Integrated control of hepatic lipogenesis versus glucose production requires FoxO transcription factors

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Insulin integrates hepatic glucose and lipid metabolism, directing nutrients to storage as glycogen and triglyceride. In type 2 diabetes, levels of the former are low and the latter are exaggerated, posing a pathophysiologic and therapeutic conundrum. A branching model of insulin signalling, with FoxO1 presiding over glucose production and Srebp-1c regulating lipogenesis, provides a potential explanation. Here we illustrate an alternative mechanism that integrates glucose production and lipogenesis under the unifying control of FoxO. Liver-specific ablation of three FoxOs (L–FoxO1,3,4) prevents the induction of glucose-6-phosphatase and the repression of glucokinase during fasting, thus increasing lipogenesis at the expense of glucose production. We document a similar pattern in the early phases of diet-induced insulin resistance, and propose that FoxOs are required to enable the liver to direct nutritionally derived carbons to glucose versus lipid metabolism. Our data underscore the heterogeneity of hepatic insulin resistance during progression from the metabolic syndrome to overt diabetes, and the conceptual challenge of designing therapies that curtail glucose production without promoting hepatic lipid accumulation.
The integrative regulation of hepatic carbohydrate and lipid metabolism by insulin is a key biological question with important ramifications for the pathogenesis and potential treatment of type 2 diabetes. Diabetics overproduce glucose and triglycerides, contributing to the twin abnormalities of this disease, hyperglycaemia and hypertriglyceridaemia. Less clear is how these actions of insulin are mediated, and why they are inextricably linked in the pathogenesis of insulin resistance, the forerunner of type 2 diabetes.

Insulin’s repression of hepatic glucose production (HGP) is mediated by the Irs–Akt–FoxO pathway. Conversely, insulin promotes de novo lipogenesis (DNL), through Akt-mediated activation of Srebp-1c. A widely held model suggests that insulin signalling bifurcates downstream of Akt, to regulate HGP and DNL separately, through these two independent, parallel effectors. In contrast to this model, evidence suggests that FoxOs are also required for normal regulation of lipid synthesis and metabolism.

FoxOs are thought to promote HGP primarily by activating transcription of glucose-6-phosphatase (encoded by G6pc). However, there are two problems with this explanation: first, G6pc enzyme levels are at best modestly correlated with HGP in diabetes, and second, the effect of liver FoxO ablation on HGP vastly exceeds its effect on G6pc. Moreover, mice with a triple knockout of Akt1/Akt2/FoxO1 in the liver (or Irs1/Irs2/FoxO1) have a transcriptional response to fasting and refeeding (F–RF) that is similar to controls, raising the question of whether and how FoxOs are required for the F–RF response.

We expected that a major reason for the mild defects observed after FoxO1 knock out is the redundancy of FoxO3,5. We employed a combination of genetic, metabolic and flux analysis in L–FoxO1,3,4 mice to address two goals: (i) to critically test the model that FoxOs promote HGP but do not regulate DNL and (ii) to examine the requirement for FoxOs during the F–RF transition in healthy mice. We report that FoxOs exert dual control over G6pc and glucokinase (Gck), thus determining whether glucose-6-phosphate is routed towards glucose versus lipid metabolism.

Results

Hepatic FoxOs control the G6pc/Gck ratio. We found that 30% of L–FoxO1,3,4 mice die before weaning (Fig. 1a), possibly because of fatal postnatal hypoglycaemia (Supplementary Fig. 1a). Surviving adult L–FoxO1,3,4 mice presumably have the most robust compensatory mechanisms but still showed low glucose and insulin during daytime ad libitum feeding, and hypoglycaemia after prolonged fasting (Supplementary Fig. 1b–c). In hyperinsulinemic–euglycaemic clamps, L–FoxO1,3,4 mice required double the glucose infusion rate of controls (Fig. 1b). There was no significant difference in glucose disposal; however, L–FoxO1,3,4 mice showed ~60% reduction in HGP (Fig. 1c,d).

We investigated transcriptional mechanisms of metabolic control by FoxO during fasting and refeeding (F–RF). In controls, G6pc peaked at 4h of fasting; however, L–FoxO1,3,4 mice were unable to induce this peak (Fig. 1e). Consistent with this, fasting L–FoxO1,3,4 mice showed delayed glycogen depletion (Fig. 1f). Ighbp1—a canonical FoxO target—showed an expression pattern similar to G6pc; however, there was no defect in the expression of Pck1 in L–FoxO1,3,4 mice (Supplementary Fig. 1d–e). We unexpectedly identified a 40% decrease in expression of the glucose-6-phosphate transporter (Fig. 1g). Thus, this transporter may contribute to FoxO’s control of glucose release from the liver, although it was not strongly regulated by F–RF.

Next, we measured expression of glucokinase (encoded by Gck), which is critical for hepatic glucose utilization and is negatively regulated by FoxOs. In controls, Gck was strongly suppressed during fasting and induced within 1h of RF (Fig. 1h). In contrast, in L–FoxO1,3,4 mice, Gck was hardly suppressed during fasting. Notably, Gck expression in L–FoxO1,3,4 mice fasted for 24h was similar to controls RF for 1h, demonstrating that inactivating FoxOs mimics the early effect of refeeding on Gck.

As G6pc opposes Gck to control the intracellular gradients of glucose and glucose-6-phosphate, the ratio of the two enzymes may reflect the direction of glucose flux. In control mice, the G6pc/Gck ratio was potently induced during fasting, consistent with increased hepatic glucose output; however, this effect was completely absent in L–FoxO1,3,4 mice (Fig. 1i). Furthermore, the ratio of G6pc/Gck was the strongest predictor of glycaemia in L–FoxO1,3,4 pups (R = 0.75; P = 0.0001; Fig. 1j). This was not true in control pups, which showed nearly undetectable Gck, as expected.

To buttress the conclusion that FoxOs are important for regulation of the transcriptional response F–RF, we performed unbiased surveys of expressed mRNAs. Analyses of global gene expression patterns after 22h fasting or 4h RF revealed that fasted L–FoxO1,3,4 mice largely resemble RF controls (Supplementary Fig. 1h). This demonstrated that inactivating FoxOs mimics the effect of RF on a large proportion of nutritionally regulated genes. Finally, we confirmed that FoxOs are required for induction of G6pc and suppression of Gck using primary hepatocytes derived from control or L–FoxO1,3,4 mice (Supplementary Fig. 1i).

Glucose cycling and DNL in FoxO-deficient mice. To investigate glucose uptake and utilization, we injected mice with a mixture of two stable isotope-labelled glucose. In control mice, both tracers peaked in blood 30 min after injection and were cleared within 2h (Fig. 2a,b). At every time point, both tracers were reduced in L–FoxO1,3,4 mice (Fig. 2a,b), demonstrating increased glucose uptake and utilization. This can likely be attributed to the liver because glucokinase activators increase liver glucose uptake and glycolysis, and because the rate of glucose disposal in the clamp (primarily reflecting glucose uptake in muscle and fat) showed no significant difference. Total plasma glucose and insulin were reduced in L–FoxO1,3,4, as expected (Supplementary Fig. 2a–c). However, the fractional difference in enrichment of the two labels was the same in both genotypes (Fig. 2c), indicating no difference in the rate of glucose cycling.

Increased Gck is expected to increase DNL and suppress glucose uptake and utilization. We next measured the rate of DNL during F–RF. In control mice, DNL was low after an 18h fast and quadrupled after 5h RF; however, L–FoxO1,3,4 mice showed two to three times higher rates of DNL at both time points (Fig. 2d). We noted that DNL in 18h-fasted L–FoxO1,3,4 mice was nearly as high as in 5h-refed controls, indicating that FoxO inactivation mimics the effect of 5h refeeding on lipogenesis.

We next investigated lipogenic pathways that could explain the increased DNL. During prolonged fasting (when L–FoxO1,3,4 mice have ~3 times higher DNL), we found no difference between genotypes in mRNA expression of target genes of canonical lipogenic transcription factors Srebp-1c and Chrebp, including Acaca, Fasn, Elovl6 and Pklr (Fig. 2e). Thus, increases in Srebp-1c or Chrebp function cannot explain the increased lipogenesis in L–FoxO1,3,4 mice, while increased Gck can (Fig. 2f).

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Figure 1 | Glucose parameters in L–FoxO1,3,4 mice. (a) Percentage of pups surviving to weaning at 21 days. n = 70 control and 24 L–FoxO1,3,4 pups. ***P < 0.001 by Fisher’s exact test. These numbers may underestimate the true mortality of L–FoxO1,3,4 pups; at genotyping (day 9) they are already present at less than Mendelian ratios. (b–d) During hyperinsulimemic–euglycaemic clamp (n = 8 controls, 6 L–FoxO1,3,4): (b) glucose infusion rate (GIR); (c) rate of glucose disposal (Rd); (d) glucose production (GP) **P < 0.01 by Student’s t-test (two-tailed). (e–i) Liver gene expression and glycogen content during F-RF time course (n = 4–7, exact n for each time point and genotype listed in Methods): (e) G6pc expression; (f) liver glycogen; (g) Slc37a4, encoding the glucose-6-phosphate transporter; (h) Gck; (i) G6pc/Gck ratio. ***P < 0.001, **P < 0.01, *P < 0.05 for control versus L–FoxO1,3,4 mice by Student’s t-test (two-tailed). Black and white bars indicate the dark/light cycle. (j) Correlation between glucose levels and the G6pc/Gck ratio in pups at P2. Data are mean ± s.e.m.
Metabolomic and lipidomic analyses revealed that L–FoxO1,3,4 livers had increased G6P, pyruvate, triglycerides, and diglycerides (Fig. 2g,h, Supplementary Table 1), consistent with increased glycolysis and lipogenesis. Other metabolites showed no significant differences, except a trend towards reduced malate, potentially indicating that TCA cycle flux is preferentially re-routed into lipogenesis. The increased diglycerides were surprising, as they have been suggested to engender insulin resistance36, and L–FoxO1,3,4 mice are exceedingly insulin-sensitive.

Our data suggest that derepression of Gck may be an exquisitely insulin-sensitive mechanism to activate DNL23,24,35 independently of canonical lipogenic pathways. We examined this in more detail in C57BL/6J mice during F–RF. Both Elovl6 (Srebp-1c target) and Pklr (Chrebp target) were reduced during fasting and increased after refeeding, as expected (Fig. 3a). In contrast, the fluctuations of Gck were greater during F–RF, and peaked within 1 h of refeeding, hours before Srebp-1c and Chrebp targets. We confirmed that Gck protein was induced within 1 h of refeeding (Supplementary Fig. 3a).

New model of insulin control of hepatic glucose versus lipid metabolism. Our data demonstrate that FoxOs regulate both glucose and lipid production, and suggest a new model of the regulation of insulin of these two processes. Instead of parallel functions through FoxOs and Srebp-1c, we propose that insulin
Discussion

The key novel conclusions of this work are as follows: (i) FoxOs are required for the dynamic regulation of $G6pc/Gck$ during F-RF; (ii) this reciprocal regulation is a critical step in controlling hepatic glucose output and DNL; and (iii) short-term WTD-feeding induces a phenotype resembling liver FoxO inactivation.

Data presented in this study suggest a new model of glucose and lipid dysregulation in the pathophysiology of insulin resistance, based on successive defects in insulin action (Fig. 4f): in mild/early insulin resistance, portal hyperinsulinemia drives FoxO inactivation. This leads to a decrease in the $G6pc/Gck$ ratio and excess DNL at the expense of hepatic glucose output. As the disease advances, there is a further rise in insulin and glucose levels, leading to induction of Srebp-1c/Chrebp-dependent lipogenesis. In this severe disease setting, FoxOs can be reactivated through oxidative stress or an adverse kinase profile favouring their nuclear retention.

This model, while partly still speculative, reconciles our molecular understanding of hepatic insulin signalling with the known clinical features of diabetes progression. Thus, in the early phases of the disease, hyperglycaemia is mostly postprandial, but liver steatosis is already present. In the advanced disease, fasting hyperglycaemia accounts for a growing fraction of total glycaemic variation. In this setting, the combination of active transcription factors heralds the increase in both HGP and lipogenesis that is typical of the diabetic state.

Our data also help to reinterpret studies showing that hepatic insulin signalling occurs normally in the absence of Akt1/Akt2/FoxO1 (ref. 5) or Irs1/Irs2/FoxO1 (ref. 4). We show that the gene expression profile of fasted mice lacking the three FoxOs is strikingly similar to that of fed wild-type controls. This, in addition to the early postnatal death of a fraction of triple knockouts, and the profound flux abnormalities described in the survivors, should disabuse us of the notion that the FoxOs can be done away with. First, we suggest that the combined knockouts of Akt or Irs with FoxO1 did not account for the functions of FoxO3a and 4, which we show to be substantial. Second, the ability of insulin to suppress HGP in clamp studies of Akt1/Akt2/FoxO1 mutants, which we confirm in triple FoxO knockouts in the present study, likely reflects two things: (1) the extrahepatic actions of insulin, for example, through free fatty acids (FFAs) or the central nervous system (CNS)2, and (2) the contrived conditions of the clamp, wherein insulin levels are raised many fold over the physiologic range.

The novel models of insulin–FoxO regulation of HGP and DNL in normal physiology (Fig. 3b) and in the pathophysiology of diabetes (Fig. 4f) underscore the conceptual advances arising from this work. These findings should prompt a reappraisal of the heterogeneity of hepatic insulin resistance during the transition
from the metabolic syndrome to overt diabetes. They also provide a powerful illustration of the conceptual and regulatory challenges underlying the development of new treatments for this condition.

**Methods**

**Mice and diets.** L–FoxO1,3,4 mice have been described7. Only males at least 16 weeks old were studied, except in studies of 2-day-old pups, where pups of both genders were used. Male C57BL/6J mice were purchased from Jackson Laboratory and were studied when they were 9 weeks old. All mice were fed chow, except in the experiment shown in Fig. 4a–e, where C57BL/6J mice were challenged with WTD for 1 week. WTD is from Harlan Teklad (TD.88137); it contains 30% kcal from sucrose and 42% from fat, and causes insulin resistance.12 Mice were maintained on a 12-h light–dark cycle (lights on at 0700 hours). The Columbia University Institutional Animal Care and Utilization Committee approved all experiments.
Metabolic tests. Blood glucose was measured using OneTouch glucose monitor and strips (LifeScan). Insulin ELISAs were from Millipore. Hyperinsulinenic-euglycemic clamps were carried 5–6 h after removing food. We infused a solution of glucose (10%) at a variable rate to maintain plasma glucose ~8 mM. Mice received a constant infusion of [3-3H] glucose (0.1 mCi/min−1) and insulin (3.6 μIU/kg body weight per min). We collected plasma samples to determine glucose levels at times 10, 20, 30, 40, 50, 60, 70, 80 and 90 min after beginning insulin and glucose infusions, and measured the specific activities of [3-3H] glucose and [6,6-2H2] water at 40, 50, 60, 70, 80 and 90 min after beginning the insulin and glucose infusions. We achieved steady-state conditions for plasma glucose concentration and specific activity within 40 min from the beginning of the insulin and glucose infusions. 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36. Samuel, V. T. & Shulman, G. I. Mechanisms for insulin resistance: common threads and missing links. *Diabetes* **51**, 537–551 (2002).

37. Shimomura, I. et al. Decreased hepatic futile cycling compensates for increased glucose production in fasting and obesity. *Diabetes* **55**, 3372–3380 (2006).

38. Sun, Z. et al. Determination of a glucose-dependent futile recycling rate constant from an intraperitoneal glucose tolerance test. *Anal. Biochem.* **315**, 238–246 (2003).

39. Ferrer, T., Riu, E., Franckhauser, S., Agudo, J. & Bosch, F. Long-term overexpression of glucokinase in the liver of transgenic mice leads to insulin resistance. *Diabetologia* **46**, 1662–1668 (2003).

40. Samuels, V. T. & Shulman, G. I. Mechanisms for insulin resistance: common threads and missing links. *Cell* **148**, 852–871 (2012).

41. Shimomura, I. et al. Decreased IR-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).

42. Sun, Z. et al. Hepatic HdaC3 promotes gluconeogenesis by repressing lipid synthesis and sequestration. *Nat. Metab.* **5**, 934–942 (2022).

43. Qiang, L. & Accili, D. Uncoupling of acetylation from phosphorylation regulates FOXO1 function independent of its sub-cellular localization. *J. Biol. Chem.* **285**, 27396–27401 (2010).

44. Kitamura, Y. I. et al. FoxO1 protects against pancreatic beta cell failure through NeuroD and MafA induction. *Cell Metab.* **2**, 153–163 (2005).

45. Ozcan, L. et al. Calcium signaling through CaMKII regulates hepatic glucose production in fasting and obesity. *Cell Metab.* **15**, 739–751 (2012).

46. Bugianesi, E. et al. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. *Diabetologia* **48**, 634–642 (2005).

47. Monnier, L., Colette, C., Dunseath, G. J. & Owens, D. R. The loss of postprandial glycemic control precedes stepwise deterioration of fasting with worsening diabetes. *Diabetes Care* **30**, 263–269 (2007).

48. Okamoto, H., Obici, S., Accili, D. & Rossetti, L. Restoration of liver insulin signaling in Insr knockout mice fails to normalize hepatic insulin action. *J. Clin. Invest.* **115**, 1314–1322 (2005).

49. Haas, J. T. et al. Hepatic insulin signaling is required for obesity-dependent expression of SREBP-1c mRNA but not for feeding-dependent expression. *Cell. Metab.* **15**, 873–884 (2012).

50. Vaitheesvaran, B. et al. Peripheral effects of FAAH deficiency on fuel and energy homeostatic role of dysregulated lysine acetylation. *PLoS ONE* **7**, e33717 (2012).

51. Barber, M. N. et al. Plasma lysophosphatidylcholine levels are reduced in obesity and type 2 diabetes. *PLoS ONE* **7**, e41456 (2012).

Acknowledgements

This work was supported by the US National Institutes of Health grants HL111206 (R.A.H., DK57539, DK58282 [D.A.], DK58132 [I.J.K.], DK45024 [R.G.-J.], DK63608 [Columbia Diabetes Research Center], and DK020541 [Einstein Diabetes Research and Training Center]. Additional support was from 3R07DK058282, a grant for Collaborative Activities to Promote Metabolomics Research (NOT-RM-11-024). M.A.F. is supported by a Senior Principal Research Fellowship (APP1021168) and Project Grant (APP1007465) from the National Health and Medical Research Council of Australia. We thank Rudy Leibel, Utpal Pajvani, Alan Tall, Ira Tabas, as well as members of the Accili, Febbraio and Kurland laboratories, for insightful discussion of the data. We acknowledge excellent technical support from A. Flete, T. Kolar and J. Lee.

Author contributions

R.A.H. designed and performed experiments, analysed data and wrote the manuscript. K.H., B.V., I.A.-C., C.M.K, H.L.K. and R.G.-J. designed and performed experiments and analysed data. M.A.F., I.J.K. and D.A. designed the experiments, analysed the data and wrote the manuscript.

Additional information

Accession codes: Microarray data have been deposited in Gene Expression Omnibus under accession code GSE60527.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Haeseler, R. A. et al. Integrated control of hepatic lipogenesis versus glucose production requires FoxO transcription factors. *Nat. Commun.* 5:5190 doi: 10.1038/ncomms6190 (2014).