A liver Hif-2α–Irs2 pathway sensitizes hepatic insulin signaling and is modulated by Vegf inhibition

Kevin Wei1,10, Stephanie M Piecewicz1,10, Lisa M McGinnis1,10, Cullen M Taniguchi2, Stanley J Wiegand3, Keith Anderson4, Carol W-M Chan1, Kimberly X Mulligan4, David Kuo1, Jenny Yuan1, Mario Vallon1, Lori C Morton3, Etienne Lefai5, M Celeste Simon6, Jacquelyn J Maher7, Gilles Mithieux8, Fabienne Rajas8, Justin P Annes9, Owen P McGuinness4, Gavin Thurston3, Amato J Giaccia2 & Calvin J Kuo1

Insulin initiates diverse hepatic metabolic responses, including gluconeogenic suppression and induction of glycogen synthase and lipogenesis1,2. The liver possesses a rich sinusoidal capillary network with a higher degree of hypoxia and lower gluconeogenesis in the perivascular area as compared to the rest of the organ3. Here, we show that diverse vascular endothelial growth factor (VEGF) inhibitors improved glucose tolerance in nondiabetic C57BL/6 and diabetic db/db mice, potentiating hepatic insulin signaling with lower gluconeogenic gene expression, higher glycogen storage and suppressed hepatic glucose production. VEGF inhibition induced hepatic hypoxia through sinusoidal vascular regression and sensitized liver insulin signaling through hypoxia-inducible factor-2α (Hif-2α, encoded by Epas1) stabilization. Notably, liver-specific constitutive activation of HIF-2α, but not HIF-1α, was sufficient to augment hepatic insulin signaling through direct and indirect induction of insulin receptor substrate-2 (Irs2), an essential insulin receptor adapter protein4,5. Further, liver Irs2 was both necessary and sufficient to mediate Hif-2α and Vegf inhibition effects on glucose tolerance and hepatic insulin signaling. These results demonstrate an unsuspected intersection between Hif-2α–mediated hypoxia signaling and hepatic insulin action through Irs2 induction, which can be co-opted by Vegf inhibitors to modulate glucose metabolism. These studies also indicate distinct roles in hepatic metabolism for Hif-1α, which promotes glycolysis7–9, and Hif-2α, which suppresses gluconeogenesis, and suggest new treatment approaches for type 2 diabetes mellitus.

The liver regulates systemic energy reserves by controlling carbohydrate and lipid metabolism in response to dietary and systemic cues. Hepatic insulin stimulation recruits insulin receptor substrate (IRS) proteins to the insulin receptor, activating protein kinase B (AKT), glycogen synthase kinase-3β (GSK-3β) and mammalian target of rapamycin; these signals coordinate suppression of gluconeogenesis and induce glycogen synthesis and lipogenesis1,2. The liver perivascular zone experiences relative hypoxia accompanied by suppression of gluconeogenesis3. During normoxia, prolyl hydroxylase domain–containing enzymes (PHD1, PHD2 and PHD3) and FIH (factor inhibiting hypoxia-inducible factor (HIF)) hydroxylate members of the HIF transcription factor family (HIF-1, HIF-2 and HIF-3), resulting in von Hippel-Lindau tumor suppressor (VHL)-dependent proteosomal degradation; hypoxic inhibition of this hydroxylation stabilizes HIFs and induces HIF transcriptional targets10.

The VEGF family contains VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF), each with distinct affinities for VEGF receptors 1–3 (VEGFR1, VEGFR2 and VEGFR3) and neuropilins. VEGFR1 (FLT1) is a high-affinity receptor for VEGF-A, VEGF-B and PIGF, whereas VEGFR2 (Flk1/KDR) is a low-affinity receptor for VEGF-A, VEGF-C and VEGF-D11,12. VEGF inhibitors have been reported to decrease fasting blood glucose levels and improve glucose tolerance in mice and humans through unclear mechanisms13,14, and specific Vegf-b inhibition improves glucose tolerance through enhanced peripheral glucose uptake15. Here, we used single intravenous (i.v.) injections of adenosviruses encoding the soluble extracellular ligand-binding domains of mouse Vegfr1 (Ad-sFlt1) or of Vegfr2 fused to an antibody Fc (Ad-sFlk1) to achieve hepatic secretion of Flt1 or Flk1 ectodomains into the circulation; both ectodomains elicit potent and durable Vegf-a neutralization in vivo16,17. An adenosvirus encoding a mouse immunoglobulin IgG2a Fc fragment (Ad-Fc) was used as a negative control16,17. In C57BL/6 mice, Ad-sFlt1–mediated Vegf inhibition improved the response to intraperitoneal (i.p.) glucose injection compared to Ad-Fc injection in both glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) (Fig. 1a). Moreover, in...
diabetic db/db mice, the results of GTT and ITT tests were again markedly improved by either Ad-sFlt1 or Ad-sFlk1 compared to control Ad-Fc treatment (Fig. 1b); all results were confirmed by area-under-the-curve (AUC) analysis (Supplementary Fig. 1a–d). Recombinant aflibercept (VEGF Trap) encodes a fusion of human VEGFR1 and VEGFR2 ectodomains with human IgG1 Fc that potently sequesters and neutralizes VEGF-A, VEGF-B and PIGF18,19. Severe combined immunodeficient (SCID) and db/db mice treated with aflibercept also showed greater improvement in glucose tolerance compared to controls treated with a control human Fc immunoglobulin fragment (hFc) (Fig. 1c,d and Supplementary Fig. 1e,f); SCID mice were used to eliminate potential neutralizing antibody responses against aflibercept. Similar results were obtained with both the anti-VEGF-A monoclonal antibody (mAb) B20.4.1.1 (ref. 20) and the anti-VEGF2 mAb DC101 (ref. 21) (Supplementary Fig. 1g,h), neither of which interferes with VEGF-B signaling.

Ad-sFlt1, aflibercept or DC101 decreased fasting or fed glucose levels in C57BL/6 (Ad-sFlt1 and DC101), SCID (aflibercept) or db/db (aflibercept) mice (Supplementary Fig. 2a–e), and aflibercept did not increase plasma insulin or decrease glucagon (Supplementary Fig. 2f,g). In a hyperinsulinemic-euglycemic clamp study, C57BL/6 mice treated with aflibercept for 2 weeks exhibited greater insulin sensitivity and enhanced insulin-induced suppression of hepatic glucose production (HGP) (Fig. 1e and Supplementary Fig. 3). Further, in db/db mice, aflibercept substantially lowered hyperinsulinemia compared to control hFc treatment (Fig. 1f). This occurred without altering insulin-stimulated whole-body glucose disposal, tissue-specific glucose uptake in the periphery or hepatic AMP response element–binding protein or AMP-activated protein kinase (AMPK) signaling (Supplementary Fig. 4a,b).

The insulin-potentiating effects of VEGF inhibition on HGP prompted us to evaluate insulin receptor signaling in mouse liver. Ad-sFlt1 treatment increased phosphorylation of Akt (p-Akt) and Gsk-3β (p-Gsk-3β), as did aflibercept for p-Akt (Fig. 1g,h). Both Ad-sFlt1 and aflibercept augmented expression of Irs2 and suppressed phosphoenolpyruvate carboxykinase (Pck1) and glucose-6-phosphatase.
Vegf inhibition induces hepatic vascular regression, liver hypoxia and HIF-2α stabilization to augment hepatic insulin signaling. (a,b) Liver CD31 immunofluorescence and vessel area density percentage following Ad-Fc, Ad-sFlt1, Ad-sFlk1 or aflibercept treatment after 14 d. Scale bars, 100 μm. (c) FACS determination of hypoxia in mouse hepatocytes 14 d after Ad-sFlt1, Ad-Fc (blue) or control Ad-Fc (red) treatment. Hypoxo probe (HP) injection in mice was followed by anti-hydroxy probe FITC-conjugated secondary antibody in disaggregated hepatocytes. The gray curve indicates a CD31 immunofluorescence and vessel area density percentage following Ad-Fc, Ad-sFlt1, Ad-sFlk1 or aflibercept treatment after 14 d. Scale bars, 100 μm. (d) Densitometric quantification of G6pc liver qRT-PCR from f. Data are expressed as mean ± s.e.m. *P < 0.05.

Vegf inhibition induces capillary regression in both normal adult organs and solid tumors, although effects in liver have not been described.13,22,23 Ad-sFlt1, Ad-sFlk1 and recombinant aflibercept induced marked and reversible regression of CD31+ hepatic sinusoids compared to Ad-Fc or hFc controls (Fig. 1g–j); however, alterations of Irs1 or insulin receptor β-chain expression were not observed in aflibercept- and Ad-sFlt1-treated mice, respectively (Fig. 1g,h). We observed similar effects with Ad-sFlk1 treatment (Fig. 1i and Supplementary Fig. 4c,d). Both sFlt1 and sFlk1 repressed liver G6pc and elevated glucose in fasted liver compared to Ad-Fc–treated mice (Supplementary Fig. 4e), again consistent with hepatic insulin signaling sensitization.

Vegf inhibition induces capillary regression in both normal adult organs and solid tumors, although effects in liver have not been described.13,22,23 Ad-sFlt1, Ad-sFlk1 and recombinant aflibercept induced marked and reversible regression of CD31+ hepatic sinusoids compared to Ad-Fc– and Ad-hFc–treated mice, respectively (Fig. 1g,h). A FACS-based hypoxo probe method detected a hypoxic shift in liver upon in vivo Vegf antagonism (Fig. 2c) that was not seen in Fc-treated animals. Ad-sFlt1 and aflibercept also decreased functional perfusion in mouse liver upon intravascular biotin infusion (Fig. 2d). Further, microarray analysis of aflibercept-treated mouse liver revealed upregulation of several hypoxia-inducible genes, including Cited2, Loxl2 and Pjkl (Supplementary Fig. 6a), whereas Hif-2α but not Hif-1α protein was stabilized in Ad-sFlt1–treated mouse liver (Fig. 2c). Notably, Ad-sFlt1 improved glucose tolerance in unexcised Epas1flox/flox mice (here called Hif-2α flox mice), but this effect was significantly blunted in mice with liver-specific Epas1 deletion (Epas1flox/flox; albumin-Cre mice, here called Hif-2α LKO mice)9 (Fig. 2f and Supplementary Fig. 7a). Similarly, Ad-sFlt1–mediated induction of hepatic Irs2 expression, Akt or Gsk-3β phosphorylation and Ad-sFlt1 suppression of G6pc were all blunted in Hif-2α LKO in comparison to Hif-2α flox mice (Fig. 2g,h), indicating that Vegf inhibition utilizes hepatic Hif-2α to sensitize liver insulin signaling. Additionally, adeno viral shRNA knockdown of liver Hif-2α also reversed the effects of sFlt1 on glucose tolerance and insulin signaling (Supplementary Fig. 7b–c).

We next examined whether hepatic Hif-2α activation was sufficient to augment liver insulin receptor signaling. Hepatic adeno viral expression of a constitutively active HIF-2α variant mutated at inhibitory hydroxylase sites Pro531 and Asn847 (Ad-HIF-2αPN) or lacking the inhibitory oxygen–dependent degradation domain (ODD) (Ad-HIF-2αODD) significantly decreased blood glucose under fed or fasted conditions and improved both glucose tolerance and insulin sensitivity compared to mice treated with either Ad-Fc or adenovirus encoding a HIF-1αODD mutant (Ad-HIF-1αODD) (Fig. 3a and Supplementary Fig. 8a–f). Similarly, Ad-HIF-2αPN or Ad-HIF-2αODD was much more effective than Ad-HIF-1αODD or Ad-Fc at inducing hepatic Irs2 protein and amounts of p-Akt, p–Gsk-3β and
p-Foxo1 under fasted or fed conditions (Fig. 3b,c and Supplementary Fig. 8g). In parallel, HIF-2α constitutive activation repressed G6pc and Pck1 expression more strongly than did HIF-1α (Fig. 3d and Supplementary Fig. 8h). In primary mouse hepatocytes, HIF-2α but not HIF-1α activation markedly induced Irs2 expression and synergized with insulin to increase Akt phosphorylation compared to controls, indicating a cell-autonomous mechanism (Fig. 3e).

We explored the functional relevance of Irs2 to HIF-2α regulation of hepatic insulin signaling both in vitro and in vivo. In primary hepatocytes, the synergistic activation of Akt phosphorylation by Ad-HIF-2αPN and insulin was attenuated by Irs2 knockdown (Ad-shRNA Irs2) (Fig. 3f). Further, in db/db mice, the ability of Ad-HIF-2αPN to improve glucose tolerance, increase liver Akt phosphorylation and repress G6pc was attenuated by concomitant Irs2 knockdown (Fig. 3g,h and Supplementary Fig. 8i). Hepatic IRS2 overexpression through Ad-IRS2 (ref. 24) markedly improved glucose tolerance in db/db mice compared to Ad-Fc–treated controls in agreement with previous results24. The ability of liver IRS2, Ad-HIF-2αPN and VEGF inhibition to phenocopy each other in GTT (Fig. 3i and Supplementary Fig. 8j) is consistent with liver Irs2 induction being itself sufficient to mediate the effects of Ad-HIF-2αPN or Vegf inhibition on glucose tolerance. Similarly, Ad-sFlt1 improvement of glucose tolerance was also attenuated by liver Irs2 shRNA knockdown (Fig. 3). HIF-2α induced liver Irs2 mRNA expression both in vivo and in primary hepatocytes (Fig. 4a,b), which suggested that Hif-2α could directly regulate Irs2 transcription. Canonical hypoxia response elements (HREs) are present at nucleotide positions –900 and –123 in the IRS2 promoter (Fig. 4c). The proximal –123 but not the distal –900 HRE was required for HIF-2α transactivation of an IRS2-luciferase reporter construct in insulin-starved H2O2 cells (Fig. 4d, 5.8 fold increase, 300 nM HIF-2αPN + shRNA Irs2 compared to 300 nM HIF-2αPN + shRNA Luc) without affecting glucose tolerance or inducing IRS2 (Fig. 4e) (Sensor IRS2: 300 nM HIF-2αPN + shRNA Irs2 compared to 300 nM HIF-2αPN + shRNA Luc). Similarly, HIF-2α knockdown (Ad-shRNA Irs2) (Fig. 4f) reversed HIF-2α-induced Irs2 induction and decreased IRS2 expression and IRS2 activity (Fig. 4g). This downregulation of IRS2 was also observed in vivo in mice fasted or fed Ad-HIF-2αPN (Fig. 4h, Supplementary Fig. 6b). The proximal –123 but not the distal –900 HRE was required for HIF-2α transactivation of an IRS2-luciferase reporter construct in insulin-starved H2O2 cells (Fig. 4d). The proximal –123 but not the distal –900 HRE was required for HIF-2α transactivation of an IRS2-luciferase reporter construct in insulin-starved H2O2 cells (Fig. 4d). 5.8 fold increase, 300 nM HIF-2αPN + shRNA Irs2 compared to 300 nM HIF-2αPN + shRNA Luc) without affecting glucose tolerance or inducing IRS2 (Fig. 4e). This downregulation of IRS2 was also observed in vivo in mice fasted or fed Ad-HIF-2αPN.
suggesting that Srebf1 repression was not itself sufficient for HIF-2α induction of Irs2. Forced adenoviral overexpression of a cleaved active form of SREBP-1c (Ad-nSREBP1c) reversed Ad-HIF-2αPN stimulation of Irs2 both in insulin-starved primary hepatocytes (Fig. 4f) and in vivo under re-fed conditions (Fig. 4g). Thus, HIF-2α suppression of the Irs2 repressor Srebf1, although not itself sufficient, appears to facilitate HIF-2α–Irs2 direct transactivation (Fig. 4h).

These studies reveal a previously unsuspected cross-talk between the liver hypoxia and insulin signaling pathways whereby HIF-2α strongly augments insulin-dependent Akt activation and gluconeogenic suppression (Fig. 4h). Here, complementary in vitro and in vivo studies demonstrate that HIF-2α positively regulates hepatic insulin signaling by upregulating Irs2. This Irs2 induction is specific to HIF-2α and not HIF-1α activation, paralleling studies where the former and not the latter has a dominant role in regulation of hepatic Epo9,28, Pou5f1, Ccnd1 and Dmrt1 (refs. 29–31) and of liver lipogenesis32,33. In knockdown and overexpression studies, liver Irs2 is both necessary and sufficient for HIF-2α–mediated improvement of glucose tolerance and hepatic insulin signaling. The improved glucose tolerance upon liver IRS2 overexpression is in agreement with prior studies where increased hepatic IRS2 expression is fully sufficient to ameliorate diabetes in db/db mice and activate fasted liver insulin signaling24.

Although we have established a clear functional role for Irs2, we cannot fully exclude parallel Irs2-independent pathways. Possibilities for Irs2-independent Hif-2α effects include insulin-independent direct association of pERK1/2 with the HIF-2α coactivator-1α, hepatocyte nuclear factor-4 or CCAAT enhancer-binding protein with PCK1 and G6pc promoter elements32,33. Alternatively, erythropoietin has been variously reported to either improve peripheral glucose uptake30 or inhibit gluconeogenesis by modulation of inflammation35, although erythropoietin does not induce liver IRS2 (S.M.P. and C.J.K., unpublished data). Our demonstration that the HIF-2α–Irs2 axis couples hypoxia sensing to gluconeogenic repression through potentiation of hepatic insulin signaling complements observations of hypoxic repression of IRS2 and invasion in breast cancer cells36. Notably, upon hepatic Vhl deletion, which stabilizes both HIF-2α and HIF-1α, a Srebf1 gene signature is induced by superimposed deletion of Eps1 but not Hif1α, suggesting the possibility of HIF-2α–specific Srebf1 repression32. HIF-2α induced Irs2 but decreased Irs1 expression, suggestive of potential reciprocal regulation; however, liver Irs1 downregulation typically impairs rather than improves glucose tolerance4,5.

The beneficial effects of HIF-2α on animal models of insulin resistance suggest the potential therapeutic utility of prolyl hydroxylase inhibitors that block VHL-dependent HIF degradation for type 2 diabetes mellitus therapy. This could occur through isofrom-specific PHD3 pharmacologic inhibition (see accompanying paper by Taniguchi et al.37), which could circumvent the hepatic lipid accumulation associated with conventional liver HIF-2α activation32,38. A second strategy for pharmacologic manipulation of the hepatic HIF-2α–Irs2 axis is represented by Vegf inhibition, which decreases pancreatic islet microvesSEL density13 with specific Vegf-b...
antagonism in mice enhancing peripheral glucose uptake\textsuperscript{25}. However, aflibercept, a clinically used therapeutic that binds VEGF-A, VEGF-B and PIGF\textsuperscript{18}, here acts on liver through insulin sensitization rather than on skeletal muscle. Such alternative mechanisms are further supported by the abilities of sFlt1 and of non–VEGF-b–targeting inhibitors such as sFlk1, B20.4.1.1 and DC101 to modulate glucose tolerance, hepatic insulin signaling or both. Conceivably, inhibition of VEGF-a or VEGF-b could coordinategly regulate glucose metabolism through hepatic insulin signaling and peripheral glucose uptake, respectively\textsuperscript{15}, with additional mechanisms certainly possible. Because HIF-2α and HIF-1α exhibit similar hypoxic stabilization profiles\textsuperscript{39}, the specific VEGF-inhibitor stabilization of HIF-2α over HIF-1α could occur through selective Phd3 inhibition (see accompanying manuscript by Taniguchi et al.\textsuperscript{37}) or alternative nonhypoxic pathways\textsuperscript{17} and could underlie observations of improved glucose control in VEGF inhibitor–treated patients with diabetes\textsuperscript{14}. As constitutive HIF-2α activation induces insulin receptor signaling endpoints more strongly than VEGF inhibitors, additional as-yet-undetermined pathways may contribute to effects of VEGF blockade and hypoxic signaling on hepatic glucose metabolism. Finally, any therapeutic use of either prolyl hydroxylase inhibitors or VEGF inhibitors for diabetes would need to be carefully balanced against known dose-limiting and chronic toxicities of these agents.

Overall, our data indicate distinct roles in energy metabolism for hepatic HIF-2α, which sensitizes liver insulin signaling and suppresses gluconeogenesis, and hepatic HIF-1α, which promotes glycyls\textsuperscript{7–9}. \textit{In vivo}, physiologic perivascular liver hypoxia\textsuperscript{3} could trigger the HIF-2α–Irs2 axis, preferentially enhance insulin signaling and suppress perivascular Pck1 and G6pc, explaining the previously observed enrichment of gluconeogenesis in the periporal zone\textsuperscript{3}. Finally, the mechanistic interplay in liver between the hypoxia/HIF-2α and insulin receptor signaling pathways, as demonstrated here, has potentially broad implications for the study of metabolism and therapeutic approaches to type 2 diabetes mellitus.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Microarray data were deposited in the NCBI Gene Expression Omnibus (GEO) with accession code GSE50519.

Note: Any Supplementary Information and Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank R. DePinho, L. Harshman, B. Tam, C. Chartier, D. Suchet and members of the K.W. and L.M.M., NIGMS US National Institutes of Health (NIH) GM-07365 and 3. Jungermann, K. Zonation of metabolism and gene expression in liver. Histochem. Cell Biol. 103, 81–91 (1995).

4. Kubota, N. et al. Dynamic functional relay between insulin receptor substrate 1 and 2 in hepatic insulin signaling during fasting and feeding. Cell Metab. 8, 49–64 (2008).

5. Dong, X. et al. Inactivation of hepatic Foxo1 by insulin signaling is required for adaptive nutrient homeostasis and endocrine growth regulation. Cell Metab. 8, 65–76 (2008).

6. Zhong, L. et al. The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1α and Hif-2α in stem cells. Mol. Cell. Biol. 26, 3514–3526 (2006).

7. Rankin, E.B. et al. Hypoxia-inducible factor-1α (HIF-1α) and HIF-2α in vivo. J. Clin. Invest. 117, 1068–1077 (2007).

8. Kaelin, W.G. Jr. & Ratcliffe, P.J. Oxygen sensing by metazoans: the central role of the HIF hypoxia pathway. Mol. Cell 30, 393–402 (2008).

9. Ferrara, N., Gerber, H.P. & LeCouter, J. The biology of VEGF and its receptors. Nat. Med. 9, 669–676 (2003).

10. Allitano, K. & Carmeliet, P. Molecular mechanisms of lypangiogenesis in health and disease. Cancer Cell 1, 219–227 (2002).

11. Kamba, T. et al. VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature. Am. J. Physiol. Heart Circ. Physiol. 290, H560-H576 (2006).

12. Bilimoria, B. et al. Blood glucose levels in patients with metastatic renal cell carcinoma treated with sunitinib. Br. J. Cancer 99, 1380–1382 (2008).

13. Hagberg, C.E. et al. Targeting VEG-F as a novel treatment for insulin resistance and type 2 diabetes. Nature 490, 426–430 (2012).

14. Kuo, C.J. et al. Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer. Proc. Natl. Acad. Sci. USA 98, 4605–4610 (2001).

15. Tam, B.Y. et al. VEGF modulates erythropoiesis through regulation of adult hepatic erythropoietin synthesis. Nat. Med. 12, 793–800 (2006).

16. Papadopoulos, N. et al. Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab. Angiogenesis 15, 171–185 (2012).

17. Holash, J. et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc. Natl. Acad. Sci. USA 99, 11393–11398 (2002).

18. Liang, W.C. et al. Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. J. Biol. Chem. 281, 991–961 (2006).

19. Prewett, M. et al. Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res. 59, 5209–5218 (1999).

20. Fan, X. et al. VEGF blockade inhibits angiogenesis and reepithelialization of endometrium. FASEB J. 22, 3571–3580 (2008).

21. Mancuso, M.R. et al. Rapid vascular regrowth in tumors after reversal of VEGF inhibition. J. Clin. Invest. 116, 2610–2621 (2006).

22. Canettieri, G. et al. Dual role of the coactivator TORC2 in modulating hepatic glucose output and insulin signaling. Cell Metab. 2, 331–338 (2005).

23. Ide, T. et al. SREBP2 suppresses IRS-2-mediated insulin signalling in the liver. Nat. Cell Biol. 6, 351–357 (2004).

24. Shimomura, I. et al. Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. Mol. Cell 6, 77–86 (2000).

25. Lecomte, V. et al. A new role for sterol regulatory element binding protein 1 transcription factors in the regulation of muscle mass and muscle cell differentiation. Mol. Cell. Biol. 30, 1182–1190 (2010).

26. Yeo, E.J., Cho, Y.S., Kim, M.S. & Park, J.W. Contribution of HIF-1α or HIF-2α to erythropoiesis in vivo evidence based on charomatin immunoprecipitation. Ann. Hematol. 87, 11–17 (2008).
29. Covello, K.L. et al. HIF-2α regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes Dev.* **20**, 557–570 (2006).

30. Mastrogiannaki, M. et al. HIF-2α, but not HIF-1α, promotes iron absorption in mice. *J. Clin. Invest.* **119**, 1159–1166 (2009).

31. Shah, Y.M., Matsubara, T., Ito, S., Yim, S.H. & Gonzalez, F.J. Intestinal hypoxia-inducible transcription factors are essential for iron absorption following iron deficiency. *Cell Metab.* **9**, 152–164 (2009).

32. Rankin, E.B. et al. Hypoxia-inducible factor 2 regulates hepatic lipid metabolism. *Mol. Cell Biol.* **29**, 4527–4538 (2009).

33. Wang, X.L. et al. Ablation of ARNT/HIF1β in liver alters gluconeogenesis, lipogenic gene expression, and serum ketones. *Cell Metab.* **9**, 428–439 (2009).

34. Scully, M.S. et al. A novel EPO receptor agonist improves glucose tolerance via glucose uptake in skeletal muscle in a mouse model of diabetes. *Exp. Diabetes Res.* **2011**, 910199 (2011).

35. Meng, R., Zhu, D., Bi, Y., Yang, D. & Wang, Y. Erythropoietin inhibits gluconeogenesis and inflammation in the liver and improves glucose intolerance in high-fat diet-fed mice. *PLoS ONE* **8**, e53557 (2013).

36. Mardilovich, K. & Shaw, L.M. Hypoxia regulates insulin receptor substrate-2 expression to promote breast carcinoma cell survival and invasion. *Cancer Res.* **69**, 8894–8901 (2009).

37. Taniguchi, C.M. et al. Cross-talk between hypoxia and insulin signaling through Phd3 regulates hepatic glucose and lipid metabolism and ameliorates diabetes. *Nat. Med.* doi:10.1038/nm.3294.

38. Qu, A. et al. Hypoxia-inducible transcription factor 2α promotes steatohepatitis through augmenting lipid accumulation, inflammation, and fibrosis. *Hepatology* **54**, 472–483 (2011).

39. Bracken, C.P. et al. Cell-specific regulation of hypoxia-inducible factor (HIF)-1α and HIF-2α stabilization and transactivation in a graded oxygen environment. *J. Biol. Chem.* **281**, 22575–22585 (2006).
ONLINE METHODS

Animals. All mice were males on a 12-h light-dark cycle in a pathogen-free animal facility, and unless otherwise specified, were C57BL/6 (Jackson Laboratory). The generation of Hi2tα (Epsa1) conditional alleles and albumin-Cre transgenic mice have been previously described40,41. Hepatocyte-specific deletion of Hi2tα was achieved through generating mice that were homozygous for the Hi2tα 2-lox alleles and expressed the albumin-Cre transgene. Littermates not carrying the albumin-Cre transgene were used as control animals. Mutant mice were generated in a mixed genetic background (BALB/c, 129Sv and C57BL/6). 8- to 10-week-old Leprdb/db and Leprdb+ mice were purchased from Jackson Laboratory. 8- to 10-week-old C57BL/6 mice were purchased from Taconic. Mice were injected with adenoviruses through the tail vein at between 1x10⁵ to 5x10⁵ PFU in 0.1 ml PBS (Ad-sFlt1, Ad-sFlk1, Ad-Fc: 1x10⁵ PFU; Ad-HIF-2αODD, Ad-HIF-1ODD, Ad-IRS2 (ref. 24), Ad-Fc: 3x10⁵ and 1.5x10⁶; Ad-shRNA Irs2, Ad-shRNA luciferase: 5x10⁴ and 6x10⁴). Mice received VEGF Trap protein (alibibercept) subcutaneously two times per week at 25 mg per kg body weight. For control groups, mice were treated with humanized Fc (Regeneron). DC101 (a rat IgG1 mAb targeting mouse VEGF) was produced by Antibody Solution from conditioned medium of the cognate hybridoma ATCC HB-11534. SCID mice received subcutaneous injections of DC101 or control rat IgG (Sigma) at 40 mg per kg body weight three times per week for 2 weeks. The anti–VEGF-A mAb B20.4.1.1 (ref. 20), or control anti-ragweed mAb (Genentech) were administered to C57BL/6 mice at 5 mg per kg body weight, three times per week i.p. All animals studies were performed in accordance with the NIH guidelines for use and care of live animals and were approved by the Stanford University Institutional Animal Care and Use Committee, A3213-01.

Adenoviruses. The construction of Ad-sFlt1 (encoding N-terminal hemagglutinin-tagged murine soluble Vegf1 capturing the first three immunoglobulin domains), Ad sFlk1 (encoding a full length murine soluble VEGF-R2 ectodomain fused to mouse IgG2α Fc, also known as Ad Flk1-Fc) and Ad-Fc (encoding IgG2α Fc alone) have been previously described16,42. Human HIF2α-2pTpN was generated by site-directed mutagenesis replacing Pro531 and Asn847 with alanines. The human HIF1α allele lacking the inhibitory ODD (HIF1α-ODD) was a gift from C. Chartier; similar methods were used to generate human HIF2αODD. Ad-IRS2 (encoding human IRS2 with Flag tag) was provided by M. Montminy24. To generate adenoviruses expressing various shRNAs, an adenoviral shRNA shuttle vector was generated in which a cytomegalovirus promoter drives a GFP 3′ UTR miR-30 expression cassette in an Ad-strain 5 backbone. miR-30-flanked shRNA sequences were inserted against mouse Epas1 (the gene encoding Hif-2α) and Irs2 were obtained from Open Biosystems and cloned into the miR-30 expression cassette through XhoI-EcoRI restriction sites. Sequence information available upon request. The human nSREBP-1c adenovirus for in vitro primary hepatocyte infection was from Eton Bioscience. The human nSREBP-1c adenovirus used for in vivo mouse experiments has been described27. All adenoviral inserts were cloned into the E1 region of E1α-E3α Ad-strain 5 by homologous recombination. Adenoviruses were produced in 293 cells and purified by double cesium chloride gradient purification as described43. Insulin clamps were performed on 5-h fasted mice. [3-3H]glucose was primed (0.8 μCi) and continuously infused for a 120-min equilibration period (0.04 μCi/min) and a 2-h clamp period (0.08 μCi/min). Baseline blood or plasma parameters were determined in blood samples collected at –10 and –5 min. At t = 0, insulin infusion (2.0 μU per kg body weight per min) was started and continued for 155 min. Blood glucose was clamped using a variable rate of glucose infusion (GIR). Mice received heparinized saline-washed erythrocytes from donors at 4.5 μL/min to prevent a fall of hematocrit. Insulin clamps were validated by assessment of glucose infusion over time. Blood glucose was monitored every 10 min, and the GIR was adjusted as needed. Blood was taken at 80–120 min for the determination of [3-3H]glucose. Clamp insulin was determined at t = 120 and t = 155 min. At 120 min, 13 μCi of [2-14C]deoxyglucose ([14C]2DG) was administered as an i.v. bolus. Blood was taken at 2–35 min for the determination of [14C]2DG. After the last sample, mice were anesthetized, and tissues were collected. Plasma insulin was determined by the Vanderbilt Hormone Assay and Analytical Services Core. Radioactivity of [3-3H]glucose, [14C]2DG, and [14C]2DG-6-phosphate were determined by liquid scintillation counting. Glucose appearance (Rg) and disappearance (Rd) rates were determined using non–steady-state equations44. Endogenous glucose production (endogenous Rg) was determined by subtracting the GIR from total Rg. The glucose metabolic index (Rd) was calculated as previously described45.

FACS-based hypoxia detection. C57BL/6 mice were injected i.v. with Flc, sFlt1 and sFlk1 adenoviruses as described above. At day 14, mice were injected i.p. with pimonidazole hydrochloride 100 mg per kg body weight (Hyoxprobe, Burlington, MA), and liver was harvested after 2 h and disassociated into a single-cell suspension using collagenase IV (Sigma), permeabilized with Triton-X and incubated for 1 h with FITC-conjugated anti-hypoxyprobe antibody (Hyoxprobe, Burlington, MA) (HP2-100, 1:100). Cells were analyzed on a BD LSR II Flow Cytometer (Becton Dickinson) using FlowJo software.

Quantification of microvessel density. Vessel area density percentage of CD31 immunohistochemical and immunofluorescence sections (n ≥ 5 fields) was analyzed by pixel intensity measurements in ImageJ.

RNA isolation and RT-PCR. Total RNA was isolated using Trizol (Invitrogen). RNA samples were reverse transcribed into cDNA using a Reverse Transcription Kit (Invitrogen). Individual gene expression was quantified by SYBR Green RT-PCR (Bio-Rad) and normalized to β-actin. Primer sequences for RT-PCRs are available upon request.

ELISA. Serum insulin was measured by ELISA (ALPCO). Serum glucagon was measured by mouse glucagon ELISA (ALPCO).

Protein immunoblot. The antibodies used were specific for β-actin (8227, 2,000,000) and Gapdh (Abcam ab9485, 1,000). Irs1 (06-248, 1,500) and Irs2 (9,52, 1,250) (Millipore), Akt (1K67, 1,100), p-Akt (4058, 1,100), Gsk-3β (9316, 1,100), p-Gsk-3β (5558, 1,100), Foxo1 (9461, 1,100) (Cell Signaling), Hif-2α (NB100-122, 1,100) (Novus Biologicals) and Hif-1α (A300-286A, 1,500) (Bethyl). The antibodies specific for the following proteins were from Cell Signaling: p-Acc1 (3661, 1,100), Acc1 (3676, 1,100), Akt (2603, 1,100), p-Akt (2535, 1,100), Gsk-3β (4150, 1,100) and p-Gsk-3β (4181, 1,100). Liver lysates were prepared in RIPA buffer. Liver nuclear extracts were prepared using previously described nuclear extraction protocols8. Quantifications were performed by chemiluminescence and densitometric scanning of the films under linear exposure conditions. p-Akt was normalized to total Akt, p-Gsk-3β was normalized to total Gsk-3β and Irs2 was normalized to actin loading control.

Isolation and culture of primary mouse hepatocytes. Primary hepatocytes were isolated from 10- to 12-week-old C57BL/6 mice using a collagenase perfusion method from University of California–San Francisco Liver Center (San Francisco General Hospital, San Francisco, CA). Isolated hepatocytes were resuspended in William’s E medium and plated on collagen I-coated plates in serum-free medium. For hypoxia chamber experiments, cells were incubated at the specific oxygen concentration for 24 h followed by RNA
extraction and gene expression analysis. For in vitro insulin stimulation assay, freshly harvested mouse primary hepatocytes were cultured in serum overnight. Adenoviruses were added to serum-free medium 12 h after plating for 24 h at a multiplicity of infection (m.o.i.) of 10 (Ad-Fc, Ad-HIF-2αPN and Ad-HIF-1αΔODD) or MOI of 20 (Ad-shRNA Irs2 and Ad-shRNA luciferase). Following adenoviral infection, cells were stimulated with 100 nM bovine pancreas insulin (Sigma) for 10 min followed by protein extraction and western blotting. For in vitro Irs2 and Srebf1 expression analysis, primary hepatocytes were infected with adenovirus 12 h after plating for 24 h at an m.o.i. of 10 in serum-free medium. After 24 h, cells were stimulated with 10 nM insulin for 6 h. RNA was extracted and qRT-PCR was performed as described above.

**Lectin perfusion.** Adult C57BL/6 mice received single i.v. injection of Ad-Fc, Ad-sFlt1 or Ad-VEGF Trap (aflibercept)17 (encoding the aflibercept coding sequence) at 1 × 10⁹ PFU each. At day 22, mice were anesthetized with Avertin. 100 µl of biotinylated *L. esculentum* lectin (1 mg/ml in 0.9% NaCl; Vector Laboratories) was injected by tail vein and allowed to circulate for 2 min, and then the mouse was perfused with 1% PFA through the left ventricle. Paraffin sections of liver were developed with streptavidin-HRP and photographed at 20x magnification.

**Transfections.** Human IRS2 promoter-luciferase constructs containing wild-type sequence or mutations of the distal (~900mut) or proximal (~123mut) HREs were generated by site-directed mutagenesis and transfected into mouse H2 hepatocytes with internal *Renilla* luciferase control in the presence of Ad-GFP, Ad-HIF-2αΔODD or Ad-HIF-1αΔODD.

**Microarray analysis.** For comparison of gene expression, 8- to 10-week-old female SCID C17 mice were injected with VEGF Trap (n = 5) or hFc (n = 4) at 25 mg per kg body weight twice weekly for 9 weeks. RNA from liver was extracted as described above and 30 µg of total RNA was used to generate cDNA. Hybridization of sample cDNA to Mouse Exonic Evidence Based Oligonucleotide (MEEBO) arrays was performed by the Stanford Functional Genomics Facility (SFGF). Statistical analysis of gene expression was performed using Significance Analysis of Microarrays software (SAM) and heatmap representation was generated through TreeView.

**Statistical analyses.** All bars show mean ± s.e.m. Significance was calculated using a Student’s *t*-test or one-way analysis of variance with Newman-Keuls post hoc test for comparison of groups greater than two. *P < 0.05.

40. Gruber, M. et al. Acute postnatal ablation of Hif-2α results in anemia. *Proc. Natl. Acad. Sci. USA* **104**, 2301–2306 (2007).
41. Postic, C. et al. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell–specific gene knock-outs using Cre recombinase. *J. Biol. Chem.* **274**, 305–315 (1999).
42. Jacobi, J. et al. Discordant effects of a soluble VEGF receptor on wound healing and angiogenesis. *Gene Ther.* **11**, 302–309 (2004).
43. Ayala, J.E. et al. Hyperinsulinemic-euglycemic clamps in conscious, unrestrained mice. *J. Vis. Exp.* **57**, 3188 (2011).
44. Steele, R., Wall, J.S., De Bodo, R.C. & Altszuler, N. Measurement of size and turnover rate of body glucose pool by the isotope dilution method. *Am. J. Physiol.* **187**, 15–24 (1956).
45. Mulligan, K.X., Morris, R.T., Otero, Y.F., Wasserman, D.H. & McGuinness, O.P. Disassociation of muscle insulin signaling and insulin-stimulated glucose uptake during endotoxemia. *PLoS ONE* **7**, e30160 (2012).