP/LoxP}$*, *Pdx-1-Cre^{ER}*;*Vhlh$^{+/LoxP}$*, and *Vhlh$^{LoxP/LoxP}$*) are normoglycemic (Fig. 1A). However, the ability of tamoxifen-treated *Pdx-1-Cre^{ER}*;*Vhlh$^{LoxP/LoxP}$* mutant mice to recover after the glucose challenge is impaired (Fig. 1B). Tamoxifen-treated *Pdx-1-Cre^{ER}*;*Vhlh$^{LoxP/LoxP}$* mutant mice do not reach normoglycemia until 3 h after the glucose challenge (data not shown). Interestingly, the glucose-intolerant phenotype develops 6–8 weeks after the start of the tamoxifen regimen. Thus, VHL function in the adult β-cell affects glucose homeostasis within the mouse.

Successful excision of the *Vhlh* floxed allele was demonstrated by PCR on genomic DNA isolated from islets. Distinct sets of primers allow for detection of the genotype (Fig. 1C, *top panel*) and the extent of excision (Fig. 1C, *bottom panel*) in islets. Both mice were injected with tamoxifen and display a robust signal for amplification of the excised allele. To correlate the severity of the phenotypes observed with the efficiency of Cre-mediated recombination of the floxed alleles, we also performed an expression analysis of Cre activity in *Pdx-1-Cre^{ER}*; *Rosa26R* transgenic mice. Histological analysis of pancreatic tissue from mice that underwent tamoxifen treatment reveals ∼80–90% excision (supplementary Fig. 1, found in an online appendix at http://dx.doi.org/10.2337/db08-0749; data not shown).

To further confirm the role of VHL in β-cell function, specific deletion of *Vhlh* in β-cells during embryogenesis was achieved using a transgenic mouse line expressing
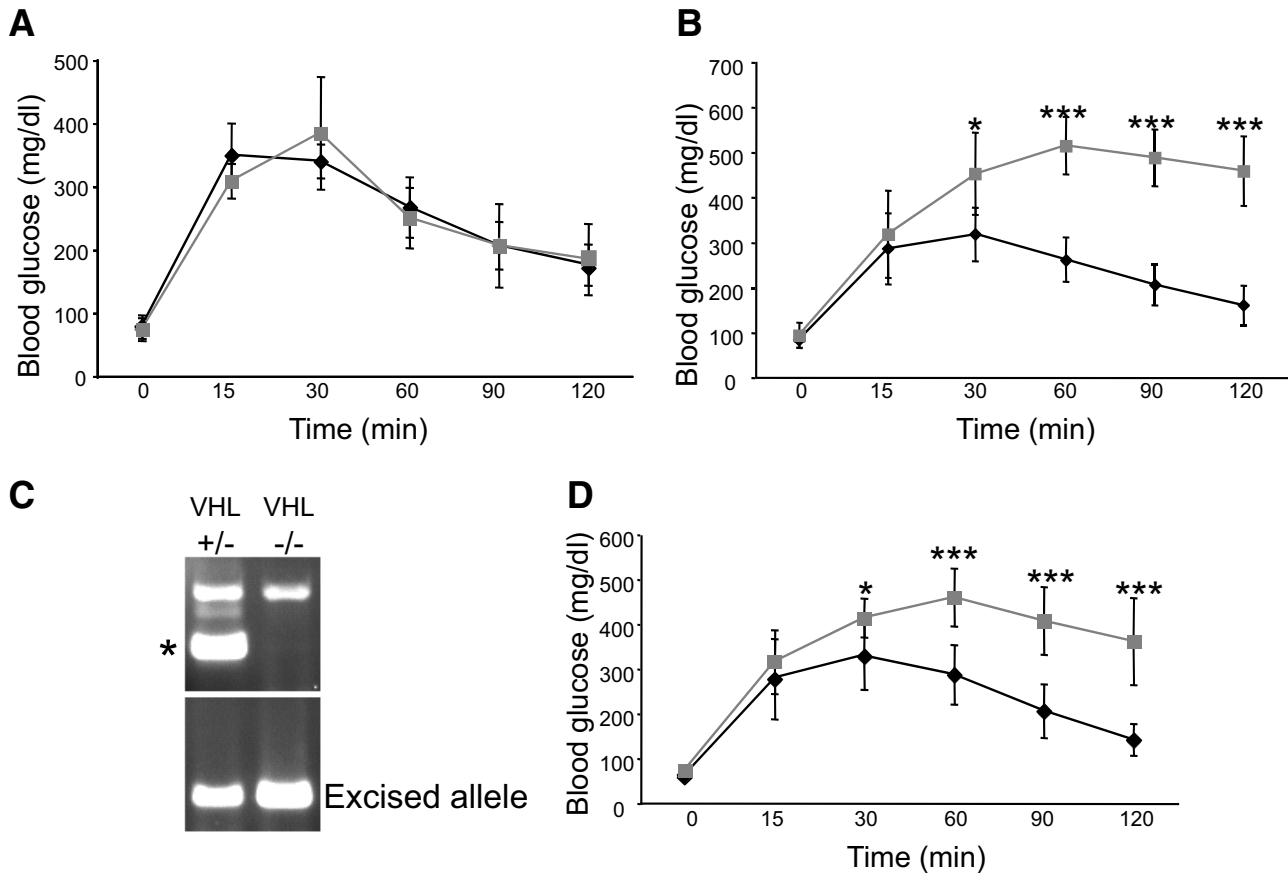
FIG. 1. VHL inactivation in β-cells leads to glucose intolerance. *A*: In the absence of tamoxifen, control (black line, *Pdx-1-Cre^{ER};VHL$^{+/LoxP}$*, *n* = 6) and *Pdx-1-Cre^{ER};VHL$^{LoxP/LoxP}$*(gray line, *n* = 3) mice normalize blood glucose 120 min after challenge. *B*: At 120 min after glucose challenge, *Pdx-1-Cre^{ER};VHL$^{LoxP/LoxP}$* mice (gray line) injected with tamoxifen have elevated glucose levels compared with control mice (black line). Eleven animals were analyzed per group from eight independent cohorts. *P < 0.001, ***P < 10^{-8}. *C*: PCR on genomic DNA from transgenic islets heterozygous and homozygous for the *VHL$^{LoxP/LoxP}$* allele demonstrates the extent of Cre-mediated excision. Both samples are positive for *Pdx-1-Cre^{ER}*. *Top panel*: The genotype of the islets. The wild-type allele in the heterozygous sample (*) runs below the floxed allele. Both mice were injected with tamoxifen and display a robust signal for the excised allele (*lower panel*). *D*: Adult *Ins-Cre;VHL$^{LoxP/LoxP}$* mice are glucose intolerant. Two hours after the glucose challenge, the 8- to 10-week-old *Ins-Cre;VHL$^{LoxP/LoxP}$* mice (gray line, *n* = 14) have elevated glucose levels compared with the control littermates (black line, *n* = 11). Control and mutant animals from nine independent cohorts were used for the analysis. *P < 0.001, ***P < 10^{-8}. Error bars represent SD in all cases.

Cre under control of the insulin promoter (*Ins-Cre*) (20). *Ins-Cre;Vhlh$^{LoxP/LoxP}$* mice develop to adulthood, with no overt defects. However, upon glucose challenge, mice with no functional VHL in β-cells are unable to normalize their blood glucose levels, revealing a glucose-intolerant phenotype (Fig. 1*D*). Thus, inactivation of VHL in β-cells either early in development (*Ins-Cre;Vhlh$^{LoxP/LoxP}$* mice) or after β-cells have acquired maturity (*Pdx-1-Cre^{ER};Vhlh$^{LoxP/LoxP}$*) points to a role for VHL in β-cell function.

Increased vasculature in islets of *Pdx-1-Cre^{ER}; Vhlh$^{LoxP/LoxP}$* mice. The defect in glucose homeostasis that results from the inactivation of *Vhlh* in β-cells raises the question of whether islet formation and/or architecture could be altered in tamoxifen-treated *Pdx-1-Cre^{ER}; Vhlh$^{LoxP/LoxP}$* mutant mice. Histological analysis of pancreatic tissue from tamoxifen-treated *Pdx-1-Cre^{ER};Vhlh$^{LoxP/LoxP}$* mutant mice does not reveal any morphological changes compared with control tissue (Fig. 2*A* and *B*). A hallmark of the VHL syndrome is increased vasculature within tumors, which correlates with a HIF-1α–mediated increase in expression of vascular endothelial growth factor (*Vegf*) (10). In agreement with this, islets in tamoxifen-treated *Pdx-1-Cre^{ER};Vhlh$^{LoxP/LoxP}$* mutant mice display a dramatic increase in blood vessels, as marked by PECAM-1/CD31 immunostaining (Fig. 2*C–H*). The increase in *Vegf* expres-

sion was confirmed by quantitative real-time PCR analysis (Fig. 2*I*). Immunostaining analysis for the hormones insulin, glucagon, and somatostatin reveal that the overall architecture of the islets is maintained in tamoxifen-treated *Pdx-1-Cre^{ER};Vhlh$^{LoxP/LoxP}$* mutant mice (Fig. 3*A–D*). The increased vasculature may explain the apparent perturbation of islet morphology in tissue lacking functional VHL, observed as gaps or holes when stained for hormones alone (Fig. 3*B* and *D*). In addition, expression of mature endocrine markers including Nkx6.1 and Pax6 (26) appears normal (Fig. 3*E–H*). β-Cell mass quantification in *Ins-Cre;Vhlh$^{LoxP/LoxP}$* pancreas revealed no difference between the control and mutant groups (supplementary Fig. 3). Thus, VHL is not essential for islet formation and β-cell differentiation.

Impaired insulin secretion in β-cells lacking VHL. To determine whether the lack of recovery to normoglycemia after glucose challenge in *Vhlh* mutant mice is due to defective insulin secretion, blood serum from resting and challenged mice was collected for insulin quantification (Fig. 4*A* and supplementary Fig. 2). Control mice (either without the *Pdx-1-Cre^{ER}* transgene or harboring the transgene but not injected with tamoxifen) display elevated insulin levels in the serum 30 min after glucose challenge. Strikingly, a significant decrease in circulating insulin is
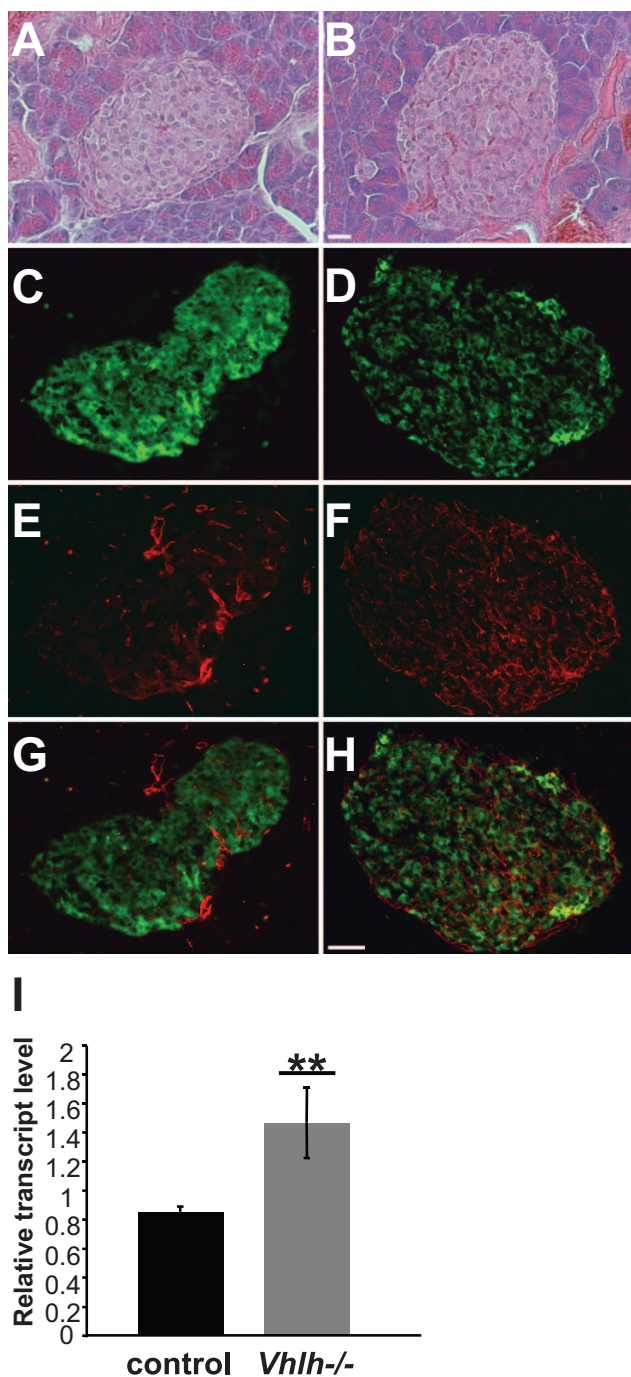
FIG. 2. Islet morphology upon VHL inactivation in adult β-cells. Hematoxylin and eosin (H&E) analysis of control (*A*) and *Vhlh*⁻/⁻ (*B*) tissue shows aberrant islet architecture due to increased blood vessel density. Bar, 20 μm. Immunostaining for insulin (*C* and *D*) and PECAM-1 (*E* and *F*) reveals that islet morphology in tamoxifen-injected *Pdx-1-Cre^ER;VHL^LoxP/LoxP* mice (*D*, *F*, and *H*) displays increased vasculature compared with the control mice (*C*, *E*, and *G*). The overlay images are shown in *G* and *H*. Bar, 50 μm. *I*: Real-time PCR analysis on islets isolated from tamoxifen-injected *Pdx-1-Cre^ER*; *VHL^LoxP/LoxP* mice shows an increase in *Vegf* expression (error bars represent SD, *n* = 5. **P < 0.005). (Please see http://dx.doi.org/10.2337/db08-0749 for a high-quality digital representation of this image.)
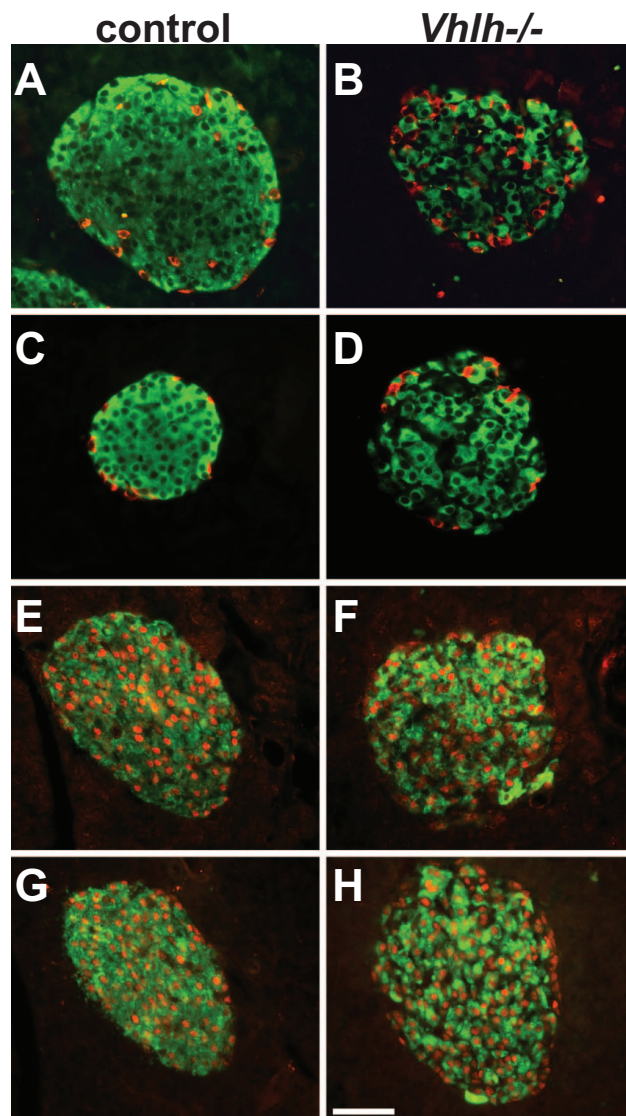

FIG. 3. VHL is not essential for islet formation and β-cell differentiation. Adult mice lacking VHL in β-cells maintain islet architecture. Insulin-expressing (green, *A–H*), glucagon-expressing (red, *A* and *B*), and somatostatin-expressing (red, *C* and *D*) cells are found in the control (*A* and *C*) and mutant tissue (*B* and *D*). Mature β-cell markers Nkx6.1 (red, *E* and *F*) and Pax6 (red, *G* and *H*) continue to be expressed in the absence of VHL. Bar, 50 μm. (Please see http://dx.doi.org/10.2337/db08-0749 for a high-quality digital representation of this image.)

observed in tamoxifen-treated *Pdx-1-Cre^ER*;*Vhlh^LoxP/LoxP* mutant mice after a 30-min glucose challenge. To determine the basis of the defect in glucose-stimulated insulin secretion in *Vhlh*-deficient mice, insulin secretion assays were carried out on islets isolated from control and mutant (tamoxifen-injected *Pdx-1-Cre^ER*;*VHL^LoxP/LoxP*)

mice. Resting insulin levels for control and mutant mice are comparable (Fig. 4*B* and *C*). However, the insulin secretory response to glucose is markedly reduced in islets with β-cells lacking VHL (Fig. 4*B* and *C*). To determine whether β-cells were primed for secretion, control and mutant islets were incubated with a nonglucose secretagogue, KCl. In the presence of KCl, mutant islets secrete insulin (Fig. 4*C*), indicating that β-cells lacking VHL possess a functional secretion machinery. Summarily, these results show that loss of VHL in β-cells leads to a severe inability to secrete insulin in response to increased glucose concentration.

Two likely explanations for the decrease in glucose-stimulated insulin secretion could be envisioned—either insulin production is reduced, or insulin secretion is affected. By immunohistochemistry, insulin levels in the mutant islets do not appear significantly diminished (Fig.
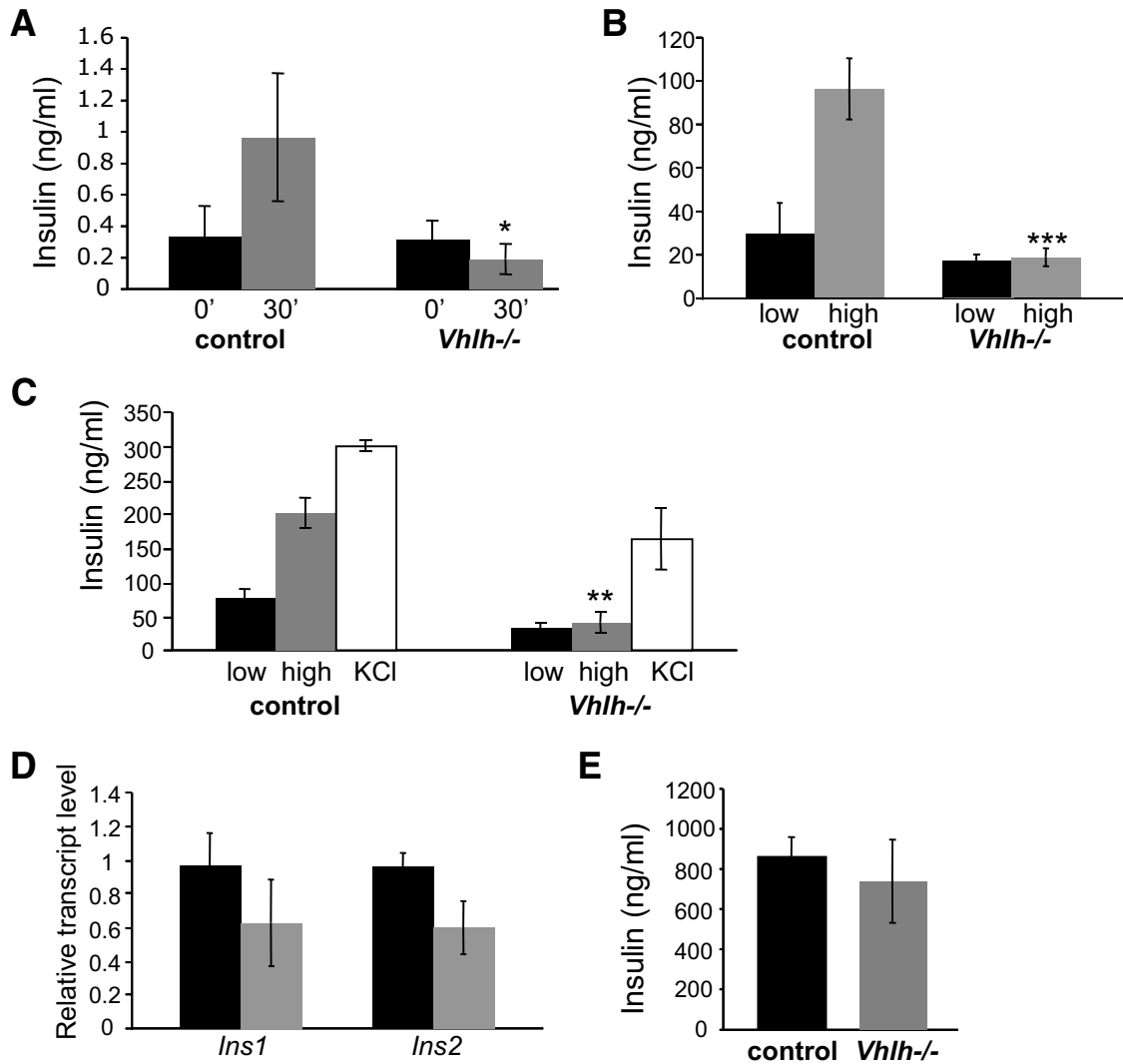
FIG. 4. VHL inactivation in adult β-cells leads to a block in insulin secretion. *A*: Serum insulin measurements 30 min after glucose challenge show an absence of circulating insulin in tamoxifen-injected *Pdx-1-Cre^ER;VHL^LoxP/LoxP* mutant mice compared with control littermates. Four animals per group were included in the analysis from four independent cohorts. *P < 0.05. *B*: In vitro insulin secretion assay carried out on isolated islets demonstrates a block in insulin secretion upon incubation with high (16.67 mmol/l) glucose in the absence of VHL. Basal secretion (low glucose, 1.67 mmol/l) was comparable between control and mutant islets. n = 3. ***P < 0.0005. *C*: Incubation of mutant islets with 40 mmol/l KCl in the presence of high (16.67 mmol/l) glucose induces insulin secretion in vitro. n = 3. **P < 0.001. Significance (*A–C*) was determined between the amount of insulin secreted under high glucose conditions from control and mutant islets using the Student's *t* test. *D*: Real-time PCR of insulin transcript levels shows a moderate decrease of insulin in mutant animals (gray bars) when compared with the control animals (black bars) (*Ins1*, P > 0.1; *Ins2*, P > 0.01). n = 3. *E*: Total insulin levels were calculated in islets isolated from control (black bar) or mutant animals (gray bar). P > 0.1, n = 3. Error bars represent SD in all cases.

3). By quantitative PCR analysis, there is a mild reduction in insulin transcript levels, although it is not statistically significant (Fig. 4*D*). This is reflected in the minor reduction of total insulin protein observed in islets of *Vhlh*-deficient mice (Fig. 4*E*). It appears unlikely that the modest decrease in insulin level could account for the complete lack of secretion upon glucose stimulation observed in *Vhlh*-deficient mice. Thus, we decided to identify additional changes in β-cells that might contribute to the defect in insulin secretion.

Modulation of glycolytic metabolism in the absence of VHL. In human cancers and mouse models, a loss of VHL is accompanied by an increase in the expression of several genes related to glucose metabolism (1,3). These genes include glucose transporters and enzymes of the glycolytic pathway. This coordinated increase in gene expression results in a switch from oxidative phosphorylation to glycolytic metabolism within cells. Such a dra-

matic shift in glucose metabolism presumably allows cells to maintain energy homeostasis and reduce the buildup of reactive oxygen species (1). Perturbation of glucose metabolism in β-cells has been linked to defects in insulin secretion (27–29). Therefore, we decided to test whether the expression of genes involved in glucose metabolism is affected in *Vhlh*-deficient mice. Gene expression of several enzymes involved in glucose metabolism, as assessed by quantitative PCR analysis is significantly increased in islets lacking VHL (Fig. 5*A*). These include glyceraldehyde-3-phosphaste-dehydrogenase (*Gapdh*), phosphoglyceratekinase (*Pgk*), phosphofructokinase (*Pfk*), glucose phosphate isomerase (*Gpi*), aldolase A (*AldoA*), and phosphoglucomutase (*Pgm2*) (Fig. 5*A*). Inactivation of *Vhlh* has also been shown to affect the expression of glucose transporters GLUT1 and GLUT2 (14,16). In agreement with these reports, we observe an increase in *Glut-1* expression in *Vhlh*-deficient islets (Fig. 5*A*). Interestingly, GLUT2, the
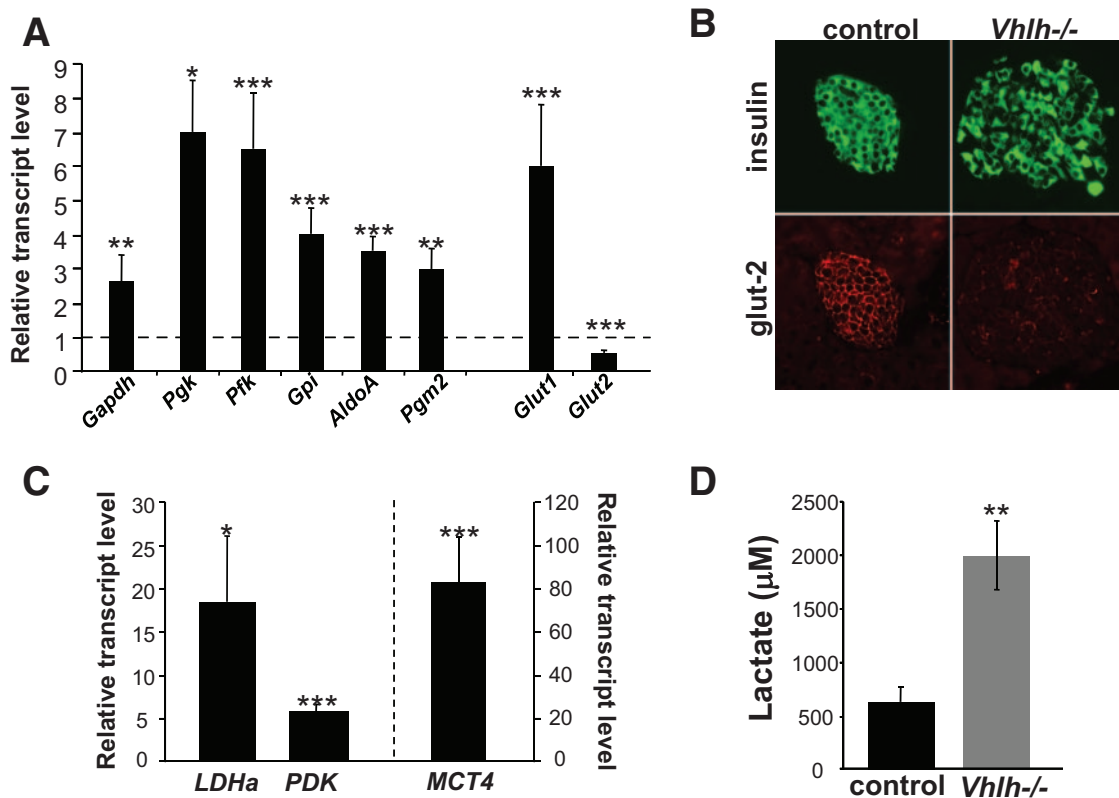
FIG. 5. Glucose metabolism genes dysregulated in islets upon VHL inactivation. *A*: Quantitative PCR analysis of relative transcript levels of glycolytic enzymes reveals an upregulation of several members of the metabolic machinery in the absence of VHL. The housekeeping gene cyclophilin A was used to normalize gene expression. Changes in glucose transporters were also observed. The canonical HIF target gene *Glut-1* is upregulated, whereas *Glut-2* is downregulated. The change in expression of genes in *Vhlh*$^{-/-}$ islets was normalized to expression in control animals that was set to one (represented by the dotted line). Between three and five mutant and control animals were used for isolation of islets for RNA. *B*: Immunostaining for GLUT2 confirms significant downregulation of the transporter in tamoxifen-injected *Pdx-1-Cre*^{ER};*VHL*$^{LoxP/LoxP}$ islets. *C*: Increased expression of genes regulating the formation and export of lactate in islets. Transcript levels of lactate dehydrogenase (*Ldha*), pyruvate dehydrogenase kinase (*PDK*), and the monocarboxylate transporter *MCT4* are dramatically increased in *Vhlh*$^{-/-}$ islets, as detected by quantitative PCR. *D*: β-Cells lacking VHL secrete an increased amount of lactate into the surrounding medium (gray bar graph) compared with control islets (black bar graph). Islets isolated from three animals per group from three independent cohorts were included in the analysis. *P < 0.05, **P < 0.01, ***P < 0.005. Error bars represent SD in all cases. (Please see http://dx.doi.org/10.2337/db08-0749 for a high-quality digital representation of this image.)

transporter that enables facilitative transport of glucose into β-cells, is downregulated in *Vhlh*-deficient islets (Fig. 5*A*). Immunohistochemical analysis confirms the dramatic reduction of GLUT2 (Fig. 5*B*). Thus, loss of VHL in β-cells appears to disturb the cellular machinery for glucose sensing and metabolism.

Gene expression assessment by quantitative PCR also revealed a significant increase in lactate dehydrogenase (*LDHa*, Fig. 5*C*) and pyruvate dehydrogenase kinase (*PDK*, Fig. 5*C*). *LDHa* catalyzes the conversion of pyruvate to lactate and is maintained at low levels in islets during homeostasis (30), presumably to ensure maximal amounts of pyruvate being shunted toward oxidative phosphorylation. PDK is an inhibitor of pyruvate dehydrogenase, which normally catalyzes the conversion of pyruvate to acetyl CoA for entry into the tricarboxylic acid cycle. Upregulation of PDK leads to a decrease in pyruvate dehydrogenase activity, consequently decreasing oxidation of pyruvate in the mitochondria and increasing the pyruvate-to-lactate conversion in the cytosol. Indeed, VHL mutant islets show a drastic increase in the expression of *MCT4* (Fig. 5*C*), a monocarboxylate transporter that mediates efflux of lactate from glycolytically active cells (31). Quantitative PCR analysis indicates that loss of *Vhlh* in β-cells results in increased glycolytic metabolism. In agreement with these results, we detected increased lac-

tate in the growth medium upon culturing mutant islets (Fig. 5*D*). Thus, given that *LDHa*, *PDK*, and *MCT4* are all downstream targets of HIFα proteins, the concerted increased activity of these genes points to a modification of intracellular respiration that may lead to a block in glucose-stimulated insulin secretion.

DISCUSSION

The von Hippel-Lindau tumor suppressor gene product (VHL) is an essential component of the cellular response to hypoxia (8,9). To gain insight into the role of VHL in β-cell formation and function, we have specifically inactivated *Vhlh* in β-cells by *Cre-loxP* mutagenesis. Elimination of *Vhlh* in embryonic and mature β-cells reveals that VHL is dispensable for β-cell formation and differentiation. However, loss of VHL has profound effects on β-cell function, demonstrating a novel role for VHL in maintaining glucose homeostasis.

Mice lacking *Vhlh* in adult β-cells develop severe glucose intolerance. The phenotype onset occurs with a slight delay that is not completely understood, and future work could address whether slow turnover of the existing stores of VHL might provide an explanation. Our results show that total insulin protein content in *Vhlh*-deficient islets is not significantly reduced. These results point to a specific

defect in insulin secretion as the underlying cause of the glucose intolerance phenotype. Basal unstimulated insulin secretion in *Vhlh*-deficient mice parallels that of control littermates. In fact, when challenged with high glucose, mice lacking VHL in β-cells normalize blood glucose ~3 h after the control group, an effect that might be explained by basal insulin secretion. In vitro, *Vhlh*-deficient islets fail to secrete elevated levels of insulin when incubated with high glucose, uncovering a role for VHL in regulating the metabolic response. The ability of *Vhlh*-deficient islets to secrete insulin in the presence of KCl indicates the presence of a competent secretory system, placing the defect further upstream. As absolute levels of insulin secreted in the presence of KCl are lower in *Vhlh*-deficient islets, a defect in the exocytotic machinery cannot be ruled out. It is, however, important to note that the relative increase in insulin secretion in the presence of KCl compared with basal levels is similar between control and mutant mice.

These results raise the question of how the loss of VHL affects insulin secretion. Previous work has demonstrated that hypoxia impairs insulin secretion (32–34). VHL inactivation, via HIF-1α stabilization, mimics certain aspects of the cellular response to hypoxia, and canonical target genes of the HIF complex, including *Vegf* and *Glut1*, are significantly upregulated in VHL-depleted islets. Similar to our in vivo studies, in vitro experiments performed on isolated islets have shown that hypoxic conditions lead to an almost complete block in glucose-stimulated insulin secretion, while basal secretion is only slightly reduced (32,34). While the mechanism by which hypoxia impairs insulin secretion is not completely understood, it is generally believed to involve the depletion of energy stores in β-cells. During hypoxia, and through HIF-1α function, a switch from oxidative phosphorylation to aerobic glycolysis occurs, resulting in impaired ATP production (1). The ATP-to-ADP ratio has been proposed as a major regulator of insulin secretion (rev. in 29). Defects in ATP production have been linked to impairment of insulin secretion in response to glucose (27,33,35). Furthermore, mitochondrial mutations that result in decreased ATP production have been linked to diabetes in humans (36–38).

Both hypoxia and loss of VHL induce the expression of genes related to the glycolytic pathway that could lead to decreased ATP production. We observe upregulation of several genes that indicate a switch from oxidative phosphorylation to glycolytic metabolism in *Vhlh*-deficient islets. Among other genes, increased expression of *Gapdh*, *Pfk*, and *Pgm2* is observed. Further evidence of a shift away from oxidative phosphorylation as the primary energy source in *Vhlh*-deficient islets was the dramatic increase in expression of genes involved in lactate formation (*LDHa*), regulation of lactate formation (*PDK*), and lactate secretion (*MCT4*) as well as increased lactate in the culture medium. Overexpression of *LDHa* has been shown to attenuate glucose-induced insulin secretion in the mature β-cell line MIN6 (39). Together, these observations point to defects in glucose metabolism as a possible cause for β-cell dysfunction in *Vhlh*-deficient mice. The VHL–HIF-1α axis controls the expression of a large number of genes. We cannot formally exclude that alterations in other genes might influence β-cell function. Indeed, expression of the glucose transporter, *Glut-2*, is reduced in *Vhlh*-deficient islets. Decreased expression of *Glut-2* has been reported in several animal models of diabetes

(40–42). In vitro, islets of *Glut-2*–deficient mice display impaired glucose-stimulated insulin secretion (43). Therefore, defective islet glucose uptake could play a role in β-cell impairment in *Vhlh*-deficient mice. However, a reduction but not elimination of *Glut-2* expression (Fig. 5*B*) might not hinder glucose uptake below the K_m threshold for glucokinase. Under these circumstances, the contribution of *Glut-2* in the development of the observed phenotype might be minor. Further analysis will elucidate the precise consequence of decreased levels of *Glut-2* in *Vhlh*$^{-/-}$ islets.

Additional evidence points to defective signaling due to hypoxia as a contributor to diabetes. Upregulation of hypoxia-related genes has been observed in pre-diabetic and diabetic Zucker diabetic fatty (ZDF) rats (44). Recent studies suggest that inhibition of the hypoxic response also affects β-cell function. A dramatic decrease in HIF-1β (ARNT), the partner of HIF-1α, was reported in islets obtained from type 2 diabetic patients, indicating an involvement of the hypoxia genes in β-cell dysfunction (45). Furthermore, the authors showed that a β-cell–specific knockout of HIF-1β in transgenic mice leads to abnormal glucose tolerance, and the genetic changes in islets overlap with those found in islets from diabetic patients. In agreement with our results, some of the genes affected in β-cells lacking HIF-1β are involved in glucose sensing and metabolism. It is possible that dysregulation of the glycolytic pathway (either by upregulation or downregulation) impairs β-cell function.

Although our results strongly suggest that cell autonomous changes in β-cells impair glucose regulation, we cannot formally exclude the scenario that the aberrations in islet architecture caused by increased *Vegf* expression might influence β-cell function. However, previously reported studies in other existing mouse models that ectopically express *Vegf* either in β-cells or in the pancreas have failed to report glucose-intolerant phenotypes (46,47). Nonetheless, it cannot be excluded that inappropriately increased vascularization might impede insulin secretion, possibly through incorrectly established contacts between the β-cells and the endothelium. Future work will need to address this issue.

VHL is a tumor suppressor gene. Germline *VHL* mutations in humans predispose to certain types of tumors, affecting several organs, including the kidney and pancreas (10). Although the most frequent pancreatic manifestation of VHL disease is serous cysts, a small percentage of VHL patients develop nonfunctional islet cell tumors (17,18). We have not observed islet tumor formation in *Vhlh*-deficient mice. However, it is important to note that in our studies, inactivation of *Vhlh* has been restricted to β-cells. The cell of origin of islet tumors in VHL patients is not known. Therefore, islet tumor formation might require loss of VHL in other non–β-cells. Alternatively, islet tumor formation in *Vhlh*-deficient β-cells might involve a second event, as has been described for other VHL-related tumors such as in the kidney (48).

In summary, we have shown that loss of VHL in β-cells leads to defects in glucose homeostasis. We believe that the β-cell–specific *Vhlh*-deficient mice might be a useful tool as a "genetic hypoxia" model, to unravel the possible link between hypoxia signaling and impairment of β-cell function. This is particularly important, since the β-cell response to hypoxia could also be relevant for therapeutic approaches to diabetes. Islet transplantation studies have

noted a dramatic increase in HIF-1 levels soon after transplantation that causes apoptosis and a block in insulin secretion (49). Thus, preventing hypoxic conditions might not only affect transplant survival but also prevent changes in insulin secretion.

ACKNOWLEDGMENTS

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