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

Yan Huang^a, Li Lei^a, DingGang Liu^a, Ion Jovin^a, Raymond Russell^a, Randall S. Johnson^b, Annarita Di Lorenzo^c, and Frank J. Giordano^{a,1}

^aDepartment of Medicine, Section of Cardiovascular Medicine, Yale University School of Medicine, New Haven, CT 06510; ^cDepartment of Pharmacology, Yale University School of Medicine, New Haven, CT 06510; and ^bDepartment of Biology, University of California at San Diego, La Jolla, CA 92093

Edited* by Vincent T. Marchesi, Yale University School of Medicine, New Haven, CT, and approved September 18, 2012 (received for review June 7, 2012)

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 -1 α (HIF-1 α) plays an important role in coordinating these endothelial functions. To test these hypotheses, we generated mice with endothelial cell-specific deletion of HIF-1 α . 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-1 α knockout mice also exhibited a reduced cerebrospinal fluid/blood glucose ratio, a finding consistent with reduced trans-endothelial 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-1 α is a critical mediator of this function.

transcription | transporter | microvascular

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-1 α 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 1 α (HIF-1 α) in these processes.

HIF-1 α 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-1 α 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-1 α 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-1 α 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-1 α can be regulated at the mRNA level, the major mechanism controlling HIF-1 α expression in response to oxygen is posttranslational, involving prolyl hydroxylation, polyubiquitylation by the von Hippel-Lindau protein, and destruction by the proteasome (17, 22). Rapid induction of HIF during hypoxia involves decreased prolyl hydroxylation and consequent stabilization of HIF-1 α protein. Exposure to high glucose concentrations diminishes this hypoxia-induced stabilization and

Author contributions: Y.H., A.D.L., and F.J.G. designed research; Y.H., L.L., D.L., I.J., R.R., A.D.L., and F.J.G. performed research; R.S.J. contributed new reagents/analytic tools; Y.H., L.L., D.L., I.J., R.R., R.S.J., A.D.L., and F.J.G. analyzed data; and Y.H., A.D.L., and F.J.G. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

¹To whom correspondence should be addressed. E-mail: frank.giordano@yale.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1209281109/-DCSupplemental.

dehydrogenase and decreased lactate production that we previously documented in these hearts.

HIF-1 α regulates the expression of all major protein classes, including intracellular, membrane-associated, and secreted proteins. We hypothesized that endothelial HIF-1 α would likely modulate glucose metabolism by two separate general mechanisms, altered endothelium-dependent transport of glucose, and paracrine modulation of parenchymal cell glucose metabolism. To address transport, we investigated the expression of the predominant glucose transporter present in the endothelium, GLUT-1. GLUT-1, an established HIF-responsive gene, demonstrated reduced expression in the EC of EC-HIF-1 $\alpha^{-/-}$ mice at both the protein and mRNA levels (Fig. 3 *A* and *B* and Fig. S2). The immunohistochemical pattern of GLUT-1 expression in the heart demonstrates the vascular predominance of this transporter and shows its reduced vascular expression in EC-HIF-1 $\alpha^{-/-}$ hearts (Fig. 3C). To corroborate the role of HIF-1 α in regulating GLUT-1 expression and glucose transport in EC, human umbilical vein EC (HUVEC) were transduced with an adenovirus encoding a stabilized/activated HIF-1 α . HIF transduction markedly increased GLUT-1 protein levels, and this increase was

accompanied by a significant increase in glucose uptake into the HUVECs, supporting a rate-limiting role of GLUT-1 in determining endothelial glucose transport (Fig. 3*D* and *E*). GLUT-1 is an insulin-independent glucose transporter. Consistent with this, the increase in glucose uptake observed did not require concomitant administration of insulin, and the administration of insulin did not further augment glucose uptake. Conversely, when insulin was administered to the control HUVECs (transduced with an adenovirus encoding GFP), glucose uptake increased significantly. Together, these data demonstrate that HIF-1 α can regulate endothelial glucose uptake in an insulin-independent manner, but also suggest that endothelial glucose uptake is regulated ultimately by a balance between HIF activity, GLUT-1 expression, and insulin levels.

To further explore the relationship between HIF-1 α , GLUT-1, and endothelial glucose transport, we used viral vectors to manipulate HIF-1 α and GLUT-1 expression in human dermal microvascular EC (HDMEC). Lentivirus-mediated shRNA knockdown of HIF-1 α reduced HIF-1 α levels to <40% of control virus-transduced HDMEC within 48 h (Fig. 3*F*). Interestingly, forced expression of a GLUT-1 cDNA, concomitant with shRNA knockdown of HIF-1 α , resulted in a further reduction of HIF-1 α (Fig. 3*F* and *G*). This GLUT-1-induced decrease of HIF-1 α has been previously reported (26). shRNA knockdown of HIF-1 α in HDMEC resulted in a significantly reduced 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. 3*H*), strongly supporting a prominent role of GLUT-1 deficiency in the reduced glucose uptake observed in the absence of endothelial HIF-1 α . Finally, to further support the role of GLUT-1 deficiency in the EC-HIF-1 α ^{-/-} phenotype, we analyzed simultaneous glucose levels in cerebrospinal fluid (CSF) and blood. The CSF/BG ratio was significantly reduced in EC-HIF-1 α ^{-/-} mice, consistent with reduced transendothelial glucose transport and congruent with the reduced CSF/BG ratios that are used as a diagnostic criterion for the clinical GLUT-1 deficiency genetic syndrome (Fig. 3*I*, $P < 0.005$).

The HIF pathway regulates the expression of multiple secreted proteins that could potentially alter cellular metabolism via paracrine or endocrine mechanisms, including apelin, angiotensin-like peptide 4, leptin, several IGF-binding proteins, and thrombospondin 1, among others. To evaluate whether HIF-1 α -mediated gene regulation in the endothelium could, in a paracrine manner, alter parenchymal cell metabolism, we exposed cultured H9C2 cells to conditioned media from HIF-1 α -transduced or control-transduced HUVECs. Conditioned media from HIF-transduced HUVECs significantly increased H9C2 glucose uptake in the absence and presence of insulin (Fig. 4A), suggesting that HIF regulates the expression of an endothelium-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-1 α markedly increased apelin mRNA, and shRNA knockdown of HIF-1 α significantly decreased apelin mRNA in HUVECs (Fig. 4B). In HIF-1 $\alpha^{-/-}$ 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-1 $\alpha^{-/-}$ mice (Fig. 4D), demonstrating that the endothelium can define circulating levels of apelin in a HIF-1 α dependent manner. Apelin binds a g-protein coupled receptor (APJ) that can signal through AMPK. After demonstrating that HIF-1 α induces apelin expression in the endothelium, we examined whether HIF-1 α -activated EC could activate AMPK in a paracrine fashion and found that conditioned media from HIF-

Fig. 3. Reduced whole-organ glucose uptake in EC-HIF-1 α ^{-/-} mice correlates with reduced CSF/blood glucose ratios and GLUT1 expression in the endothelium. (A, Upper) Western blot showing decreased GLUT1 in EC isolated from EC-HIF-1 α ^{-/-} mice (WT = littermate controls). These findings paralleled HIF-1 α deletion from EC (but not from non-EC) in EC-HIF-1 α ^{-/-} mice (PCR; A, Lower), reduced GLUT-1 and Hif-1 α mRNA levels (qRT-PCR) in EC from EC-HIF-1 α ^{-/-} vs. control mice (B), and reduced GLUT-1 endothelial immunostaining in EC-HIF-1 α ^{-/-} hearts vs. controls (C). (D and E) HUVEC transduced with adenovirus encoding a stabilized HIF-1 α (Adv-HIF) showed higher GLUT-1 protein levels and commensurately higher insulin-independent glucose uptake vs. control-transduced HUVECs (Ad-GFP). (F) Lentivirus-mediated shRNA (LVshHIF) in HDMEC resulted in efficient knockdown of HIF-1 α mRNA. Concomitant transduction with adenovirus encoding GLUT1 (AdGlut1) further reduced HIF-1 α (LVshCtrl and AdCtrl = controls) and markedly increased GLUT1 mRNA expression (G). (H) LVshHIF knockdown of HIF-1 α in HDMEC decreases glucose uptake, and concomitant adenovirus-mediated expression of GLUT1 rescues this LVshHIF-mediated decrease in glucose uptake. (I) The ratio of CSF/BG is significantly reduced in EC-HIF-1 α ^{-/-} mice vs. control littermates ($P < 0.005$; $n \geq 4$ /group). For in vitro experiments a single asterisk indicates significance of at least $P < 0.05$; “#” indicates $P < 0.05$ basal vs. insulin stimulated.

1 α -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-1 α 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-1 α plays a critical role in defining whole-organ glucose metabolism. We show that in the absence of endothelial HIF-1 α 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-1 α -responsive gene. These

data support a mechanism whereby HIF-1 α 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-1 α -GLUT-1 glucose transport mechanism, we found that the CSF/blood glucose ratio was significantly decreased in EC-HIF-1 α ^{-/-} 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-1 α -deficient endothelial cells is rescued by forced expression of a GLUT-1 cDNA, the cumulative data are consistent with an important role of HIF-1 α in controlling transendothelial glucose transport via transcriptional control of GLUT-1. The clinical implications of these findings are

Fig. 4. Endothelial HIF-1 α paracrine effects. (A) Conditioned media (CM) from HUVEC transduced with adenovirus encoding stabilized HIF-1 α (Ad-HIF) increased glucose uptake in cardiac muscle-derived H9C2 cells vs. CM from control transduced (Ad-GFP) HUVEC ($*P < 0.05$ vs. control under identical conditions; # $P < 0.05$ vs. control without insulin). (B) Transduction of HUVEC with Ad-HIF increased, and lentivirus shRNA knockdown of HIF-1 α (LV shHIF1) decreased, apelin and adrenomedullin (ADM) mRNA ($*P < 0.05$ for increase; # $P < 0.05$ for decrease). (C) Apelin mRNA is reduced in normoxic EC isolated from EC-HIF-1 α ^{-/-} mice, and does not demonstrate induction under hypoxia. (D) Plasma apelin levels (determined by ELISA) in EC-HIF-1 α ^{-/-} mice and control littermates under normoxia (21% O₂) and after 3 d exposure to hypoxia (10% O₂) (# $P < 0.05$ hypoxia vs. normoxia; $*P < 0.05$ EC-HIF-1 α ^{-/-} vs. EC-HIF-1 α ^{+/+}; $n \geq 5$ /group). (E and F) CM from HIF-1 α -transduced EC induces AMPK phosphorylation (representative Western).

Fig. 5. Endothelial HIF-1 α regulation of glucose transport and parenchymal cell metabolism. Shown is a representation of how endothelial expression of HIF-1 α can regulate parenchymal cell glucose metabolism. (A) HIF-1 α 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-1 α is a major regulator of GLUT-1 expression, and, at higher glucose concentrations, HIF-1 α levels decrease (17, 18). Thus, HIF-1 α -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 a HIF-dependent link between parenchymal cell metabolism and transvascular glucose transport.

The abnormal GTT and delayed insulin response in EC-HIF-1 α ^{-/-} 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-1 α ^{-/-} 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-1 α ^{-/-} 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-1 α ^{-/-} 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-1 α . Thus, the HIF pathway plays crucial cell-type-specific roles in glucose homeostasis, and 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 mechanisms may also contribute to the reduced glucose uptake and oxidation exhibited by EC-HIF-1 α ^{-/-} mice. HIF-1 α regulates the expression of a significant array of genes that encode secreted proteins. Several, including leptin, apelin, angiopoietin-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-1 α 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 (VEGFR1) (34). There are HIF-response elements in the human and mouse *Vegf-b* promoters (antisense strand), and our data (Fig. S3) demonstrate *Vegf-b* induction by HIF-1 α . Furthermore, flt-1 is HIF-1 α responsive. Thus, endothelial HIF-1 α might have metabolic effects beyond glucose metabolism.

Although our data support reduced endothelial Glut1 in the absence of HIF-1 α 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 pancreatic β -cell microcirculations. The effects that we observed using conditioned media from HIF-1 α -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-1 α ^{-/-} mice and littermate controls. We assessed vascularity, which, in agreement with our previously published data demonstrated no significant differences between genotypes. To exclude a potential metabolic contribution of circulating cells of hematopoietic origin, which might be HIF-1 α 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