HHS Public Access

Author manuscript

Nat Med. Author manuscript; available in PMC 2012 September 01.

Published in final edited form as:

Nat Med. ; 18(3): 388–395. doi:10.1038/nm.2686.

Insulin regulates liver metabolism in vivo in the absence of hepatic Akt and Foxo1

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Abstract

Considerable data support the idea that Foxo1 drives the liver transcriptional program during fasting and is inhibited by Akt after feeding. Mice with hepatic deletion of *Akt1* and *Akt2* were glucose intolerant, insulin resistant, and defective in the transcriptional response to feeding in liver. These defects were normalized upon concomitant liver–specific deletion of *Foxo1*. Surprisingly, in the absence of both Akt and Foxo1, mice adapted appropriately to both the fasted and fed state, and insulin suppressed hepatic glucose production normally. Gene expression analysis revealed that deletion of *Akt* in liver led to constitutive activation of Foxo1–dependent gene expression, but once again concomitant ablation of *Foxo1* restored postprandial regulation, preventing its inhibition of the metabolic response to nutrient intake. These results are inconsistent with the canonical model of hepatic metabolism in which Akt is an obligate intermediate for insulin's actions. Rather they demonstrate that a major role of hepatic Akt is to restrain Foxo1 activity, and in the absence of Foxo1, Akt is largely dispensable for hepatic metabolic regulation *in vivo*.

INTRODUCTION

Eukaryotic organisms have developed mechanisms of varying complexity to deal with periods of starvation and nutritional abundance. In mammals, the obligate requirement for at

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AUTHOR CONTRIBUTIONS

M.L. conceived the hypothesis, designed and performed the experiments, and analyzed the data. M.W. and K.F.L. performed experiments. Q.C., B.R.M. and S.F. provided technical assistance. The R.S.A. lab performed the hyperinsulinemic–euglycemic clamps experiments. K.U. and C.R.K. generated the *Akt1*$^{loxP/loxP}$ mice. M.J.B. conceived the hypothesis and directed the project. M.L. and M.J.B. prepared the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

least some simple carbohydrate at all times is fulfilled during fasting by liver, which initially breaks down glycogen before transitioning to gluconeogenesis as a means to release glucose into circulation. Just as phylogenetically conserved adaptations to fasting are critical to survival, a complex interplay of hormonal, neural and nutritional signals drive the coordinated response to feeding. Ultimately, the ability of an organism to survive dietary deprivation depends on the effectiveness of its nutrient storage during the postprandial state as well as the mobilization of those nutrients.

Almost since its discovery, researchers have recognized the importance of insulin to the metabolic transition that accompanies feeding[1]. In recent years, there has emerged a clear picture of the insulin signaling pathway, beginning with insulin's interaction with its receptor, which phosphorylates the scaffolding protein family, insulin receptor substrate. This initiates a linear signaling cascade that culminates in the phosphorylation of Akt protein kinases[1–10]. Once activated, Akt kinases utilize several distinct downstream pathways to modulate metabolism. One branch involves phosphorylation and inactivation of the Tsc1–Tsc2 complex, leading to activation of the mTorc1 complex and its immediate downstream target p70 S6 kinase, which promotes protein translation and cell growth[11,12]. Activation of mTorc1 correlates with and is most likely necessary for insulin–induced accumulation of the sterol regulatory element binding transcription factor 1 (Srebf1, also known as Srebp1c), which drives the lipogenic program[12–17]. On another branch, Akt phosphorylates and inactivates glycogen synthase kinase 3 (Gsk3α, Gsk3β), resulting in glycogen synthase activation and glycogen accumulation, as well as reduced phosphorylation and degradation of Srebf1[18]. On the third branch, Akt phosphorylates and inactivates the Foxo family of transcription factors, which is responsible for the decrease in transcription of genes encoding gluconeogenic enzymes[19–23].

In liver, Foxo1 collaborates with the co–activators peroxisome proliferator–activated receptor gamma co–activator 1α (Ppargc1a) and Creb regulated transcription co–activator 2 (Crtc2) to increase coordinately the expression of the genes *glucose–6–phosphatase, catalytic subunit* (*G6pc*) and *cytosolic phosphoenolpyruvate carboxykinase 1* (*Pck1*)[24–26]. It is generally accepted that Foxo1 is active during fasting, inactivated by Akt–mediated phosphorylation after feeding, and that the antagonism of Foxo1 by Akt is the predominant mechanism by which insulin suppresses hepatic glucose output after a meal[1,26–29]. Data contributed by a number of laboratories in support of this model include the following observations: 1) insulin induces phosphorylation and activation of Akt after feeding; 2) activated Akt phosphorylates Foxo1 at T24, S253, and S316 of the mouse protein; 3) phosphorylated Foxo1 translocates out of nucleus; and 4) transcription of Foxo1–dependent genes, such as *G6pc*, *Pck1*, *insulin–like growth factor binding protein 1* (*Igfbp1*), and *insulin receptor substrate 2* (*Irs2*), is reduced[19–22,25–27,30]. Nonetheless, there are several gaps and apparent contradictions in the knowledge concerning Foxo1. For example, liver–specific ablation of *Foxo1* results in mildly improved insulin sensitivity and hypoglycemia occurs only after prolonged fasting in adult mice. In addition, in these mice there is a decrease in expression of *Irs2*, *G6pc*, *Pck1*, *Ppargc1a*, and *Igfbp1*, but little or no change in the expression of *glucokinase* (*Gck*) or lipogenic genes[31,32]. Perhaps one reason for confusion regarding the physiological role of Foxo1 is that much of the information about its functions

in hepatic metabolism has been obtained from insulin resistant livers, in which unchecked Foxo1 activation results in profound metabolic abnormalities in gluconeogenesis as well as lipid metabolism and glycogen storage[6,31]. Antagonizing or reducing hepatic Foxo1 activity in insulin–resistant mice significantly improves glucose tolerance and insulin responsiveness[6,23,28,31,33,34]. Dramatic examples of this are studies using the *insulin receptor* knockout mice or mice with liver–specific knockout of both *insulin receptor substrate*, *Irs1* and *Irs2*, for which concomitant deletion of hepatic *Foxo1* reverses much of the metabolic defect[6,31].

Current data support a model in which Akt is an obligate insulin signaling intermediate that suppresses expression of genes encoding gluconeogenic enzymes via inhibition of Foxo1, which is active during fasting. In this study, we test an alternative interpretation of these data. We found that inhibition of Foxo1 is a major role of hepatic Akt, such that once Foxo1 is removed, most of normal metabolic regulation is maintained in the absence of liver Akt. These data refute the notion that insulin signals through Akt under all conditions but rather suggest an alternative, previously unrecognized mechanism through which liver responds to nutrients and insulin.

RESULTS

Liver–specific deletion of *Akt1* in *Akt2* null mice results in hyperglycemia

In mouse liver, Akt2 accounts for 84% of total Akt protein, the remainder being Akt1 with Akt3 not detectable[35]. Whole body *Akt2* null mice ($Akt2^{-/-}$) display a mildly diabetic phenotype on a mixed 129–C57BL/6J background[4]. A plausible explanation for the relative mildness of the glucose intolerance is that residual Akt1 in liver of the $Akt2^{-/-}$ mice partially rescued insulin responsiveness. To address this question, we bred $Akt1^{loxP/loxP}$;$Akt2^{-/-}$ mice to *Afp–Cre* mice, which express *Cre* recombinase specifically in liver[36], to generate mice with systemic *Akt2* and liver–specific *Akt1* deletion. *Akt1* deletion in liver of $Akt2^{-/-}$ mice led to severe hyperglycemia, whether compared to wildtype or $Akt2^{-/-}$ mice (Fig. 1a). In addition, these mice lacked Gck protein in liver, and had increased Igfbp1 protein both in liver and in serum (Fig. 1b). Since *Igfbp1* is a major target gene for Foxo1[6], these data suggested Foxo1 activation in these livers.

Insulin signaling defects in the liver–specific *Akt1*, *Akt2* double knockout mice

To understand in more detail the hepatic functions of Akt kinases without the complications of peripheral insulin resistance[4], we injected $Akt2^{loxP/loxP}$ mice and $Akt1^{loxP/loxP}$;$Akt2^{loxP/loxP}$ mice with adeno–associated virus expressing *Cre* recombinase under the control of the *Tbg* promoter (AAV–*Tbg–Cre*), which led to the liver–specific deletion of *Akt2* alone (*2LKO*) and both *Akt1* and *Akt2* (*DLKO*), respectively (Fig. 2a, b). $Akt2^{loxP/loxP}$ mice (*2LWT*) and $Akt1^{loxP/loxP}$;$Akt2^{loxP/loxP}$ mice (*DLWT*) injected with virus expressing GFP served as controls. Expression of GFP or Cre in wildtype livers using this viral delivery system did not result in any detectable change in carbohydrate or lipid metabolism (data not shown).

Western blot confirmed efficient deletion of *Akt1* and *Akt2* in hepatocytes from the *DLKO* mice 2 weeks after virus injection (Fig. 2a). There was no change in Akt1 or Akt2 protein in muscle or adipose tissue. To investigate the effect of removing Akt kinases on hepatic insulin signaling, we fasted the mice overnight and then fed the mice with normal chow. In comparison to the *2LWT* control mice, indices of insulin–dependent signaling, such as phosphorylation of ribosomal protein S6, phosphorylation of Gsk3α and Gsk3β, and decrease in Igfbp1 protein, remained relatively unchanged in *2LKO* livers after feeding (Fig. 2b). In spite of Akt2 contributing the majority of Akt protein in liver, postprandial phosphorylation of Akt at S473 was unchanged in *2LKO* livers. This is likely a reflection of the low stoichiometry of Akt phosphorylation in the prandial state and the ability of Akt1 to compensate for deficiency in Akt2. To test this possibility, we injected a supraphysiological dose of insulin into the *2LWT* and *2LKO* mice. 20 minutes after insulin injection, Akt phosphorylation at S473 was notably lower in the *2LKO* livers compared to the control livers (Supplementary Fig. 1). These data indicate that chow feeding led to phosphorylation of only a small fraction (< 4%) of the endogenous Akt.

In the *DLKO* livers, the feeding–induced phosphorylation of Akt at S473 was decreased to virtually undetectable levels (Fig. 2b). S6 phosphorylation was diminished but phosphorylation of Gsk3α and Gsk3β was preserved. The normal phosphorylation of Gsk3α and Gsk3β likely reflected higher expression in the *DLKO* livers of *serum–glucocorticoid regulated kinase 1* (*Sgk1*) (Fig. 2c), which is known to phosphorylate Gsk3α and Gsk3β in response to insulin[37]. Erk2 kinases, which are downstream of insulin receptor in a pathway parallel to Akt, were activated normally in the *DLKO* livers after feeding (Fig. 2b).

Disrupted expression of the Foxo1–regulated genes in the *DLKO* livers

The Foxo1 transcription factor represents a major target of Akt. Expression of *Irs2*, a direct Foxo1 target gene[6], was significantly higher in the *DLKO* compared to the control livers (Fig. 2c). Other Foxo1–dependent genes, such as *Ppargc1a* and *Igfbp1*[38–40], were also induced in the *DLKO* livers (Fig. 2c). The expression of *Gck*, a Foxo1–suppressed gene[6,27,41], was markedly lower in the *DLKO* livers compared to the control livers. Expression of *G6pc* and *Pck1* in the *DLKO* livers was not different from the control livers during fasting, but the normal suppression of these genes after feeding was lost in the *DLKO* livers (Fig. 2c). Consistent with altered gene expression, Igfbp1 protein was substantially more abundant in both liver and serum upon deletion of both *Akt1* and *Akt2* from liver, and Gck protein was almost undetectable (Fig. 2b). Taken together, these data indicate strongly that activation of Foxo1 is an important consequence of deleting all Akt from the liver.

Hyperglycemia, glucose intolerance and insulin resistance in the *DLKO* mice

2 weeks after virus injection, the fasting blood glucose concentration in the *DLKO* mice trended to be higher than the control mice but the difference did not reach statistical significance (Fig. 3a). However, the blood glucose concentration after overnight fast and 4 hours feeding was significantly higher in these mice (Fig. 3b). Glycogen content was considerably lower in the *DLKO* livers 4 hours after feeding (Supplementary Fig. 2). 3 weeks after virus injection, blood glucose concentrations in the *DLKO* mice during both fasting and refeeding were higher than in control mice (not shown). To minimize the

potential secondary effects resulting from hyperglycemia, subsequent experiments were performed 2 weeks after virus injection.

When challenged with glucose, the *DLKO* mice were glucose intolerant (Fig. 3c). Serum insulin concentration during both fasting and the glucose tolerance test were higher than the control mice (Fig. 3d), suggesting insulin resistance in these mice. This was confirmed by a hyperinsulinemic–euglycemic clamp (Fig. 3e), which revealed a much lower glucose infusion rate and failure to suppress liver glucose output by insulin in the *DLKO* mice. Glucose disposal rate in the *DLKO* mice was also lower than in the control mice (Fig. 3e and Supplementary Fig. 3), indicating that these mice developed peripheral insulin resistance as a result of removal of *Akt1* and *Akt2* in liver. In most of these assays, *2LKO* mice were indistinguishable from control mice, though there was a small but significant blunting of postprandial glycogen storage (Supplementary Fig. 2) and insulin–dependent suppression of hepatic glucose output (Fig. 3f).

Effects of liver–specific *Foxo1* deletion from wildtype and *DLKO* mice

Since Akt suppresses Foxo1 activity, if activation of Foxo1 were responsible for the glucose intolerance and altered gene expression in *DLKO* mice, deletion of *Foxo1* should largely reverse the abnormal metabolism. Mice with liver–specific chronic deletion of *Foxo1* developed hypoglycemia after prolonged fasting[31,32]. To assess the effect of acute removal of *Foxo1* from liver, we injected the *Foxo1*$^{loxP/loxP}$ mice[31] with AAV–*Tbg–Cre*. Efficient deletion of *Foxo1* was confirmed by the decrease in both mRNA and protein (Supplementary Fig. 4a and 4b). These mice displayed hypoglycemia after overnight fasting but euglycemia after feeding (Supplementary Fig. 4c and 4d). To investigate whether unrestrained activation of Foxo1 in the *DLKO* livers was indeed responsible for the metabolic disturbances in the *DLKO* mice, we deleted *Foxo1* from the *DLKO* livers. Injection of AAV–*Tbg–Cre* into *Akt1*$^{loxP/loxP}$;*Akt2*$^{loxP/loxP}$;*Foxo1*$^{loxP/loxP}$ mice resulted in the liver–specific recombination of all three genes (*TLKO*), as confirmed by Western blotting (Fig. 4a). Mice injected with virus expressing GFP instead of *Cre* served as controls (*LWT*). In the *TLKO* mice, fasting blood glucose concentration was indistinguishable from that in control mice (Fig. 4b). Hyperglycemia present 4 hours after feeding in the *DLKO* mice was normalized in the *TLKO* mice (Fig. 4c). Deletion of *Foxo1* also reversed the glucose intolerance and fasting hyperinsulinemia in the *DLKO* mice (Fig. 4d, 4e). In contrast, liver glycogen 4 hours after feeding remained lower in the *TLKO* livers than the control livers (Supplementary Fig. 5), despite *Foxo1* deletion normalizing the suppressed glucose ↔ glucose–6–phosphate cycling in the *DLKO* livers (Supplementary Fig. 6).

In hepatocytes from both *DLKO* and *TLKO* mice, phosphorylation of Akt at S473, Foxo1 at T24, Foxo4 at T28 was not detectable (Fig. 4a). In control fasted mice, insulin injection quickly induced robust phosphorylation of Akt, S6, and direct Akt substrates such as Foxo1, Tsc2[42], and Akt1s1 (also known as Pras40)[43] in liver (Fig. 4f). These phosphorylation events were either lost or markedly blunted in both the *DLKO* livers and the *TLKO* livers. In line with these observations, no compensatory increase in Akt3 was detected in either the *DLKO* livers or the *TLKO* livers (Supplementary Fig. 7). Phosphorylation of Gsk3α and

Gsk3β was maintained in both *DLKO* livers and *TLKO* liver, though to lesser extent in the latter (Fig. 4f).

Similar to insulin injection, feeding elicited enhanced phosphorylation of Akt at S473, Foxo1 at S253 and T24, and Akt1s1 in the control livers, but not in the *DLKO* or *TLKO* livers (Fig. 4g). Gsk3α and Gsk3β phosphorylation was induced after feeding in the *DLKO* liver but not in the *TLKO* livers, correlating with the increase of *Sgk1* in the *DLKO* livers and its normalization in the *TLKO* livers (Supplementary Fig. 8). In the *TLKO* livers, Gck protein levels were partially normalized, and Igfbp1 protein was restored to the same level as in the control mice (Fig. 4g), consistent with Foxo1 activity being considerably higher in the *DLKO* livers than the control or *TLKO* livers.

Normal response to feeding and insulin in livers lacking both Akt and Foxo1

The above data are consistent with the prevailing view that Akt drives adaptation to the fed state by suppressing Foxo1–dependent gene expression, such that defects resulting from ablation of Akt signaling are reversed by concomitant removal of Foxo1 (Fig. 4). Analogous data have been interpreted as reflecting a linear pathway in which Foxo1 promotes transcription of those genes encoding rate–determining gluconeogenic enzymes. However, a plausible alternative view that is compatible with both published and our new data is that Foxo1 also represses a parallel, Akt–independent signaling pathway that controls the prandial response (Fig. 5a). To distinguish between these models, expression of critical metabolic genes was measured in the fasted state and after feeding. If the canonical view is correct, deletion of *Foxo1* alone or deletion of both *Akt* and *Foxo1* should “lock” gene expression into the fed state, mimicking the continual presence of insulin. However, if insulin–dependent inhibition of Foxo1 serves primarily a permissive function, then deletion of *Foxo1* alone or deletion of both *Akt* and *Foxo1* would restore normal postprandial regulation. Acute deletion of *Foxo1* from liver did not significantly alter the expression of *G6pc*, *Pck1* and *Igfbp1* during fasting or after feeding, and the fasting to feeding transition was largely normal (Fig. 5b). As shown in Figure 5c, in control animals feeding induced the expected regulation of gene expression, as indicated by decreases in *Ppargc1a*, *Igfbp1*, *Pck1*, and *G6pc*, and increases in *Srebf1*, *HMG-CoA reductase* (*Hmgcr*), *HMG-CoA synthase* (*Hmgcs*) and *Gck*. In the *DLKO* livers, the expression of these genes under the fasted and the fed state resembled that of the fasted state in the control livers, though *Ppargc1a*, *Igfbp1*, *Srebf1*, and *Hmgcr* continued to be regulated to some extent by feeding. Of note, for most genes tested, the *TLKO* livers responded to feeding in a manner indistinguishable from control livers, indicating that their regulation in these livers was through a mechanism independent Akt and Foxo1. These data support a permissive role for Akt–mediated suppression of Foxo1 rather than or in addition to Akt acting as an obligate intermediate in the postprandial signaling pathway.

In order to assess the scope of Foxo1’s effects on expression of metabolically responsive genes, mRNA from *LWT*, *DLKO* and *TLKO* livers were subjected to genome–wide expression analysis. 688 genes were classifies as “metabolically responsive”, as defined by having at least 2–fold change from fasting to feeding in control livers (Supplementary Table 1). Of these, 298 genes showed altered expression in the *DLKO* livers compared to control

livers four hours after feeding (Supplementary Table 2), indicating that Akt was critical to their regulation. As shown in the "heatmap" in Supplementary Figure 9, for most members of the 298 metabolically regulated genes, their expression in the *DLKO* livers in both fasted and fed state behaved like the fasted state in control livers (compare columns 3 and 4 to column 1) and the dysregulated gene expression was largely reversed in the *TLKO* livers (compare columns 1, 2 to columns 5, 6). Whether expression was induced or suppressed in the control livers after feeding, the disruption in regulation in the *DLKO* livers was at least partially reversed in the *TLKO* livers, reestablishing appropriate postprandial adaptation. Given that an analogous experiment has been performed with liver–specific *Irs1*;*Irs2* double knockout mice[6], we compared the ratio of gene expression in mutant to wildtype mice for liver–specific *Irs1*;*Irs2*[6] and *Akt1*;*Akt2* knockout mice in the fasted and fed states (Supplementary Fig. 10). As predicted, there was a striking concurrence between the changes resulting form deletion of the two *Irs* genes and the two *Akt* genes in liver. The largely appropriate switch in gene expression from the fasted to fed state in the *TLKO* and the *Irs1*;*Irs2*;*Foxo1* triple knockout livers demonstrates that livers lacking *Foxo1* and much of the proximal insulin signaling pathway are capable of mounting a normal transcriptional response to nutritional intake.

The restoration of normal regulation in *TLKO* liver raised two important questions: 1) does the appropriate postprandial response in livers null for *Akt* and *Foxo1* reflect a reestablishment of insulin action? and 2) are the changes in gene expression in *TLKO* mice accompanied by a corresponding suppression of hepatic glucose output? To dissociate the effects of insulin and nutrients on gene expression, mRNA encoding key regulatory proteins was measured in control and *TLKO* livers during euglycemic clamp with and without infusion of insulin. As shown in Figure 5d, expression of *G6pc* and *Pck1* was suppressed by insulin to the same extent in the *TLKO* livers as in the control livers, indicating that insulin was able to suppress the transcription of these two genes independent of Akt and Foxo1 in liver. Notably, insulin also suppressed glucose production in the *TLKO* mice in a manner that was indistinguishable from the control mice, as ascertained by euglycemic clamp (Fig. 5e). These data further support the permissive role for Akt–dependent inhibition of Foxo1, as normal regulation persists in the absence of both proteins.

The data presented above indicated that Foxo1 exerts a suppressive effect on postprandial insulin–responsive pathway that signals independent of Akt. To ask whether this alternative pathway is the sole mediator of insulin action or exists in parallel to the canonical pathway, hepatocytes were isolated from the *TLKO* livers and tested for their response to insulin in vitro. As a comparison, hepatocytes from the *Foxo1*$^{-/-}$ livers were also tested. As shown in Figure 6, in both *Foxo1*$^{-/-}$ and *TLKO* primary hepatocytes, the expression of *G6pc*, *Pck1* and *Igfbp1* was substantially lower than in control cells and did not respond to addition of hormone. Thus, when studied *ex vivo*, liver cells behaved as if insulin signals through the canonical linear pathway of Akt–dependent inhibition of Foxo1–driven gluconeogenic gene expression (Figure 5a).

DISCUSSION

In the present study, we used genetic manipulation of the mouse genome to study the tissue–specific, physiological functions of Akt and Foxo1 in insulin–regulated metabolic responses in liver. We established that hepatic Akt is essential in maintaining whole body glucose homeostasis and insulin responsiveness, and that Akt kinases execute these functions primarily through suppressing Foxo1. However, we also made several unexpected observations that cannot be explained by the prevalent, long–held model in which insulin regulates glycemia via Akt–dependent suppression of Foxo1–driven metabolic gene expression. First, and most surprisingly, feeding, and to a significant extent insulin alone, suppresses hepatic glucose output and appropriately regulates gene expression in the absence of Akt and Foxo1, indicating that the former is not an obligate intermediate in insulin's actions to control liver metabolism under some conditions *in vivo*. This conclusion necessitates a significant reevaluation of the current view of how insulin utilizes Akt as a "downstream" signaling molecule in liver[44]. Second, Foxo1 is activated under both fasting and feeding conditions upon loss of Akt in liver, indicating that there is sufficient Akt signaling during fasting to repress a substantial portion of Foxo1 activity towards many genes. The functional consequences of this is illustrated by the rather small decreases in blood glucose when Foxo1 is deleted from wildtype liver, but the greater metabolic changes when excised from an Akt–deficient liver (Fig. 4, 5 and Supplementary Fig. 4).

Though about 84% of the Akt protein in liver is represented by the Akt2 isoform, *2LKO* mice are almost indistinguishable from control mice in these studies (Fig. 2 and 3). In contrast, in a previous publication we reported protection from steatosis in mice with liver–specific deletion of *Akt2* and wildtype levels of *Akt1*[45]. However, there are two critical differences in the design of those experiments compared to the current study: 1) in the previous study, the metabolic assays focused on lipid synthesis and accumulation and 2) the role of liver Akt2 was ascertained in obese, insulin–resistant mice. In contrast, when fed with normal chow, insulin dependent signaling and glucose tolerance are largely wildtype in *2LKO* mice, indicating the capacity of residual Akt1 to compensate under these conditions. Consistent with this idea, 4 hours postprandial Akt phosphorylation is nearly the same in control and *2LKO* livers (Fig. 2b, Supplementary Fig. 1), indicating that only a small fraction of total hepatic Akt is phosphorylated after normal chow feeding, but this is largely sufficient to convey insulin's signal. In contrast to *2LKO* mice, *DLKO* mice are hyperglycemic, glucose intolerant and insulin resistant (Fig. 3 and 4). These phenotypes are similar to those produced by liver–specific *insulin receptor* knockout (*LIRKO*)[5] and liver–specific *Irs1* and *Irs2* double knockout[6]. Hyperglycemia in the *DLKO* mice is milder than that in the other two insulin signaling deficient models, though this might have been due to acute deletion of *Akt1* and *Akt2* from the livers of *DLKO* mice, rather than *Cre*–dependent recombination early in life, which would enhance the development of secondary peripheral insulin resistance. The similar phenotypes between the *Irs1*, *Irs2* liver–specific double knockout mice and the *DLKO* mice suggest that the signaling is linear between these two nodes without major branch points.

In *TLKO* livers, despite the lack of Akt and Foxo1 the liver adapts appropriately to the fed state, successfully modulating its gene expression and maintaining euglycemia (Fig. 4, 5).

The appropriate regulation in these mice indicates that there is a pathway independent of Akt and Foxo1 that signals the liver's adaptation to feeding. This parallel pathway may have been partially functional even in the *DLKO* livers, as expression of some genes, such as *Ppargc1a*, *Igfbp1*, *Srebf1* responds to feeding; however, many more lose regulation in *DLKO* only to regain in the *TLKO* livers. Perhaps most notably, in *TLKO* mice insulin suppresses hepatic glucose production in a manner indistinguishable from that in control mice (Fig. 5e). These results demonstrate clearly that Akt is not required for insulin to regulate hepatic metabolism under all conditions and thus the kinase does not represent an obligate intermediate only in a simple, linear pathway. However, the disrupted glucose metabolism and significant insulin resistance in the *DLKO* livers indicate that under normal conditions Akt is indispensable to the preservation of metabolic homeostasis. Furthermore, normalization of the metabolic disturbances in the *DLKO* liver by additional deletion of *Foxo1* argues that the predominant role of Akt is to suppress Foxo1. To reconcile these seemingly conflicting data, we propose that active Foxo1 exerts a general repressive effect on many of the early physiological adaptations to feeding (Supplementary Fig. 11). We suggest that Akt participates in the liver's response to postprandial insulin and nutrient abundance by maintaining the suppression of Foxo1 activity, thus allowing the full prandial response. Importantly, even during the fasting state, a repressive function of Akt towards Foxo1 is still largely operational, such that the changes in Foxo1 activity that accompany normal physiological fluctuations in insulin levels are rather modest. We propose that Akt maintains a persistent suppressive effect on a large fraction of Foxo1 throughout routine daily periods of fasting and feeding. Though this interpretation represents a departure from the traditional model of insulin action, it is nonetheless consistent with the substantial body of relevant published data as well as the results presented herein. As discussed above, expression of Foxo1 target genes in response to change in metabolic states can occur in the absence of Akt and Foxo1 (Fig. 5). Moreover, there appear to be striking differences in the impact of *Foxo1* deletion in normal versus insulin–resistant livers. In mice not stressed by insulin resistance, acute deletion of *Foxo1* does not significantly reduce the expression of Foxo1 target genes (Fig. 5b), although chronic deletion of multiple *Foxo* family members including *Foxo1* results in modest reduction of expression of these genes[31,32]. In contrast, in this and previous reports[6,31], the effects of *Foxo1* deletion on the expression of these genes are more dramatic in mice with insulin resistance, whether induced genetically or by nutritional overabundance, and the scope of *Foxo1* deletion is broader, affecting not only the gluconeogenic but many anabolic programs as well (Supplementary Fig. 9). In fact, many of the studies that have supported a major role for Foxo1 in the control of glucose homeostasis are performed in mouse models of insulin resistance[23,33,34], or by overexpressing a gain–of–function Foxo1 mutant[28]. Even in model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*, the functional importance of Foxo orthologs is best revealed on genetic backgrounds with defective insulin signaling[46–48]. These data are compatible with the idea that Foxo1 becomes active during insulin resistance but is largely repressed in normal mice.

At present, it is unclear how Akt exerts a repressive effect on Foxo1 during fasting. The established mechanism by which Akt directly inhibits Foxo1 involves its phosphorylation at T24, S253 and S316 of the mouse protein by Akt. Both feeding and insulin injection into the

fasted mice induced Foxo1 phosphorylation at these sites, indicating that the majority of Foxo1 is not phosphorylated during fasting and therefore cannot be used to explain the Akt–dependent repression of Foxo1. Foxo1 is phosphorylated at sites in addition to those mentioned above, and its activity is modulated through acetylation[39], methylation[49], glycosylation[50] and ubiquitination[51]. It remains to be determined if Akt affects Foxo1 activity through these modifications, or via a cofactor that functions cooperatively with Foxo1[52].

One trivial possibility that could be raised to explain the lack of major effect of deletion of *Foxo1* on fasting metabolism is functional rescue by other Foxo family members. This is unlikely for several reasons. First, there is little enhancement of fasting hypoglycemia in liver–specific *Foxo1* null mice when additional family members are deleted[32]. Second, if Foxo family members other than Foxo1 were capable of transmitting a signal to gluconeogenic gene expression and enhanced hepatic glucose output, then deletion of *Foxo1* alone would be unable to suppress the abnormalities incurred by removal of *Akt1* and *Akt2* from liver. In this study we found a more profound effect of deletion of *Foxo1* alone in the liver on fasting glycemia than has been reported previously[31], possibly due to the acute nature of the recombination in our protocol. Of note, the hypoglycemia in the liver–specific *Foxo1* knockout is reversed by concomitant deletion of *Akt* (Fig. 4 and Supplementary Fig. 4). These data suggest that the phenotypes in the liver–specific *Foxo1* deletion mice depend on the presence of functional Akt, though the underlying mechanism is not clear.

A major question to be addressed in future studies is the nature of the regulatory signal(s) triggered by feeding that are suppressed by the unrestrained activity of Foxo1 (Supplementary Fig. 11). Since mice with liver–specific knockout of *Irs1* and *Irs2* display a pattern of gene expression similar to *DLKO* mice[6], including suppression by concomitant deletion of *Foxo1*, it is likely that any Akt–independent, cell–autonomous pathway for control of hepatic metabolism is also not signaled through the Irs proteins. In addition, the loss of phosphorylation of recognized Akt substrates in the *DLKO* as well *TLKO* livers argues strongly against the idea that Akt–like kinases are rescuing insulin signaling under these conditions (Fig. 4f, 4g). These data are consistent with the absolute requirement for Foxo1 for insulin regulation of gene expression in primary hepatocytes, results that contrast sharply with the relatively normal insulin action *in vivo*. Taken together, these data suggest strongly that insulin's regulation of hepatic function in the *TLKO* mice is cell non–autonomous, *i.e.* the direct insulin target organ is not liver. Moreover, as shown in Figure 6, hepatocytes studied *ex vivo* behave like cells that utilize a linear pathway in which Akt suppresses Foxo1–dependent gene expression. Thus it is likely that the canonical Akt–Foxo pathway operates in a cell autonomous manner in response to insulin, while there is a parallel pathway in which insulin signals to liver through another intermediary tissue. Consistent with the idea of cell non–autonomous regulation of hepatic metabolism, restoration of hepatic insulin signaling alone is not sufficient to reverse the diabetic phenotype associated with systemic loss of insulin receptor[53]. One candidate target organ for insulin is the central nervous system, as a number of studies have provided data supporting a role of insulin signaling through brain to control hepatic glucose output[53–57]. It will be of great interest to determine conclusively if the apparent normal suppression of liver

glucose production in the *TLKO* livers is mediated through insulin signaling in liver or at some other sites, such as brain or adipocytes.

These findings have implications for understanding the pathophysiology of Type 2 diabetes mellitus (T2DM) and potentially designing effective therapeutics. Given the substantial evidence cited above that Foxo1 is inappropriately active in rodent models of insulin resistance, the idea has emerged that, like Type 1 diabetes mellitus, T2DM resembles a physiological fasting state during periods of nutritional abundance. However, the data presented herein reveal that Foxo1 is largely inactive in healthy animals and thus the metabolic state of liver in T2DM represents a true pathological condition. Moreover, we predict that adequate suppression of Foxo1 through targeted therapeutics would restore much of the normal regulation in individuals with T2DM. This strategy has advantages over the current treatments that produce a state mimicking constitutive hyperinsulinemia with all of the attendant adverse consequences such as increased lipid synthesis and hyperlipidemia. Lastly, but perhaps most importantly, the demonstration of an insulin–dependent, Akt–independent pathway for suppressing liver glucose output raises the possibility of novel targets for the treatment of hyperglycemia.

METHODS

Mice

We used male mice in all the experiments, except for those as indicated. The *Akt1*$^{loxP/loxP}$ mice[58], *Akt2*$^{-/-}$ mice[4], *Akt2*$^{loxP/loxP}$ mice[45], *Foxo1*$^{loxP/loxP}$ mice[31] were described previously. We backcrossed the *Akt2*$^{-/-}$ mice, *Akt1*$^{loxP/loxP}$ mice, *Akt2*$^{loxP/loxP}$ mice onto *C57/BL6J* background and further intercrossed these mice to generate the desired genotypes. We generated The *Akt1*$^{loxP/loxP}$;*Akt2*$^{loxP/loxP}$;*Foxo1*$^{loxP/loxP}$ mice by crossing the *Akt1*$^{loxP/loxP}$;*Akt2*$^{loxP/loxP}$ mice with the *Foxo1*$^{loxP/loxP}$ mice. These mice were on the 129–C57BL–6J/FVB mixed background. We recovered the *Akt1*$^{loxP/loxP}$;*Akt2*$^{loxP/loxP}$ mice on the same background and used them to compare the *DLKO* against *TLKO*. For fasting–refeeding experiments, we fasted mice 16 hours (4 PM to 9 AM), then sacrificed the mice (for fasted groups), or refed the mice with normal chow (Laboratory Rodent Diet, Cat. 5001. Percentage calories provided by protein: 29%, by Fat: 13%, by carbohydrates: 58%) for additional 4 hours before sacrifice (for refed group). For insulin injection, we fasted the mice for 16 hours, injected the mice with either phosphate buffered saline or insulin at 2 mU g^{-1} body weight and waited for 20 minutes before sacrificing the mice and freeze clamping the livers. All mice experiments were reviewed and approved by the University of Pennsylvania IACUC in accordance with NIH guidelines.

Adeno–associated virus injection

We injected 6 to 8 weeks old mice with adeno–associated virus at 10^{11} genomic copies per mouse. Experiments were performed 2 weeks after virus injection.

Liver lysates extraction and Western blotting

We freeze–clamped and stored livers at −80 °C until the time of further processing. We extracted lysates from frozen livers with RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.6,

1% Triton X–100, 0.5% sodium deoxycholate, 0.1% SDS, supplemented with protease and phosphatase inhibitors). We used the cleared lysates for Western blot. We used the following antibodies: antibody to Akt2 was described previously[59]; antibodies to Akt1, phospho–Akt S473, phospho–S6 (S240/S244), S6, Foxo1, phospho–Foxo1 T24, phospho–Foxo1 S253, phospho–Gsk3 (S21/S9), phospho–Erk (T202/Y204) were from Cell Signaling Technology; antibodies to Gsk3, Igfbp1, Erk were from Santa Cruz Biotechnology; antibody to Gck was kindly provided by Mark A. Magnuson (Vanderbilt University).

Primary hepatocytes isolation and culture

We isolated primary hepatocytes as previously described[60]. We cultured the cells in M199 medium containing 100 nM T3, 100 nM dexamethasone and 1 nM insulin overnight. For gene expression analysis, we cultured the cells for additional 6 hours in the same medium with or without 1 nM insulin.

mRNA Isolation and real time PCR

We isolated total RNA from the frozen livers or primary hepatocytes using the RNeasy Plus kit from Qiagen. We synthesized cDNA using M–MLV reverse transcriptase, and quantitated the relative expression of genes of interest by real time PCR using the SYBR Green Dye–based assay.

Glucose tolerance test

We fasted the mice overnight, gave them glucose at 1 g kg^{-1} body weight by oral gavage. We monitored glucose by tail bleeding at 0, 15, 30, 60, 120 minutes after glucose injection. We measured insulin concentration in serum collected before and 15 minutes after glucose gavage.

Liver glycogen content measurement

We fasted mice overnight. We then sacrificed the mice (fasted mice), or fed the mice for 4 hours before sacrificing (fed mice). We extracted liver glycogen with 6% cold perchloric acid, which was then neutralized with potassium hydroxide. We treated glycogen in the supernatant with amyloglucosidase. We then measured glucose content before and after the amyloglucosidase treatment, and we calculated the difference as the glycogen content.

Hyperginsulinemic–euglycemic clamp

We performed the hyperinsulinemic–euglycemic clamps at the Mouse Phenotyping, Physiology and Metabolism Core at the University of Pennsylvania School of Medicine as previously described[4]. We infused human insulin at 2.5 mU kg^{-1} min^{-1}, we maintained blood glucose concentration between 120 mg dl^{-1} and 140 mg dl^{-1} by infusing 20% glucose at various rate. For gene expression analysis under the euglycemic clamp condition, we clamped the mice similarly, except that we infused the control mice with phosphate–buffered saline instead of insulin (10 mU kg^{-1} min^{-1}) and the clamp lasted for 3 hours, during which we maintained the blood glucose concentration between 120 mg dl^{-1} and 140 mg dl^{-1}.

Statistical Analysis

We presented all data as means ± s.e.m. We performed the statistical analysis with one–way ANOVA when more than two groups of data were compared, with two–way ANOVA when two conditions were involved, and with student's t–test when only two groups of data were concerned. We deemed the difference being significant when $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors would like to thank M. Magnuson (Vanderbilt University) for kindly providing the antibody to Gck, and D. Accili (Columbia University) for sharing the *Foxo1*$^{loxP/loxP}$ mice. We are grateful to J. Schug who helped with the microarray data analysis and generated the heatmap and the density plot. The Functional Genomics Core, the Transgenic, Knockout, Mouse Phenotyping, and Biomarker Cores of the University of Pennsylvania Diabetes and Endocrinology Research Center (NIH grant P30 DK19525) were instrumental in this work. This work was supported by NIH grant RO1 DK56886 (M.J.B.) and diabetes training grant T32 DK007314 (M.L.).

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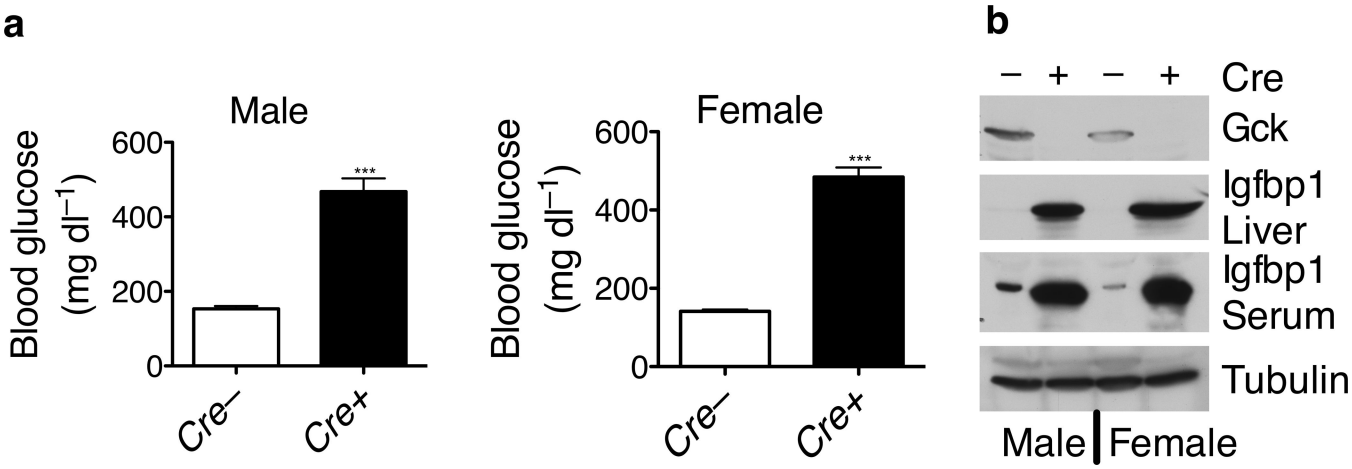


Figure 1. Liver–specific deletion of *Akt1* in the *Akt2* whole body knockout mice resulted in severe hyperglycemia and disruption of Foxo1–regulated gene expression

(**a**) Fed blood glucose in the *Akt2*$^{-/-}$;*Akt1*$^{\text{loxP/loxP}}$ mice in the presence (*Cre*$^{+}$) or absence (*Cre*$^{-}$) of *Afp–Cre* (*** $P < 0.001$, $n = 7, 8$). (**b**) Western blot for Gck in liver lysates, and Igfbp1 in both liver lysates and serum.

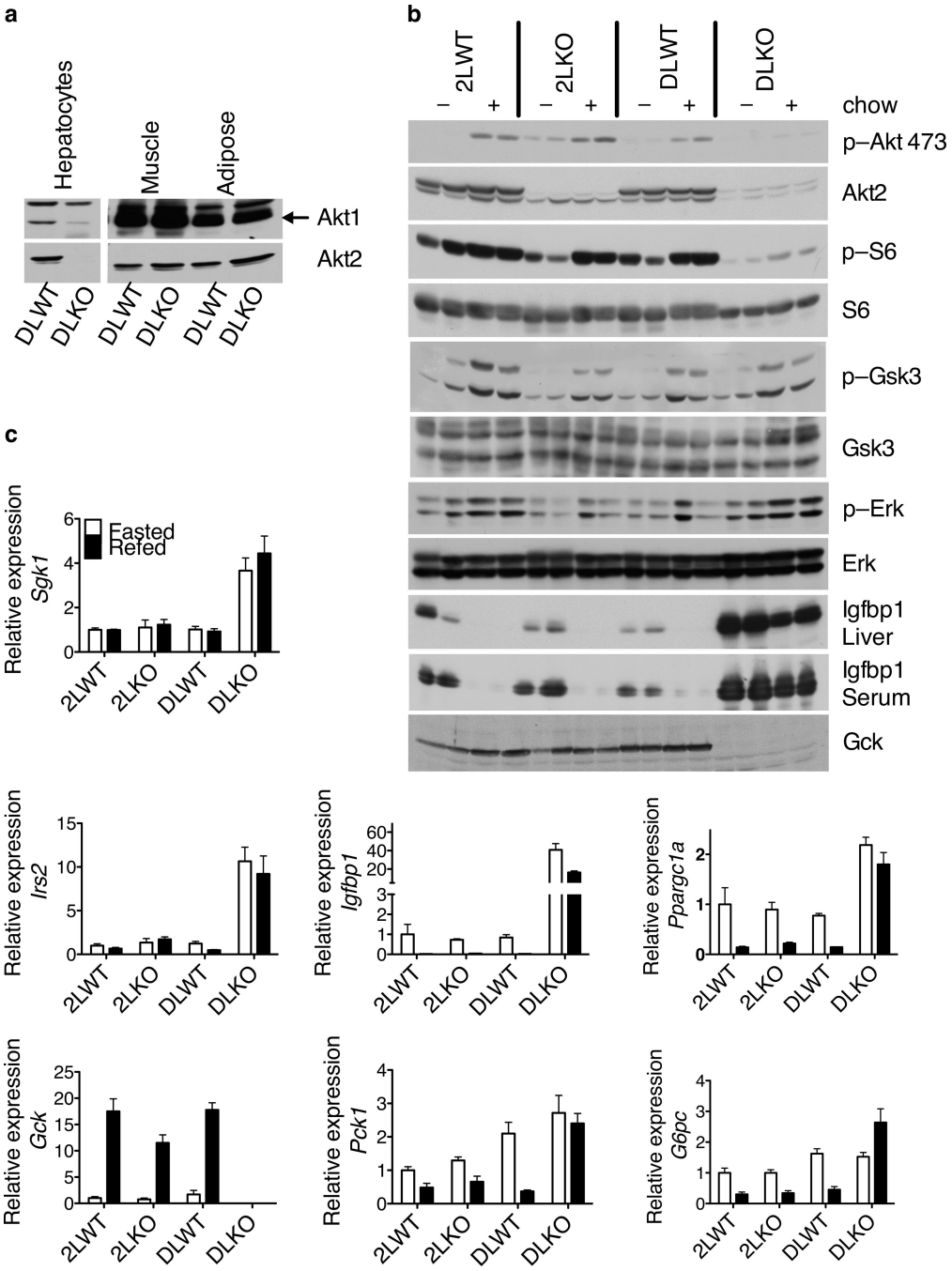


Figure 2. Aberrant insulin signaling and disrupted expression of Foxo1–regulated genes in the *DLKO* livers but not in the *2LKO* livers

(**a**) Western blot of Akt1 and Akt2 in primary hepatocytes, muscle and adipose tissues from the *DLWT* and *DLKO* mice. (**b**) Western blot of liver lysates. p–Akt 473: phosphorylation of Akt at S473. p–S6: phosphorylation of ribosomal protein S6 at S240, S244. p–Gsk3: phosphorylation of Gsk3α at S21 and Gsk3β at S9. p–Erk: phosphorylation of Erk at T202 and Y204. (**c**) Relative expression of genes in livers quantitated by real time PCR. In (**b**) and

(**c**), mice were either fasted overnight (open bars) or fasted overnight followed by 4 hours feeding with normal chow (filled bars).

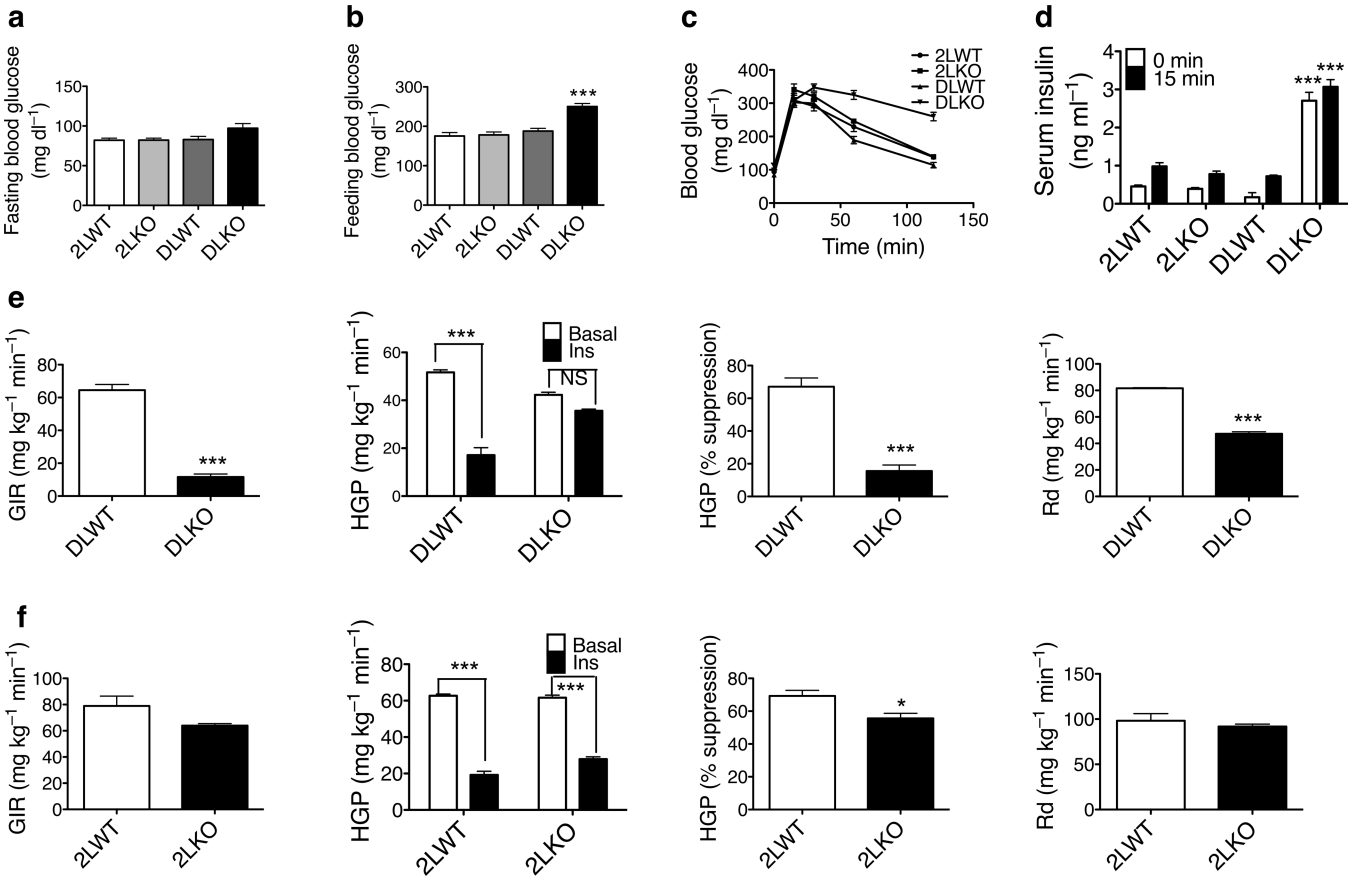


Figure 3. Deletion of both *Akt1* and *Akt2* in livers results in hyperglycemia, glucose intolerance and insulin resistance

Blood glucose in mice after overnight fast (**a**) and 4 hours feeding (**b**). (*** $P < 0.001$, $n = 7$, 8). (**c**) Oral glucose tolerance test. (**d**) Serum insulin before and fifteen minutes after glucose gavage. (**e**) Hyperinsulinemic–euglycemic clamp in the *DLWT* and *DLKO* mice. (**f**) Hyperinsulinemic–euglycemic clamp in the *2LWT* and *2LKO* mice. GIR: glucose infusion rate; HGP: hepatic glucose production; Rd: rate of glucose disposal. (* $P < 0.05$, *** $P <$ 0.001, NS: not significant, $n = 4$, 5)

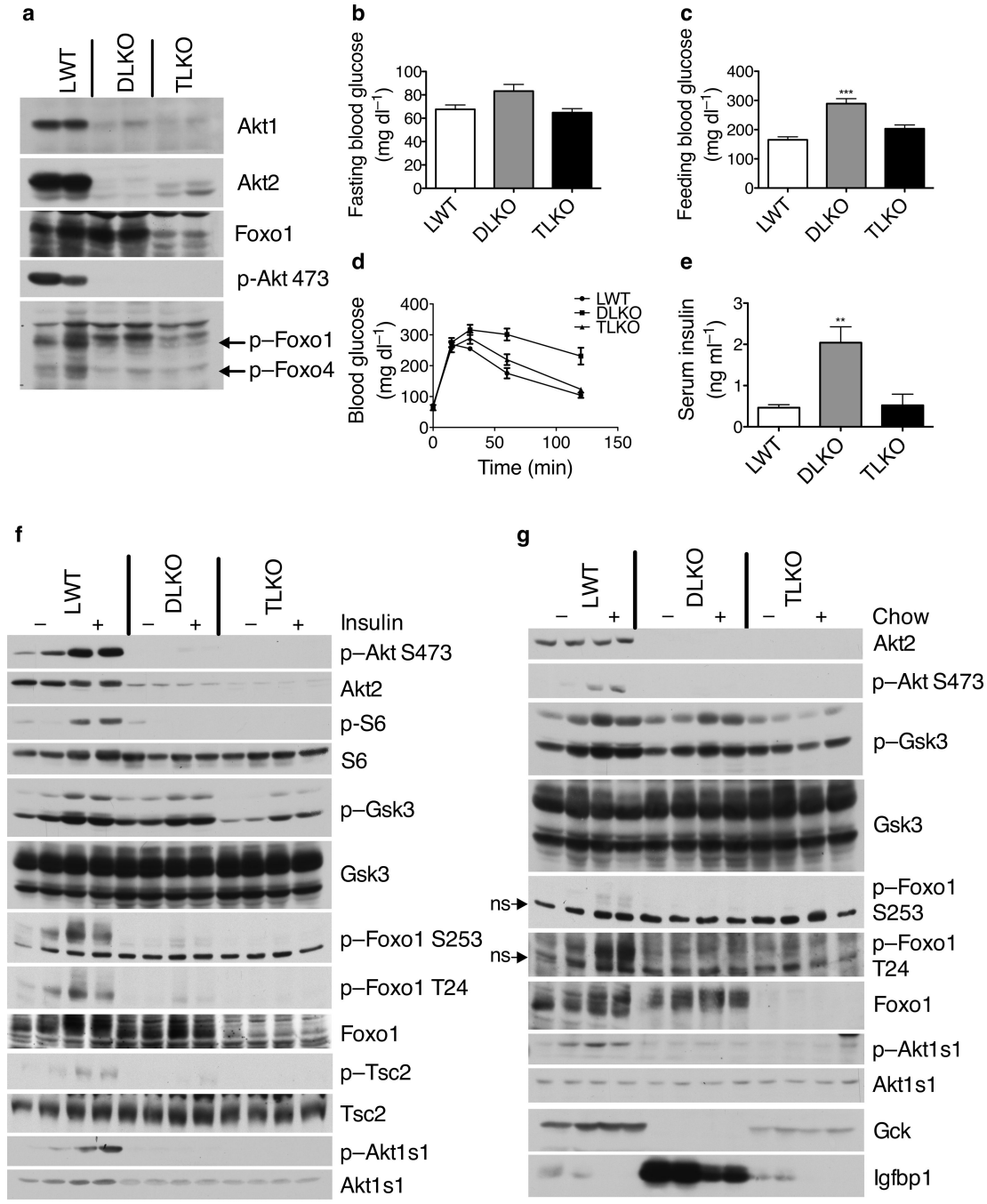
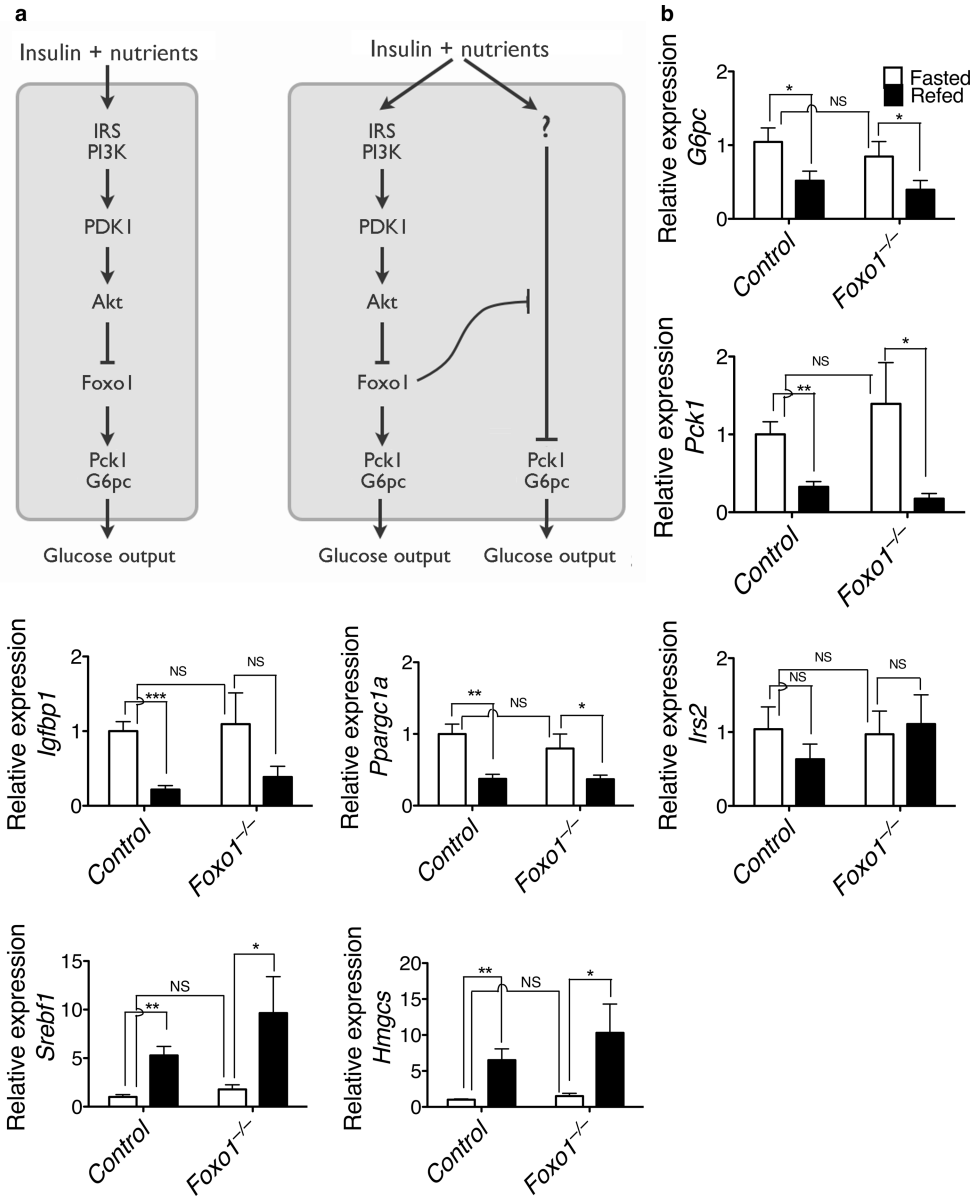


Figure 4. Deletion of *Foxo1* normalizes hyperglycemia, glucose intolerance, and hyperinsulinemia in *DLKO* livers despite defective insulin signaling

(**a**) Western blot of the lysates from primary hepatocytes isolated from fed mice. The phospho–specific antibody detected phosphorylation of Foxo1 at T24 and Foxo4 at T28. (**b**) Blood glucose after overnight fast. (**c**) Blood glucose after overnight fast followed by 4 hours feeding. (*** $P < 0.001$, $n = 9$) (**d**) Oral glucose tolerance test. (**e**) Fasting serum insulin. (** $P < 0.01$, $n = 5$). (**f**) Western blot of liver lysates from mice fasted overnight and injected with either insulin or saline. (**g**) Western blot of liver lysates from mice fasted

overnight or fasted overnight followed by 4 hours feeding with normal chow (ns: non–specific band).



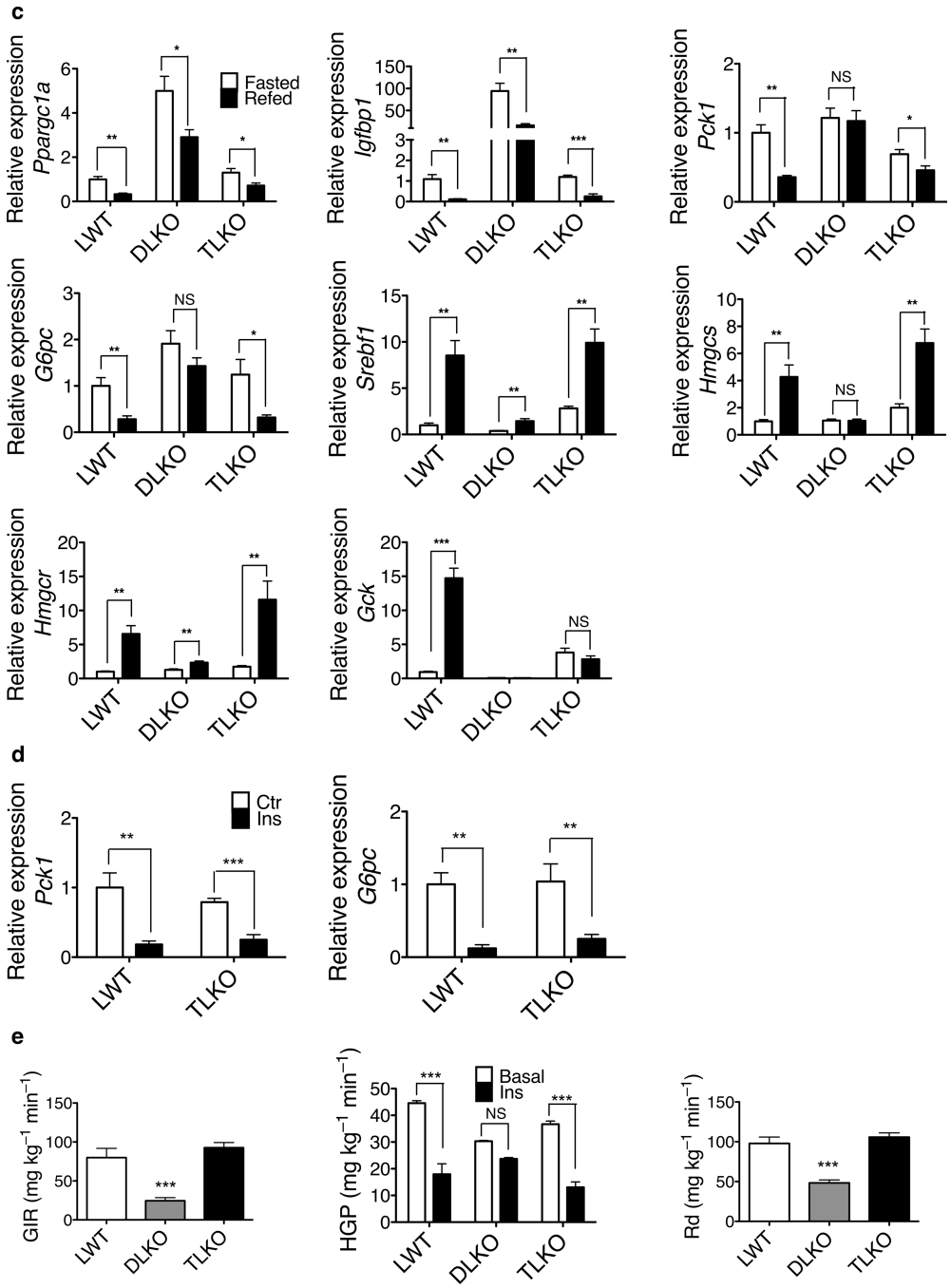


Figure 5. Nutritional regulation of hepatic gene expression in the absence of Foxo1 alone and in the absence of both Akt and Foxo1

(**a**) Two alternative models for signaling by Akt in liver. On the left is the traditional view, in which there is a linear pathway terminating at the control of gluconeogenic gene expression. On the right is a newly proposed model, in which Akt constitutively suppresses Foxo1, which is a global inhibitor of the metabolic transition in liver that accompanies food intake. (**b**) Hepatic gene expression in the control mice and the acute, liver–specific *Foxo1* knockout mice. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS, not significant, $n = 8$). (**c**)

Hepatic gene expression in *LWT*, *DLKO* and *TLKO* mice. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS, not significant, $n = 7, 8$). (**d**) Relative expression of *G6pc* and *Pck1* under euglycemic clamp condition (** $P < 0.01$, *** $P < 0.001$, $n = 6$). (**e**) Hyperinsulinemic–euglycemic clamp (*** $P < 0.001$, NS, not significant, $n = 4, 5$).

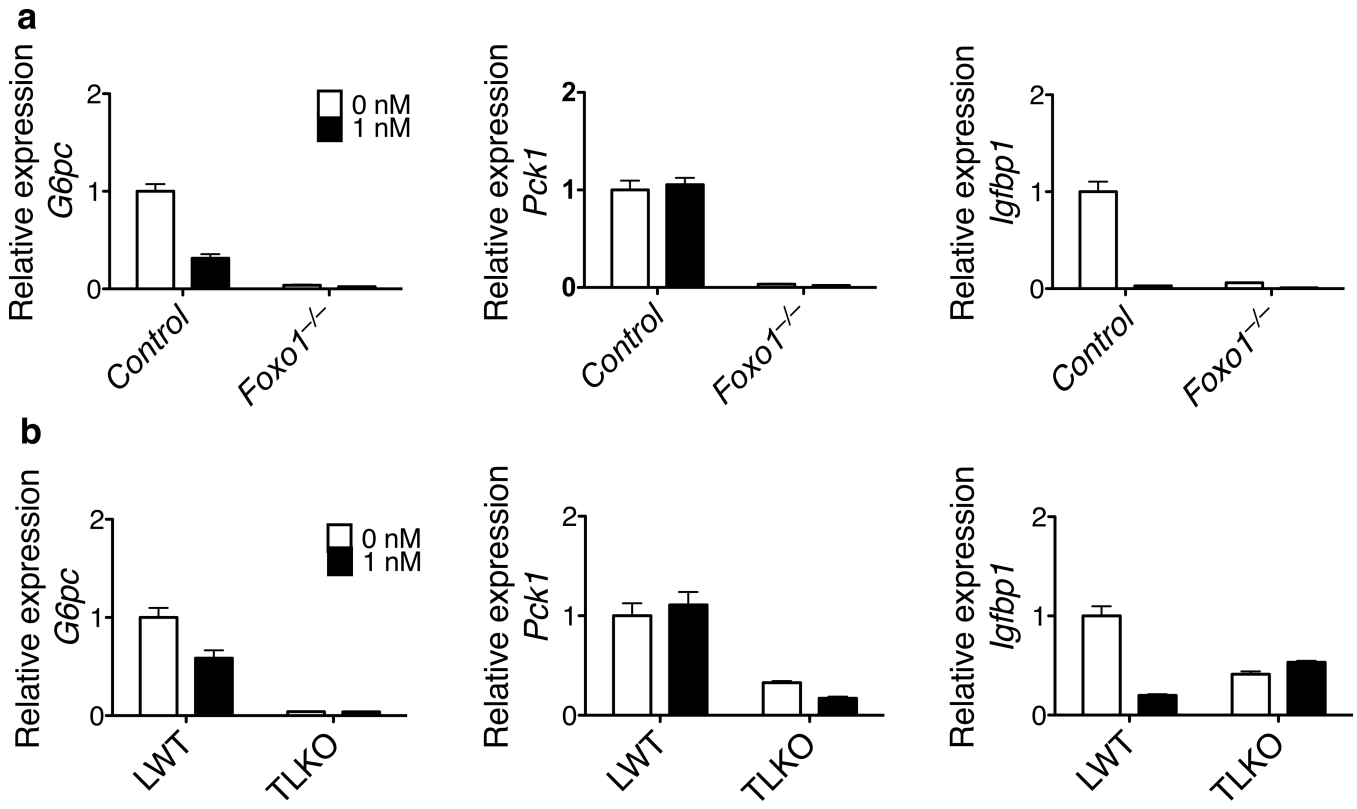


Figure 6. Insulin–regulated expression of Foxo1 targets genes was compromised in the *Foxo1*$^{-/-}$ and *TLKO* primary hepatocytes

Primary hepatocytes were isolated from control and *Foxo1*$^{-/-}$ livers (**a**), or *LWT* and *TLKO* livers (**b**). The hepatocytes were cultured overnight in 1 nM insulin–containing medium and additional 6 hours in the presence or absence of 1 nM insulin. Relative expression of the listed genes was quantitated by real time PCR.