Normal glucose uptake in the brain and heart requires an endothelial cell-specific HIF-1α–dependent function

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Although intimately positioned between metabolic substrates in the bloodstream and the tissue parenchymal cells that require these substrates, a major role of the vascular endothelium in the regulation of tissue metabolism has not been widely appreciated. We hypothesized that via control of transendothelial glucose transport and contributing paracrine mechanisms the endothelium plays a major role in regulating organ and tissue glucose metabolism. We further hypothesized that the hypoxia-inducible factor -1α (HIF-1α) plays an important role in coordinating these endothelial functions. To test these hypotheses, we generated mice with endothelial cell-specific deletion of HIF-1α. Loss of HIF in the endothelium resulted in significantly increased fasting blood glucose levels, a blunted insulin response with delayed glucose clearance from the blood after i.v. loading, and significantly decreased glucose uptake into the brain and heart. Endothelial HIF-1α knockout mice also exhibited a reduced cerebrospinal fluid/blood glucose ratio, a finding consistent with reduced transendothelial glucose transport and a diagnostic criterion for the Glut1 deficiency genetic syndrome. Endothelial cells from these mice demonstrated decreased Glut1 levels and reduced glucose uptake that was reversed by forced expression of Glut1. These data strongly support an important role of the vascular endothelium in determining whole-organ glucose metabolism and indicate that HIF-1α is a critical mediator of this function.

Normal cellular metabolism depends upon the regular delivery of fuel substrates and oxygen to organs and tissues, and the vasculature is the means by which this delivery occurs. Specifically, it is the vascular endothelium across which these substrates must pass to gain access to the parenchymal cells that will use them (1–4). Despite this unique position of the endothelium between blood and tissue, it has been generally held that the endothelium does not play a rate-limiting role in glucose metabolism. The data that we present here challenge that perception, support an important role of the endothelium in tissue glucose metabolism, and demonstrate that the oxygen-sensing transcription factor HIF-1α is a key regulator of the metabolic effects of the endothelium.

There are several ways in which the endothelium can influence glucose metabolism in the parenchyma of organs and tissues. One is via regulation of glucose transport across the endothelium, from blood to tissue. Another is via endothelial secretion of endocrine or paracrine factors that influence parenchymal cell metabolism. Clinical evidence of the former comes from studies of children and adults with mutations in the glucose transporter GLUT-1 (3, 5, 6). Glucose uptake occurs primarily by facilitated diffusion, an energy-independent process that uses a carrier protein to transport a substrate across a membrane (4). The glucose transporter proteins in mammalian tissues are members of a family of structurally related proteins expressed in a tissue-specific manner (7, 8). For example, GLUT-1 is widely expressed in endothelial cells (8), whereas GLUT-4 is the major isoform present in skeletal muscle and myocardium (9, 10). GLUT-1 is the major transporter of glucose across the endothelial blood–brain barrier (11–13). Mutations in human GLUT-1 that reduce its function are associated with reduced glucose uptake into the brain and a propensity for seizures (3, 5). GLUT-1 mutations have been proposed as a potential mechanistic link to other CNS syndromes, including some learning disabilities (12), and alterations in glucose transporter genes have been associated with Alzheimer’s disease (14). Although GLUT-1 is expressed in glia, albeit to a much lesser extent than in endothelial cells (EC), the major glucose transporter in neurons is GLUT-3 (11). Thus, it is likely that the neurological abnormalities in individuals with dysfunctional GLUT-1 are due to abnormal glucose transport across the endothelium into the brain, underscoring an important role of the endothelium in regulating brain glucose metabolism.

Although it has been held that vascular EC are “glucose-blind” and lack the ability to decrease glucose transport in response to elevated glucose levels, recent data have shown that exposure to high glucose results in decreased GLUT-1 expression and reduced glucose uptake in EC from various tissues, including brain and heart (15, 16). In this manner, it is proposed that the endothelium senses and responds to blood glucose (BG) levels and coordinates these with parenchymal cell glucose delivery and metabolism. We hypothesized involvement of the hypoxia-inducible factor 1α (HIF-1α) in these processes.

HIF-1α is a basic helix-loop–helix transcription factor that plays a crucial role in coordinating gene expression with oxygen availability and is estimated to be involved in the transcriptional regulation of ~5% of all mammalian genes (17, 18). Among these are many involved in cellular metabolism, including the glycolytic enzymes, lactate dehydrogenase, and GLUT-1. Previously, we showed that HIF-1α plays a central role in coordinating energy availability and utilization in the heart, even under normoxic conditions (19). Recently, several single-nucleotide polymorphism (SNPs) in the human HIF-1α gene were identified and a link found between the P582S coding SNP (cSNP) haplotype and susceptibility to type 2 diabetes, suggesting a crucial role of HIF-1α in normal glucose metabolism (20).

In addition to its well-characterized response to hypoxia, HIF has been shown capable of responding to glucose levels (21). The mechanism of this response, however, is not well understood. Although HIF-1α can be regulated at the mRNA level, the major mechanism controlling HIF-1α expression in response to oxygen is posttranslational, involving prolyl hydroxylation, polyubiquitylation by the von Hippel-Lindau protein, and destruction by the proteosome (17, 22). Rapid induction of HIF during hypoxia involves decreased prolyl hydroxylation and consequent stabilization of HIF-1α protein. Exposure to high glucose concentrations diminishes this hypoxia-induced stabilization and...
leads to decreased HIF-1α protein levels (21). Conversely, low glucose leads to transcription of HIF-1α-responsive genes, and deletion of HIF abrogates this effect (23). These findings establish that HIF is involved in “sensing” glucose levels. As such, we hypothesized that HIF may be involved in endothelial modulation of tissue glucose metabolism. To investigate, we used a Cre-lox approach to generate mice with EC-specific deletion of HIF-1α.

**Results**

Mice homozygous for EC deletion of HIF-1α (EC–HIF-1α+/−) were born at expected Mendelian frequencies as we have described (24). The efficiency of HIF-1α gene deletion in EC was 92 ± 7% by quantitative PCR, similar to our previous findings (24). There was no failure to thrive or increased early mortality, and body weights were not different from age- and sex-matched littermates (EC–HIF-1α+/−) (Fig. 1A).

BG after overnight fasting was significantly elevated in EC–HIF-1α+/− mice (Fig. 1B, P ≤ 0.001; n ≥ 40/group), and >40% had fasting BG levels ≥ 126 mg/dL whereas only 8% of control mice reached this value (Fig. 1C; P ≤ 0.001), suggesting a critical role for endothelial HIF-1α in maintaining glucose homeostasis. To determine whether EC deletion of HIF-1α altered glucose clearance, we performed glucose tolerance testing (GTT) by i.v. bolus injection of glucose (2 mg/g of body weight) and determination of BG and insulin levels in response to serial time points. Both groups showed the same BG peak 10 min after glucose injection, but the rate of BG decline was significantly delayed in EC–HIF-1α+/− mice vs. control littermates, indicating impaired glucose clearance in the absence of endothelial HIF-1α (Fig. 1D). Basal insulin levels were the same in both groups. Control mice had a robust insulin increase 20 and 60 min after glucose injection, but this increase was markedly delayed in EC–HIF-1α+/− mice, reaching the levels of WT mice only 60 min post injection (Fig. 1E). This delay was not associated with any histological abnormalities in pancreatic structure, nor in the histology of multiple organs and tissues assayed (heart and brain shown) (Fig. 1F). Previously, we showed that loss of HIF-1α in EC does not significantly reduce basal vessel counts (24). To corroborate this and determine if the impaired glucose clearance in EC–HIF-1α+/− mice was due to altered vascular density, we performed immunostaining for platelet endothelial cell adhesion molecule 1 (PECAM-1) or isolectin in multiple tissues from EC–HIF-1α+/− and control mice. Quantitation of vessel density in all tissues analyzed, including heart, skeletal muscle, and brain, showed no differences in basal vessel counts between groups. Representative sections are shown in Fig. 1G.

Thus, we postulated that a contributing mechanism for the increased BG levels and the impaired glucose clearance that we observed was a defect in endothelial glucose transport. To test this, we performed both in vivo and ex vivo determinations of glucose uptake. In vivo we infused [2-3H]deoxyglucose ([2-3H]DG) into the jugular vein of anesthetized mice, monitored serum-specific activity over time, and analyzed [2-3H]DG uptake into brain and heart. Glucose uptake into the brain was reduced by 34.4% (Fig. 2A; P < 0.05) and into the heart by 31.6% (Fig. 2B, P < 0.05) in EC–HIF-1α+/− mice vs. controls, despite similar serum [2-3H]DG-specific activity curves (Fig. 2C). These data were not due to altered cardiac work, as heart rate and peak developed pressure were not different between these groups, either at baseline or with progressive adrenergic stimulation (Fig. S1). To further evaluate, and to avoid any contribution of bloodborne cells or noncardiac EC to the glucose uptake determination, we analyzed glucose uptake in isolated perfused hearts. Hearts from knockout and control mice were perfused via a modified Langendorf approach at a constant flow rate using a perfusate containing glucose and free fatty acids as alternative metabolic fuels, as would be encountered clinically (25). Glucose uptake into EC–HIF-1α+/− hearts was 55.5 ± 4.3% less than control hearts (P = 0.02; Fig. 2D).

Comensurate with reduced glucose uptake, glucose oxidation was significantly reduced in isolated perfused EC–HIF-1α+/− hearts at baseline, during low-flow ischemia, and during reperfusion (vs. control littermates), demonstrating further the critical role of endothelial HIF-1α in the metabolic response to these physiologically relevant conditions (Fig. 2E). These reductions were similar to those observed in hearts from mice in which HIF-1α was deleted specifically from cardiac muscle (cmHIF-1α+/−), establishing that the endothelial HIF-1α can mediate effects on cardiac glucose metabolism of similar magnitude to those mediated by HIF-1α in cardiac myocytes. We also analyzed lactate levels in the perfusate of these hearts, a parameter that reflects lactate production within the heart, release from parenchymal cells, and transport across the endothelium into the perfusate. Interestingly, lactate levels were significantly lower under basal conditions in the perfusate from EC–HIF-1α+/− hearts than in the perfusates from cmHIF-1α+/− or control littermate hearts. Lactate rose significantly during ischemia and reperfusion in perfusate from EC–HIF-1α+/− and control hearts, but remained flat in the perfusate from cmHIF-1α+/− hearts, consistent with the reduced expression of lactate transporters.

**Fig. 1.** Endothelial deletion of HIF-1α results in elevated basal glucose levels and abnormal glucose tolerance. Mice with deletion of HIF-1α in the endothelium (EC–HIF-1α−/−) were generated by crossing HIF-1αfl/fl mice with Tie-2-Cre mice. (A) Body weights of EC–HIF-1α−/− and control littermates were not different. (B) EC–HIF-1α−/− mice demonstrated significantly elevated basal fasting blood glucose (FBG) levels (n ≥ 40 age- and sex-matched mice/group). (C) >40% of EC–HIF-1α−/− and 8% of control (EC–HIF-1α+/+) mice had FBG levels ≥ 126 mg/dL. (D) Glucose tolerance testing (i.v. glucose bolus of 2 mg/g) demonstrated a significantly reduced rate of BG clearance in EC–HIF-1α−/− mice and a delayed increase in circulating insulin levels in response to the glucose load (E), although basal insulin levels were not different between the groups. (F and G) There were no histological abnormalities in pancreatic architecture or islets or in brain or heart histology in EC–HIF-1α−/− mice (F, Upper panels) and no basal differences in vascular density in multiple tissues by immunohistochemical analysis (heart, skeletal muscle, and brain shown), consistent with our previous findings (24). *P < 0.05.
bical vein EC (HUVEC) were transduced with an adenovirus encoding GLUT-1 expression and glucose transport in EC. Human umbilical vein endothelial cells (HUVECs) were transduced with an adenovirus encoding GLUT-1 expression and glucose transport in EC. Human umbilical vein endothelial cells (HUVECs) were transduced with an adenovirus encoding GLUT-1 expression and glucose transport in EC. Human umbilical vein endothelial cells (HUVECs) were transduced with an adenovirus encoding GLUT-1 expression and glucose transport in EC. Human umbilical vein endothelial cells (HUVECs) were transduced with an adenovirus encoding GLUT-1 expression and glucose transport in EC.

To further explore the relationship between HIF-1α and GLUT-1, and endothelial glucose transport, we used viral vectors to manipulate HIF-1α and GLUT-1 expression in human dermal microvascular EC (HDMEC). Lentivirus-mediated shRNA knockdown of HIF-1α reduced HIF-1α levels to <40% of control virus-transduced HDMEC within 48 h (Fig. 3F). Interestingly, forced expression of a GLUT-1 cDNA, concomitant with shRNA knockdown of HIF-1α, resulted in a further reduction of GLUT-1 expression (Fig. 3F and G). This GLUT-1–induced decrease of GLUT-1 has been previously reported (26). shRNA knockdown of HIF-1α in HDMECs resulted in a significant reduction of glucose uptake into these cells, and concomitant restoration of GLUT-1 levels by virus-mediated GLUT-1 expression restored glucose uptake to near baseline levels (Fig. 3F), strongly supporting a prominent role of GLUT-1 deficiency in the reduced glucose uptake observed in the absence of endothelial HIF-1α. Finally, to further support the role of GLUT-1 deficiency in the EC–HIF-1α–α-glucosuria phenotype, we analyzed simultaneous glucose levels in cerebrospinal fluid (CSF) and blood. The CSF/BG ratio was significantly reduced in EC–HIF-1α−/− mice, consistent with reduced transendothelial glucose transport and congruent with the previously documented ability to increase glucose uptake. Virus-mediated increase in GLUT-1 expression restored glucose uptake to near baseline levels (Fig. 3F).

The HIF pathway regulates the expression of multiple secreted proteins that could potentially alter cellular metabolism via paracrine or endocrine mechanisms, including apelin, angioptoeitin-like peptide 4, leptin, several IGF-binding proteins, and thrombospondin 1, among others. To evaluate whether HIF-1α–mediated gene regulation in the endothelium could, in a paracrine manner, alter parenchymal cell metabolism, we transduced H9C2 cells to conditioned media from HIF-1α–transduced or control-transduced HUVECs. Conditioned media from HIF-1α–transduced HUVECs significantly increased H9C2 glucose uptake in the absence and presence of insulin (Fig. 4A), suggesting that HIF-1α regulates the expression of an endothelial-derived paracrine signal that can alter the glucose metabolism of other cells.

Given the significant repertoire of HIF-responsive genes, full characterization of the HIF-regulated factors that may mediate paracrine metabolic effects will require extensive study. To initiate studies, we concentrated on apelin, a small secreted protein with documented ability to increase glucose uptake. Virus-mediated expression of a stabilized active HIF-1α markedly increased apelin mRNA, and shRNA knockdown of HIF-1α significantly decreased apelin mRNA in HUVECs (Fig. 4B). In HIF-1α−/− EC, there was a marked decrease in apelin mRNA under normoxia and loss of the hypoxic induction seen in control EC (Fig. 4C). In vivo, there was a 59% increase in plasma apelin levels in control mice exposed to hypoxia, and this increase was absent in EC–HIF-1α−/− mice (Fig. 4D), demonstrating that the endothelium can define circulating levels of apelin in a paracrine-dependent manner. Apelin binds a γ-protein coupled receptor (APJ) that can signal through AMPK. After demonstrating that HIF-1α induces apelin expression in the endothelium, we examined whether HIF-1α–activated EC could activate AMPK in a paracrine fashion and found that conditioned media from HIF-1α–transduced EC significantly increased apelin mRNA and GLUT-1 expression in human umbilical vein EC (HUVEC).
1α-transduced endothelial cells promoted AMPK phosphorylation in muscle cells (Fig. 4 E and F).

These cumulative findings (depicted schematically in Fig. 5) demonstrate an important endothelial contribution to whole-organ glucose metabolism, a critical role of HIF-1α in mediating this endothelial function, and a mechanistic contribution of HIF-regulated GLUT-1 expression in determining these effects.

Discussion

Here we show that vascular endothelial expression of HIF-1α plays a critical role in defining whole-organ glucose metabolism. We show that in the absence of endothelial HIF-1α there is a significant defect in glucose uptake into the brain and heart and a reduction in levels of the primary endothelial glucose transporter GLUT-1, the product of a HIF-1α-responsive gene. These data support a mechanism whereby HIF-1α regulates glucose transport to the heart and brain by altering the endothelial expression of GLUT-1. This is consistent with established clinical data from patients with genetic GLUT-1 deficiency syndrome who exhibit neurological abnormalities attributed to decreased trans-endothelial glucose delivery to the brain (3, 5). Supporting this HIF-1α-GLUT-1 glucose transport mechanism, we found that the CSF/blood glucose ratio was significantly decreased in EC-HIF-1α−/− mice, an important diagnostic criterion for clinical GLUT-1 deficiency syndrome. Along with in vitro data in which we show that decreased glucose uptake into HIF-1α-deficient endothelial cells is rescued by forced expression of a GLUT-1 cDNA, the cumulative data are consistent with an important role of HIF-1α in controlling transendothelial glucose transport via transcapillary control of GLUT-1. The clinical implications of these findings are...
Endothelial HIF-1α regulation of glucose transport and parenchymal cell metabolism. Shown is a representation of how endothelial expression of HIF-1α can regulate parenchymal cell glucose metabolism. (A) HIF-1α transcriptionally regulates expression of Glut1, the major glucose transporter in the endothelium: Glut1 is a rate-limiting determinant of glucose uptake into EC and mediates the transport of glucose from blood to tissue across the endothelium (B). HIF regulates the expression of a wide repertoire of metabolism-related genes, including the major glycolytic enzymes, and plays a significant role in defining glucose utilization within the endothelium (C). HIF regulates the expression of several proteins secreted from the endothelium, including apelin, that can affect the metabolic state of neighboring cells via a paracrine mechanism and potentially of remote tissues via endocrine effects (D).

significant and suggest that vascular dysfunction should be seriously considered as a contributor to abnormal glucose handling, rather than just a consequence of it. More broadly, the data underscore the importance of considering vascular contributions to parenchymal cell metabolic perturbations.

Before the discovery of specific glucose transport proteins, glucose was thought to pass from blood to tissue by passive diffusion. It is now well established that normal glucose transport occurs by facilitated diffusion that requires specialized glucose transporter proteins, such as GLUT-1 (8, 27). As the primary EC glucose transporter, GLUT-1 transports glucose not only into EC, but also across them. This crucial function of GLUT-1 provides a potential mechanism by which the endothelium can regulate glucose delivery to parenchymal cells, thus modulating their metabolism. Quantitative modeling of glucose transport in the brain supports this rate-limiting role of transendothelial glucose transport (28). HIF-1α is a major regulator of GLUT-1 expression, and, at higher glucose concentrations, HIF-1α levels decrease (17, 18). Thus, HIF-1α-mediated expression of GLUT-1 in the endothelium might represent a feedback pathway by which blood levels of glucose and oxygen are coordinated with glucose transport to organs and tissues. HIF is also induced by pyruvate and by lactate (29). It is conceivable that production of these metabolites in tissues influences vascular HIF levels, thus providing an HIF-dependent link between parenchymal cell metabolism and transvascular glucose transport.

The abnormal GTT and delayed insulin response in EC-HIF-1α−/− mice can also be attributed to reduced transendothelial glucose transport resulting in a slower rate of BG decrease after glucose bolus and a slower delivery rate of glucose from the blood to the islet cells. There were no alterations in the vascularity or histological architecture of the pancreas in EC-HIF-1α−/− mice; thus the delayed insulin response does not appear to be due to a developmental defect in the pancreas of these mice. Basal and peak levels of insulin were normal in the EC-HIF-1α−/− mice, consistent with normal pancreatic capacity to produce insulin, despite the delayed response. Interestingly, the HIF pathway is involved substantively in pancreatic β-cell function, but the effects on glucose homeostasis and insulin secretion are the inverse of those we show in EC-HIF-1α−/− mice (30). Specifically, chronic activation of the HIF pathway secondary to deletion of von Hippel-Lindau in β cells resulted in increased blood glucose and decreased insulin levels and was corrected by simultaneous deletion of HIF-1α. Thus, the HIF pathway plays a crucial cell-type-specific role in glucose homeostasis. The role of HIF in defining vascular effects on tissue and organ metabolism is distinct from the metabolic effects of HIF in parenchymal cells.

Other EC-mediated factors may also contribute to the reduced glucose uptake and oxidation exhibited by EC-HIF-1α−/− mice. HIF-1α regulates the expression of a significant array of genes that encode secreted proteins. Several, including leptin, apelin, angiopeptin-like 4, thrombospondin 1, adrenomedullin, Vegf-b, and others, affect cellular metabolism and are made by endothelial cells (31–34). Supporting an endothelium-derived paracrine function, we found that conditioned media from HIF-activated EC activated the AMPK pathway and increased glucose uptake into muscle-derived cells. This may reflect the effects of several HIF-induced proteins, and defining the identity and role of each will require further investigation. One strong candidate is apelin, which increases glucose uptake. EC-HIF-1α deletion is sufficient to alter circulating levels of apelin, underscoring the potential importance of the endothelium in glucose metabolism. Interestingly, it was recently shown that Vegf-b regulates endothelial fatty acid transport, in part via the flt-1 receptor (ECFR1) (34). There are HIF-responsive variants in the human and mouse Vegf-b promoters (antisense strand), and our data (Fig. S3) demonstrate Vegf-b induction by HIF-1α. Furthermore, flt-1 is HIF-1α responsive. Thus, endothelial HIF-1α might have metabolic effects beyond glucose metabolism.

Although our data support reduced endothelial Glut1 in the absence of HIF-1α as the major determinant of our phenotype, we propose that endothelium-derived paracrine factors may play a major role in defining tissue metabolism, perhaps particularly in specific microcirculations, such as the hypothalamic or the basal α-cell microcirculations. The effects that we observed using conditioned media from HIF-1α-activated EC were relatively modest, but in vivo, where cell-cell distances are small and microenvironmental concentrations of paracrine factors may be much higher than in conditioned media, the effects could be much more robust. Defining the metabolically active endothelial secretome, the paracrine and/or endocrine functions of individual component factors, and the mechanisms whereby the expression and secretion of these factors is controlled could conceivably yield new therapeutic targets for metabolism-associated diseases.

To assure the specificity of our findings, we assessed a variety of potential confounding variables (Fig. S4). In vivo glucose uptake into the heart, for example, can vary with cardiac workload. We used peak left ventricular (LV) pressure as a surrogate for LV afterload and monitored heart rate, under basal conditions and with progressive adrenergic stimulation, to ensure that these parameters were not different between the EC-HIF-1α−/− mice and littermate controls. We assessed vascularity, which, in agreement with our previously published data demonstrated significant differences between genotypes. To exclude a potential metabolic contribution of circulating cells of hematopoietic origin, which might be HIF-1α deficient secondary to developmental promiscuity of Tie-2-driven Cre expression, we recapitulated our in vivo glucose uptake data, adding glucose oxidation data, in isolated hearts perfused with
Materials and Methods

Generation of EC-Hif-1α−/− Mice. Conditional EC deletion of Hif-1α was accomplished by crossing Tie-2-Cre transgenic mice with Hif-1αloxP/loxP mice, as we previously described (24).

Glucose Uptake and Oxidation Studies. In vivo glucose uptake was determined on anesthetized mice fasted for 14 h as described (SI Materials and Methods). Glucose uptake and oxidation in isolated mouse hearts was performed at the Yale Mouse Metabolic Phenotyping Center as previously described (25).

Glucose uptake in vitro was done in six-well plates with [2-3H]DG (2.5 μCi/well). The lentivirus constructs for shRNA HIF-1α were kindly provided by Dr. Mary B. Woodgett (University of Sheffield, United Kingdom) and by Dr. Soo Hiong (University of Western Australia, Australia). For transduction. For rescue studies, cells were transduced with adenovirus 24 h after lentivirus and then studied 48 h later (72 h post lentivirus transduction).

Floxing Efficiency and Gene Expression Analysis. Floxing was quantified by real-time PCR with a primer pair designed to give product only in the absence of floxing (19, 24). Quantitative RT-PCR (qRT-PCR) was performed as previously described (35).

Histology and Vessel Counts. For standard histology, formalin-fixed tissues were embedded in paraffin, sectioned, and stained (H&E) by the Yale Pathology Core Facility. Optimal cutting temperature-embedded frozen sections were used for immunohistochemistry. A monoclonal anti-PECAM antibody (Invitrogen) was used for microvessel counts as previously described (19, 24).

Statistical Analysis. Data are presented as mean ± SE. Differences between groups were determined by Student’s t test or by ANOVA where appropriate. Significance was set at P < 0.05.

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Supporting Information

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SI Materials and Methods

Generation of Endothelial Cell–Hypoxia-Inducible Factor 1α/−/− Mice. Conditional endothelial cell (EC) deletion of hypoxia-inducible factor 1α (HIF-1α) was accomplished by crossing Tie-2-Cre transgenic mice with HIF-1α−/− flox/flox mice, as we have previously described. All mice had been backcrossed into the C57BL6 background for at least six generations. Mice were fed normal chow and were exposed to normal light/dark cycles. Genotyping was performed per usual PCR methods on tail DNA, and floxing efficiency was evaluated on isolated endothelial cells by quantitative PCR.

Glucose Tolerance Testing. Glucose tolerance testing (GTT) was performed on anesthetized mice by i.v. bolus injection of glucose (2 mg/g body weight). This i.v. route was chosen rather than standard i.p. administration to ensure that differences in i.p. absorption rate would not contribute to the findings. Blood glucose was determined using a clinical grade glucometer (OneTouch Ultra LifeScan, Inc.) at baseline 10, 20, 30, 60, and 120 min post glucose administration. Serum insulin levels were determined by ELISA (Linco Research, Inc.), using mouse insulin as standards (at baseline 20 and 60 min post glucose administration). All mice were fasted overnight before GTT, and controls were sex- and age-matched Cre-negative HIF1α/−/− littermates.

In Vivo Glucose Uptake Assay. Mice were anesthetized with ketamine and xylazine after fasting for 14 h. The left common jugular vein was cannulated for sampling, and the right carotid artery was cannulated for infusion, and the right carotid artery was cannulated for sampling with polyethylene PE-10 tubing. At t = 0 min, an arterial blood sample (150 μL) was obtained and processed as the baseline blood sample. At t = 5 min, a 12-μCi bolus of 2-deoxy[3H]glucose ([2-3H]DG) was administered to determine Rg, an index of tissue-specific glucose uptake. Mice received saline-washed red blood cells from a donor mouse as required to prevent a marked fall in hematocrit. At t = 10, 15, 20, and 30 min, arterial blood (50 μL) was sampled to determine arterial blood glucose and plasma [2-3H]DG. After the 30 min time point, mice were killed, and the brain and heart were excised and immediately frozen in liquid nitrogen.

After deproteinization with Ba(OH)2 (0.3 N) and ZnSO4 (0.3N), [2-3H]DG radioactivity of plasma was determined by liquid scintillation counting (Wallac 1409/11) with Ultima Gold (Packard) as the scintillant. Brain and heart samples were weighed and homogenized in 0.5% perchloric acid. Homogenates were centrifuged and neutralized with KOH. One aliquot was counted directly to determine [2-3H]DG and [2-3H]DG-6-phosphate ([2-3H]DGP) radioactivity. A second aliquot was treated with Ba(OH)2 and ZnSO4 to remove [2-3H]DGP and any tracer incorporated into glycogen and then counted to determine radioactivity. [2-3H]DGP is the difference between the two aliquots. In all experiments, the accumulation of [2-3H]DGP was normalized to tissue weight.

Mouse Heart Perfusion and Metabolic Analysis. Glucose uptake on isolated mouse hearts was performed in the Yale Mouse Metabolic Phenotyping Center (http://mouse.yale.edu/) as previously described (1). Briefly, isolated mouse hearts from EC–HIF–1α/−/− and littermate control mice were retrograde-perfused on a modified Langendorff apparatus with Krebs–Henseleit buffer containing 7 mM glucose, 0.4 mM oleate, 1% BSA, and a low fasting concentration of insulin (10 μU/mL). To assess the effects of low-flow ischemia and reperfusion on heart glucose and fatty acid oxidation, constant flow perfusion was achieved using a peristaltic pump to produce low-flow ischemia with ~80% flow reduction. Following initial perfusion at 80 mm Hg for ~5 min to flush out residual blood from the heart and allow stabilization, hearts were perfused at a baseline flow of 4 mL/min (30 min), ischemia at 0.75 mL/min (30 min), and reperfusion at 4 mL/min (30 min). These perfusions were performed with radiolabeled glucose and oleate. The rate of glucose uptake was determined by the production of [3H2]H2O from [2-3H]glucose, and the rate of glucose oxidation was determined by the production of [14C]CO2 from [U-14C]glucose.

In Vitro Studies of Glucose Uptake. Human umbilical vein endothelial cells and human dermal microvascular endothelial cells, obtained at low passage from the Yale Vascular Biology Core, were cultured in medium M199, containing penicillin–streptomycin, glutamine, 20% (vol/vol) FBS, and 1% (vol/vol) endothelial cell growth supplement. H9C2 cells were cultured in DMEM, supplemented with penicillin–streptomycin, glutamine, and 10% (vol/vol) FBS. Cells were split into six-well plates before transduction. Cells were transduced with adenovirus encoding either HIF-1α or GFP (Adv-HIF or Adv-GFP), and 48 h later the medium was changed to serum-free DMEM. After an additional 6 h, insulin (100 nM; Sigma) was added, and the cells were cultured under these conditions for an additional 90 min. The cells were then washed four times with HBSS containing 1 mM CaCl2 and 1 mM MgCl2, [2-3H]DG (2.5-μCi/well) was added, and the cells were incubated for 30 min at 37 °C. After washing the cells five times with cold PBS, 1 mL 0.1 N NaOH was added to achieve lysis. Lysates were pH neutralized with 1 N HCl, and 100-μL samples were used for scintillation counting. H9C2 cells were similarly studied. The lentivirus constructs for lentiviral shRNA HIF-1α knockdown and controls were a kind gift from Zhong Yun (Yale University New Haven, CT). The adenovirus encoding Glut1 was obtained from the Beta Cell Biology Consortium (www.betacell.org/). For knockdown studies, cells were studied 48 and 72 h after transduction. For rescue studies, cells were transduced with adenovirus 24 h after lentivirus transduction and then studied 48 h later (72 h after lenti transduction).

Floxing Efficiency and Gene Expression Analysis. For determination of floxing efficiency, genomic DNA was isolated from endothelial cells and nonendothelial cells isolated from knockout and control lung and reproductive fat. Isolation of endothelial cells was accomplished by collagenase digestion of these tissues, followed by magnetic bead-based separation using anti-platelet endothelial cell adhesion molecule (anti-PECAM) antibody as previously described (2). Purity was documented by determination of the transfected cells and nonendothelial cells isolated from knockout and control. Following initial perfusion at 80 mm Hg for ~5 min to flush out residual blood from the heart and allow stabilization, hearts were perfused at a baseline flow of 4 mL/min (30 min), ischemia at 0.75 mL/min (30 min), and reperfusion at 4 mL/min (30 min). These perfusions were performed with radiolabeled glucose and oleate. The rate of glucose uptake was determined by the production of [3H2]H2O from [2-3H]glucose, and the rate of glucose oxidation was determined by the production of [14C]CO2 from [U-14C]glucose.

For Western blot analysis, cells were lysed in radioimmuno precipitation assay buffer supplemented with proteinase inhibitor mixture, and equally loaded protein samples underwent electrophoresis on 10% NuPAGE gels (Invitrogen). Protein bands were transferred onto nitrocellulose membranes and blocked with 5% nonfat milk. Membranes were incubated at 4 °C overnight with primary antibody (HIF-1α, anti–GLUT-1, anti–β-actin; Santa Cruz Biotechnology), washed, and incubated for 1 h with secondary antibody and developed by using chemiluminescence (Pierce). Quantitative RT-PCR was performed as previously described (4, 5).

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Histology and Vessel Counts. For standard histology, the pancreas was fixed in formalin, embedded in paraffin, sectioned, and stained (H&E) by the Yale Pathology Core Facility. Optimum cutting temperature-embedded frozen sections were used for immunohistochemistry. Sections (5 μm) from HIF-1α null and control hearts were cut and fixed with acetone/methanol. A monoclonal anti-PECAM antibody (Invitrogen) was used for microvessel counts as previously described (2, 3, 6). Digital images from five separate 40× fields were obtained from each section, and vessel counts were determined from these images by two separate blinded investigators.

Statistic Analysis. Data are presented as mean ± SE. Differences between groups were determined by Student’s t test or ANOVA where appropriate. The significance level was set at P < 0.05.


Fig. S1. Hemodynamic assessment in EC-HIF-1α−/− (KO) and EC-HIF-1α+/+ (WT) mice showed no differences in (A) heart rate or (B) peak-developed pressure at baseline and with progressive dosing of dobutamine.

Fig. S2. (A) PCR for HIF-1α on EC and tails from EC-HIF-1α+/+ and EC-HIF-1α−/− mice. (B) Densitometry of Western blot in Fig. 3A.

Fig. S3. Human umbilical vein endothelial cells were transduced with adenovirus vectors encoding either a stabilized HIF-1α (Ad HIF1) or green fluorescent protein (Ad Ctl). Real-time PCR revealed HIF-1α induction of VEGFA and VEGFB expression. *P < 0.05; n = 3.
DNA was isolated from heart and skeletal muscle from EC-HIF1⁺⁺ and EC-HIF1⁻⁻ mice, and determination of relative mitochondrial DNA abundance was made using mDNA-specific primers. Normalization was to the single-copy genomic DNA gene GAPDH.

**Fig. S4.** DNA was isolated from heart and skeletal muscle from EC-HIF1⁺⁺ and EC-HIF1⁻⁻ mice, and determination of relative mitochondrial DNA abundance was made using mDNA-specific primers. Normalization was to the single-copy genomic DNA gene GAPDH.