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Akt2 is required for hepatic lipid accumulation in models of insulin resistance

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Summary

Insulin drives the global anabolic response to nutrient ingestion, regulating both carbohydrate and lipid metabolism. Previous studies have demonstrated that Akt2/protein kinase B is critical to insulin's control of glucose metabolism, but its role in lipid metabolism has remained controversial. Here we show that Akt2 is required for hepatic lipid accumulation in obese, insulin-resistant states induced by either leptin-deficiency or high fat diet feeding. *Lep*$^{ob/ob}$ mice lacking hepatic Akt2 failed to amass triglycerides in their livers, associated with and most likely due to a decrease in lipogenic gene expression and *de novo* lipogenesis. However, Akt2 is also required for steatotic pathways unrelated to fatty acid synthesis, as mice fed high fat diet had reduced liver triglycerides in the absence of hepatic Akt2 but did not exhibit changes in lipogenesis. These data demonstrate that Akt2 is a requisite component of the insulin-dependent regulation of lipid metabolism during insulin resistance.

Introduction

Among its numerous functions, the mammalian liver serves both as a repository for stored nutrients and an organ that senses, integrates and controls the metabolic state of the organism. As a metabolic tissue, the liver responds to multiple inputs, including hormones, neuronal impulses and nutrients. Following the ingestion of food, the dominant signals to the liver are insulin and absorbed nutrients, particularly glucose, which serves as a key substrate and a first messenger providing information to the liver (Postic et al., 2007). Understanding how the liver processes these diverse inputs becomes even more challenging when one considers pathological states associated with over-nutrition, the incidence of which are reaching epidemic proportions throughout the world (Doria et al., 2008). Here it becomes difficult to distinguish

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protective from maladaptive processes, though there is little doubt that the accumulation in the liver of large quantities of macromolecules, such as neutral lipid, has unmistakable potential for serious toxicity (Browning and Horton, 2004; Savage et al., 2007). Thus the study of hepatic metabolism has justifiably attracted considerable attention in recent years.

During the transition from fasting to the fed state, insulin stimulates hepatic glycogen synthesis and suppresses hepatic gluconeogenesis, resulting in suppression of glucose production by the liver (Petersen et al., 1998; Saltiel, 2001). Loss of function experiments have shown that the protein kinase Akt (also known as protein kinase B) is central to the hepatic actions of insulin on glucose output, though a recent report has questioned the role of Akt as a cell-autonomous intermediate in insulin signaling (Chen et al., 2009; Cho et al., 2001a; Garofalo et al., 2003; Gross et al., 2008). Nonetheless, the prevalent model of hepatic insulin signaling is that activated Akt phosphorylates and inhibits the transcription factor FoxO, peroxisome proliferator-activated receptor-coactivator-1α (PGC-1α) and others, thereby terminating expression of the rate-controlling enzymes of gluconeogenesis (Gross et al., 2008; Li et al., 2007). During insulin resistance, this process is blunted and the persistence of hepatic glucose output following a meal compounds diminished glucose uptake into muscle, resulting in postprandial hyperglycemia (Saltiel, 2001). However, in a seminal essay, McGarry argued persuasively that the view of type 2 diabetes mellitus (T2DM) as a disease of glucose metabolism is purely historical, and that the lipid abnormalities are equally intrinsic to the pathophysiology (McGarry, 1992). Certainly, T2DM is associated with a characteristic dyslipidemia, and the vast majority of obese T2DM individuals with insulin resistance have abnormal accumulation of triglyceride in their livers, so-called non-alcoholic fatty liver disease (NAFLD) (Petersen et al., 2005; Postic and Girard, 2008a). Moreover, the high serum triglycerides and low HDL cholesterol of insulin resistance predispose to cardiovascular disease, the predominant morbidity associated with T2DM, and NAFLD itself can progress to hepatitis (Browning and Horton, 2004; Williams, 2008). However, our understanding of the mechanistic processes governing hepatic lipid metabolism is still rudimentary.

During times of nutritional abundance, the liver converts substrate into triglyceride for local storage as well as export in the form of very low-density lipoprotein (VLDL). This process is controlled at multiple steps by regulation of gene expression, posttranslational modification and substrate supply (Postic and Girard, 2008b). Most recent experiments have been directed towards investigating the transcriptional control of *de novo* lipogenesis, for which insulin and glucose activate overlapping sets of genes via the sterol regulatory element-binding protein-1c (SREBP1c) and the carbohydrate response element-binding protein (ChREBP), respectively (Koo et al., 2001; Postic et al., 2007; Postic and Girard, 2008a). Though each is required for the maximal accumulation of hepatic triglyceride during insulin resistance in mice, the relative roles of these two transcription factors in the development of NAFLD and under more physiological conditions remains unclear (Dentin et al., 2006; Postic and Girard, 2008a; Yahagi et al., 2002). Moreover, the relative contribution of *de novo* lipogenesis to hepatic triglyceride accumulation has been controversial, though recent data suggest that it might account for as much as 25–30% of the triglyceride in livers of patients with NAFLD (Donnelly et al., 2005). In the studies described below, we consider the downstream signaling pathway that insulin utilizes to promote hepatic lipid accumulation and, in particular, the induction of SREBP1c, a question for which a consensus answer has yet to emerge. For example, hepatic overexpression of constitutively active Akt increases lipid synthesis, resulting in NAFLD and hypertriglyceridemia, as does hepatic deficiency of PTEN, a negative regulator of PI3K-dependent protein kinases including Akt (Horie et al., 2004; Ono et al., 2003; Stiles et al., 2004). Akt mediates insulin's effect on *SREBP1c* expression in cell culture, but studies *in vivo* have argued that atypical protein kinase C (aPKC) is the responsible intermediary signaling protein (Fleischmann and Iynedjian, 2000; Hegarty et al., 2005; Matsumoto et al., 2003; Porstmann et al., 2005; Taniguchi et al., 2006). Here we present an *in vivo* model that is

inherently defective in insulin signaling due to loss of hepatic Akt2, the major isoform expressed in liver, and utilize these mice to evaluate the role of Akt2 in lipid synthesis and accumulation.

Results

Germline Akt2 Deficiency Prevents NAFLD in $Lep^{ob/ob}$ mice

The serine-threonine kinase Akt exists as three highly related isoforms, Akt1-3, each encoded by a distinct gene. Based on studies of mice with interruptions in each locus, Akt2 is the major paralog mediating insulin's effects on glucose metabolism, whereas Akt1 and Akt3 are more important to growth (Chen et al., 2001; Cho et al., 2001a; Cho et al., 2001b; Easton et al., 2005; Garofalo et al., 2003). Akt2 is the predominant isoform expressed in insulin target tissues, such as liver, muscle and adipose tissue, and $Akt2^{-/-}$ mice exhibit glucose intolerance and a mild diabetic phenotype (Cho et al., 2001a; Garofalo et al., 2003). While the role of Akt2 in glucose homeostasis has been established, its influence on lipid metabolism *in vivo* remains controversial. To resolve this, we crossed $Akt2^{-/-}$ mice with leptin-deficient $Lep^{ob/ob}$ mice. The $Lep^{ob/ob}$ $Akt2^{+/+}$ mice were substantially heavier than $Lep^{+/+}$ $Akt2^{+/+}$ littermates at 12 weeks of age and had large, lipid-laden livers. However, deletion of both alleles of *Akt2* led to a dramatic reduction of both body and liver weight in the $Lep^{ob/ob}$ mouse, with loss of one allele of *Akt2* resulting in an intermediate decrease (Figure 1A and 1B). Triglyceride accumulation in the livers of $Lep^{ob/ob}$ mice under both fed and fasted conditions was completely prevented by loss of both alleles of *Akt2*, and, as above, mice heterozygous for Akt2 demonstrated intermediate protection from fatty liver (Figure 1C and 1D). Deletion of Akt2 exacerbated the impairment of glucose homeostasis in $Lep^{ob/ob}$ mice, as indicated by an elevation in fasted glucose and insulin levels compared with $Lep^{ob/ob}$ $Akt2^{+/+}$ mice (Figure 1E and 1F). Interestingly, hyperglycemia was not worsened in the $Lep^{ob/ob}$ $Akt2^{+/-}$ mice, suggesting that redistribution of hepatic lipid to other organs was not the cause of the aggravated insulin resistance.

Accumulation of hepatic triglyceride in $Lep^{ob/ob}$ mice is associated with an increase in *de novo* lipogenesis (Bray and York, 1979; Shimomura et al., 1999). Therefore, we measured hepatic fatty acid synthesis and incorporation into triglycerides using deuterated water as a biosynthetic tracer. $Lep^{ob/ob}$ $Akt2^{+/+}$ mice demonstrated greater *de novo* lipogenesis compared to $Lep^{+/+}$ $Akt2^{+/+}$ mice, but this was reduced in both $Lep^{ob/ob}$ $Akt2^{+/-}$ and $Lep^{ob/ob}$ $Akt2^{-/-}$ mice (Figure 1G). Non-esterified free fatty acids (NEFA) were unchanged or increased in $Lep^{ob/ob}$ $Akt2^{-/-}$ compared to $Lep^{ob/ob}$ $Akt2^{+/+}$ mice, and there was an increase in serum triglyceride levels (Suppl. Table 1). In addition, loss of Akt2 resulted in a modest decrease in serum cholesterol but no change in serum ketone bodies in $Lep^{ob/ob}$ mice (Suppl. Table 1).

Generation of $Lep^{ob/ob}$ Liver-Specific Akt2 Null Mice

In the experiments described above, the non-cell autonomous effects of germline deletion of Akt2 confound the interpretation of changes in hepatic lipid content and metabolism. To clarify this, we generated mice with liver-specific deletion of Akt2. Mice containing an allele of *Akt2* in which exons 3 and 4 are flanked by loxP sires ($Akt2^{lox/lox}$) were crossed with mice expressing Cre recombinase under the control of the albumin promoter with an alpha-fetoprotein enhancer (*AFP*). Progeny were crossed with $Lep^{ob/ob}$ mice, and offspring were assessed for deletion of Akt2 selectively in the liver. As shown in Suppl. Figure 1, *AFP;*$Akt2^{lox/lox}$ and $Lep^{ob/ob}$ *AFP;*$Akt2^{lox/lox}$ mice lacked hepatic Akt2 protein, while showing no compensatory changes in the levels of Akt1 or decrease in Akt2 protein in other tissues. While *AFP;*$Akt2^{lox/lox}$ mice exhibited no difference in body weight compared to $Akt2^{lox/lox}$ mice or *AFP;*$Akt2^{+/+}$ mice, $Lep^{ob/ob}$ *AFP;*$Akt2^{lox/lox}$ mice weighed less than both $Lep^{ob/ob}$ $Akt2^{lox/lox}$ and $Lep^{ob/ob}$ *AFP;*$Akt2^{+/+}$ mice (Figure 2A and not shown). This decrease in body

weight was due to proportional reductions in both lean and fat mass, such that relative body composition remained unchanged (Suppl. Figure 2A). The decreased body weight could not be attributed to changes in food intake or energy expenditure, which were indistinguishable in *Lep*$^{ob/ob}$ mice with or without hepatic Akt2 (Suppl. Figure 2B and 2C). *AFP;Akt2*$^{lox/lox}$ mice had mildly elevated fasting glucose levels compared with *Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$ mice but did not exhibit any change in insulin levels (Figure 2C and 2D).

Loss of hepatic Akt2 reduces hepatic triglyceride levels, de novo lipogenesis and lipogenic gene expression in the Lep$^{ob/ob}$ mouse

Hepatic deletion of Akt2 resulted in decreased liver weight in the *Lep*$^{ob/ob}$ mouse, due at least in part to a reduction in triglyceride content under both fed and fasted conditions (Figure 2B, 2E and 2F). This decrement does not solely reflect the decreased body weight in *Lep*$^{ob/ob}$ *AFP;Akt2*$^{lox/lox}$ mice, as even compared to weight-matched controls, *Lep*$^{ob/ob}$ *AFP;Akt2*$^{lox/lox}$ mice exhibited reduced hepatic triglyceride content under fed conditions (*Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$: 56.7 ± 3.34 mg/g TG, 34.9 ± 1.6 g body weight;, *Lep*$^{ob/ob}$ *AFP;Akt2*$^{lox/lox}$:12.3 ± 1.43 mg/g TG, 38.7 ± 1.1 g body weight). Fasted serum triglyceride levels were decreased in *Lep*$^{ob/ob}$ *AFP;Akt2*$^{lox/lox}$: mice compared with *Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$ mice, while fed values were unchanged (Suppl. Table 2). Serum cholesterol was decreased in *Lep*$^{ob/ob}$ mice upon liver-specific deletion of Akt2, but other serum markers of lipid metabolism were largely unaffected (Suppl. Table 2). The content and distribution of long-chain fatty acyl-CoA were not significantly different among any of the genotypes, though there was a trend towards a decrease in the two monounsaturated fatty acids palmitoleate (C16:1) and oleate (C18:1) in *Lep*$^{ob/ob}$ *AFP;Akt2*$^{lox/lox}$ compared with *Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$ livers (Figure 2G). *De novo* lipogenesis was elevated in the *Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$ mice, and loss of hepatic Akt2 reversed this effect, as was previously observed with whole-body loss of Akt2 (Figure 2H). These results demonstrate that Akt2 is required in the liver for the increases in both hepatic *de novo* lipogenesis and triglyceride accumulation in *Lep*$^{ob/ob}$ mice.

The augmented *de novo* lipogenesis in the *Lep*$^{ob/ob}$ mouse is associated with an increase in lipogenic gene expression, in particular *SREBP-1c* and its transcriptional targets (Shimomura et al., 1999). In the present studies, we confirmed the predicted increases in the mRNAs for *SREBP-1c*, steroyl CoA desaturase (*SCD1*), fatty acid synthase (*FAS*), acetyl COA carboxylase (*ACC*), ATP citrate lyase (*ACL*), glycerol phosphate acyltransferase (*GPAT*) and glucokinase (*GCK*) in *Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$ livers, and moreover found that all of these increases were prevented by loss of hepatic Akt2 (Figure 3). Expression of *ChREBP* was unchanged, but its target pyruvate kinase (*L-PK*) was increased in *Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$ mice and normalized in livers from *Lep*$^{ob/ob}$ *AFP;Akt2*$^{lox/lox}$ mice. Peroxisome proliferator-activated receptor-γ (*PPARγ*) and *Cidec/Fsp27*, gene products required for development of hepatic steatosis in *Lep*$^{ob/ob}$ mice, were induced in *Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$ livers and this was prevented by loss of hepatic Akt2 (Figure 3) (Matsusue et al., 2008). *Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$ mice exhibited a slight increase in the expression of *PGC-1α*, which was further enhanced in *Lep*$^{ob/ob}$ *AFP;Akt2*$^{lox/lox}$ mice (Figure 3). However, expression of medium-chain acetyl-CoA dehydrogenase (*MCAD*), a transcriptional target of PGC-1α and rate-determining enzyme in fatty acid oxidation, was not altered in *Lep*$^{ob/ob}$ *AFP;Akt2*$^{lox/lox}$ compared with *Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$ livers (Schreiber et al., 2003). Additionally, the expression of carnitine palmitoyltransferase I (*CPT1*), another PGC-1α target, was actually decreased in *Lep*$^{ob/ob}$ *AFP;Akt2*$^{lox/lox}$ livers, while the expression of the mitochondrial gene cytochrome c oxidase subunit 7a (*Cox7a*) was not different between *Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$ and *Lep*$^{ob/ob}$ *AFP;Akt2*$^{lox/lox}$ mice (Song et al., 2004).

Germline Akt2 deletion decreases hepatic lipid accumulation resulting from Surwit high fat diet (HFD)

Though the *Lep*$^{ob/ob}$ mouse is a robust model of obesity and NAFLD, these mice also lack leptin, which itself plays a role in metabolism. To complement our studies in *Lep*$^{ob/ob}$ mice, we fed *Akt2*$^{-/-}$ mice a Surwit diet, which induces obesity and insulin resistance in mice through dietary enrichment of fat (58% kcal from fat) and simple carbohydrates (25.5% kcal from carbohydrates, approximately half from sucrose) (Surwit et al., 1995; Surwit et al., 1988). Male mice were given a Surwit HFD at approximately 5 weeks of age, and maintained on the diet for one or 4 months; chow-fed age-matched male mice served as controls. After 4 months on Surwit diet, *Akt2*$^{+/+}$ mice gained more weight than their chow-fed counterparts, an effect that was attenuated by whole-body loss of Akt2 (Figure 4A). Though liver weight was not significantly different between any of the groups (Figure 4B), the increase in fed hepatic triglycerides observed in *Akt2*$^{+/+}$ mice after 4 months on the diet was abrogated with loss of Akt2 (Figures 4C and 4D). Germline deletion of Akt2 did not change serum triglyceride levels on Surwit HFD, but did decrease fasted serum free fatty acids and serum ketone bodies (Suppl. Table 3). Interestingly, while *Akt2*$^{-/-}$ mice developed severe hyperglycemia on a *Lep*$^{ob/ob}$ background, *Akt2*$^{-/-}$ mice on a Surwit HFD for 4 months were able to maintain fasting euglycemia by increasing insulin levels (Figure 1E, Figure 4E and 4F).

AFP;Akt2$^{lox/lox}$ mice display decreased hepatic triglycerides on Surwit HFD without significant changes in de novo lipogenesis or lipogenic gene expression

As in the previous experiments, we wanted to determine if the requirement for Akt2 in hepatic triglyceride accumulation resulting from Surwit HFD was cell autonomous. Starting at approximately 5 weeks of age, we fed male *AFP;Akt2*$^{lox/lox}$ mice Surwit HFD for one or 4 months and used chow-fed age-matched male mice as controls. During this period, there were no differences in the weight gained on Surwit HFD between *Akt2*$^{lox/lox}$ mice and *AFP;Akt2*$^{lox/lox}$ mice or in liver weight between any of the groups (Figure 5A, 5B and Suppl. Figure 3A). *AFP;Akt2*$^{lox/lox}$ mice did not differ from *Akt2*$^{lox/lox}$ mice with regards to fasting glucose or insulin levels, food intake, body composition, or energy expenditure (Figures 5C and 5D and Suppl. Figure 3B–D). After one month on Surwit HFD, *Akt2*$^{lox/lox}$ mice had significantly increased fed hepatic triglyceride levels compared to their chow-fed counterparts, an effect that was partially ameliorated by loss of hepatic Akt2 (Figure 5E). The same held true after 4 months on Surwit HFD, as the 4-fold increase in fed hepatic triglycerides in *Akt2*$^{lox/lox}$ mice was reduced by approximately 25% with loss of hepatic Akt2. Total long chain fatty acyl-CoA and linoleoyl-CoA (C18:2) were decreased in livers from mice on Surwit HFD, as the latter is an essential fatty acid and its abundance is relatively low in coconut oil, the major source of fat in the Surwit diet. However, there were no differences between mice having or lacking hepatic Akt2 (Figure 5F). Fed serum triglycerides were increased in mice after 4 months on the diet with or without Akt2 in the liver (Suppl. Table 4).

While there was a significant increase in *de novo* lipogenesis in livers from mice on a Surwit HFD, this was not prevented by deletion of Akt2 (Figure 5G). Lipogenic gene expression paralleled this finding, as there was no decrease in hepatic expression of *SREBP-1c* or its targets in *AFP;Akt2*$^{lox/lox}$ fed Surwit HFD for one month; in fact there was a slight increase in *SREBP1c*, *FAS*, *ACC* and *L-PK* expression (Figure 6). However, only *SCD1* showed a significant increase in livers from *Akt2*$^{lox/lox}$ mice on Surwit HFD compared chow-fed animals. Additionally, there were not changes in the expression of *PGC-1α*, *CPT-1*, *MCAD*, or *Cox7a* in the *AFP:Akt2*$^{lox/lox}$ livers, and fasted or fed serum ketone levels were similar in *AFP;Akt2*$^{lox/lox}$ mice on Surwit HFD, suggesting that β-oxidation was not altered in these animals (Figure 6, Suppl. Table 4).

AFP;Akt2$^{lox/lox}$ mice display decreased hepatic triglycerides upon being fed a lard HFD

Given the differences in phenotype between mice deficient for leptin and those fed a Surwit HFD, we tested the generality of the requirement for Akt2 in hepatic steatosis by feeding male *AFP;Akt2*$^{lox/lox}$ mice another commonly used HFD, one enriched in fat from lard (60% kcal from fat) with a mixture of both starch and sucrose (20% kcal from carbohydrates). After one month on the lard HFD, there was an increase in hepatic triglycerides in *Akt2*$^{lox/lox}$ mice, which was ameliorated by loss of hepatic Akt2 (Figure 7A). However, unlike the results with the Surwit HFD, there was a significant increase in *SREBP-1c* expression in the livers of *Akt2*$^{lox/lox}$ mice after one month on a lard HFD, an increase that was significantly reduced in the *AFP;Akt2*$^{lox/lox}$ mice (Figure 7B). Other lipogenic genes were not increased on the lard HFD, though there was a trend for reduced expression in the absence of Akt2. Nonetheless, these data again show clearly that hepatic Akt2 is required for hepatic lipid accumulation ensuing from HFD feeding.

Loss of hepatic Akt2 decreases insulin signaling in lean mice, but does not change downstream protein phosphorylation in insulin-resistant livers

In order to gain insight into the pathways that could be mediating Akt2's effect on hepatic lipid metabolism, we performed Western blots on liver extracts from *Akt2*$^{lox/lox}$ and *AFP;Akt2*$^{lox/lox}$ mice following intraperitoneal (IP) injection of saline or insulin (Figure 7C). Phosphorylation of Akt at residues S473 and T308 was increased in *Akt2*$^{lox/lox}$ livers injected with insulin, and this increase was blunted in the absence of Akt2. The same pattern held true for the phosphorylation of Proline-Rich Akt Substrate of 40kDa (PRAS), glycogen synthase kinase 3-β (GSK3β) and phospho-tuberous sclerosis complex-2 (TSC2), while phosphorylation of FoxO1 did not appear to be decreased in the absence of hepatic Akt2. Interestingly, phosphorylation of these Akt targets was increased under basal fasted conditions in *AFP;Akt2*$^{lox/lox}$mice, suggesting relief of a negative feedback loop in the absence of Akt2 (Figure 7C). We also determined if loss of Akt2 had significant effects on hepatic insulin signaling in *Lep*$^{ob/ob}$ and HFD-fed mice as Akt2 mediates different lipid metabolic pathways in these two models (Suppl. Figure 4). In order to assess insulin signaling in both experimental groups in the presence of comparable levels of insulin and glucose, *Lep*$^{ob/ob}$ *Akt2*$^{lox/lox}$ and *Lep*$^{ob/ob}$ *AFP;Akt2*^{loxlox} mice were submitted to a hyperinsulinemic euglycemic clamp and liver extracts were prepared; glucose and insulin levels did not differ between *Akt2*$^{lox/lox}$ and *AFP;Akt2*$^{lox/lox}$ on a Surwit HFD, so liver extracts were made from these animals after one month on the diet under fed conditions. Insulin signaling was significantly impaired in both models of insulin resistance, such that phosphorylation of Akt and its targets was significantly below stimulated conditions in *Akt2*$^{lox/lox}$ animals; moreover, loss of hepatic Akt2 in either model did not reduce protein phosphorylation further (Suppl. Figure 4).

Discussion

Though insulin is well known to stimulate *de novo* lipogenesis in liver, the precise mechanism by which this is accomplished is not well understood. SREBP1c is clearly an important intermediate in the transcriptional control of lipogenesis, but the pathway by which insulin activates both processing and expression of SREBP1c remains uncertain (Raghow et al., 2008). To some extent, the difficulty in investigating this problem relates to the low rates of *de novo* lipogenesis in the normal postabsorptive liver, which have been estimated to contribute as little as 5% of total triglyceride fatty acids in humans (Diraison et al., 2003). For this reason, it has proven instructive to manipulate the metabolic state of experimental organisms in order to increase fat accumulation in the liver. In the present study, we have done so employing both genetic and dietary strategies and found a strong dependency on the presence of hepatic Akt2 for the development of steatosis and an absolute requirement for the increase in lipogenesis. Though these data are derived from mouse models of human disease, there is much to support

the notion that the obligatory role for Akt2 in lipid synthesis is not unique to the insulin-resistant, steatotic condition. We often see a trend towards lower hepatic triglycerides, fatty acid synthesis and lipogenic gene expression in livers from normal mice on a chow diet and in several cohorts these changes have reached statistical significance (Figure 6, 7). Thus, we can conclude that Akt2 is required definitively for full accretion of hepatic triglyceride during pathological states and it is likely that this is an extension of a requirement for the kinase in normal anabolic lipid metabolism.

One surprising result of these studies is that even though the necessity of Akt2 for steatosis applies to multiple models, in at least one, i.e. obesity induced by high fat and sucrose feeding, hepatic triglyceride content is reduced but lipogenesis and lipogenic gene expression are unchanged (Figure 5). This contrasts with development of NAFLD in the $Lep^{ob/ob}$ mouse, in which the protection from steatosis is mediated, at least in part, through preventing the stimulation of *de novo* lipogenesis (Figure 2). Thus, Akt2 likely mediates insulin's induction of triglyceride accumulation by stimulating fatty acid synthesis as well as processes other than *de novo* lipogenesis (Figure 5). One obvious candidate mechanism it that loss of Akt2 abrogates the normal suppression of β-oxidation produced by insulin. For example, insulin suppresses the PGC-1α-dependent stimulation of fatty acid oxidation, both by reducing hepatocyte cyclic AMP and by promoting the Akt-dependent inhibition of PGC-1α activity (Li et al., 2007). However, by several criteria, an increase in β-oxidation is unlikely to explain the protection from steatosis in our studies. First, though *PGC-1α* mRNA increased in $Lep^{ob/ob}$ $AFP;Akt2^{lox/lox}$ livers, expression of the critical targets *MCAD* and *CPT1* were not elevated in parallel (Figure 3) (Schreiber et al., 2003;Song et al., 2004). Second, there were no differences in expression of hepatic oxidative genes comparing $Akt2^{lox/lox}$ to $AFP;Akt2^{lox/lox}$ mice placed on a Surwit HFD (Figure 6). Lastly, RER and serum ketones bodies were largely indistinguishable in wildtype versus $AFP;Akt2^{lox/lox}$ mice on either an $Lep^{ob/ob}$ background or Surwit HFD (Suppl. Figures 2 and 3, Suppl. Tables 2 and 4). Another possible mechanism through which Akt2 could be decreasing hepatic lipid accumulation is increased VLDL export, as serum triglycerides were elevated in $Lep^{ob/ob}$ $Akt2^{-/-}$ mice (Suppl. Table 1). However, neither $Lep^{ob/ob}$ $AFP;Akt2^{lox/lox}$ nor $AFP;Akt2^{lox/lox}$ mice on Surwit HFD exhibited increased serum triglyceride levels under fasted or fed conditions and direct measurement of triglyceride export failed to reveal a requirement for Akt2 (Suppl. Tables 2 and 4 and not shown). Thus, the necessity for Akt2 in the development of hepatic steatosis relates to its role in mediating induction of SREBP1c and *de novo* lipogenesis as well as other presently unidentified anabolic processes.

The role of Akt as the primary mediator of insulin's action to increase SREBP1c and promote lipogenesis has been a point of some controversy. Hepatic overexpression of constitutively active Akt increases hepatic neutral lipid dramatically by a pathway only partially dependent on SREBP1c (Ono et al., 2003). Similarly, forced activation of endogenous Akt by liver-specific deletion of the lipid phosphatase *Pten* produces substantial accumulation of hepatic triglyceride and increased lipogenic gene expression, though this model is complicated by the concomitant activation of other PI3K-dependent kinases (Stiles et al., 2004). On the other hand, a dominant inhibitory Akt does not block insulin's induction of SREBP1c in tissue culture cells and $Lep^{ob/ob}$ mice have markedly increased *SREBP1c* mRNA in spite of significantly reduced levels of phospho-Akt (Matsumoto et al., 2002; Shimomura et al., 2000). Atypical PKC (PKCλ/ζ) proteins have received considerable attention as obligate mediators of the effects of insulin and PI3K on anabolic lipid metabolism, and have specifically been advanced as an alternative to Akt (Matsumoto et al., 2003; Taniguchi et al., 2006). Matsumoto et al. showed that mice with liver-specific deletion of PKCλ have decreased *SREBP1c* expression and triglyceride content, though reduced serum insulin levels complicated the interpretation of the *in vivo* findings in the study (Matsumoto et al., 2003). Kahn and colleagues undertook a different approach, eliminating both aPKC and Akt activity by ablation of all PI3K in the liver,

and then selectively introducing constitutively activate versions of the two kinases by adenovirus-mediated delivery (Taniguchi et al., 2006). They found that aPKC, but not Akt, restored *SREBP1c* mRNA, but the effects on hepatic lipids were not reported. The current studies do not address a potential role for aPKC and thus are compatible with a requirement for this kinase. However, in contrast to Taniguchi et al., they strongly support a critical role for Akt2. Importantly, $Lep^{ob/ob}$ mice heterozygous for *Akt2* displayed a reduction in liver triglyceride content and *de novo* lipogenesis intermediate between that of mice wildtype and null for *Akt2* (Figures 1C, 1D and 1G). This indicates that Akt2 is not only permissive for anabolic lipid metabolism, but is actually rate-determining. Moreover, $Lep^{ob/ob}$ $Akt2^{+/-}$ mice did not display the increase in serum glucose compared to $Lep^{ob/ob}$ mice evident in the *Akt2* null mice, so the protection in hepatic steatosis cannot be attributed to a worsening of the diabetes. Thus, these data support a obligate role for Akt2 in the development of the steatosis of obesity and insulin resistance, most likely reflecting the function of Akt2 in normal insulin signaling to lipid metabolism.

As shown in Figure 1E, deletion of both Akt2 and leptin resulted in fasting hyperglycemia considerably more severe than that in mice lacking either of the two proteins alone. This is associated with glycosuria, which likely contributes to caloric loss and reduction in body size in these mice (Figure 1A). In a recent paper, Chen and colleagues attribute the severe diabetes observed in compound $Akt1^{+/-}$ $Akt2^{-/-}$ mice to leptin deficiency secondary to lipodystrophy (Chen et al., 2009). Our findings cannot be explained by this model as Akt2 deficiency enhances the diabetes and presumably insulin resistance in spite of the complete absence of leptin in either mouse line. Rather, these data suggest that Akt and leptin control glucose metabolism in parallel pathways.

A recent study examining lipid abnormalities in humans with genetic syndromes of insulin resistance reported increased liver fat content, lipogenesis and serum triglycerides in several individuals with a dominant-negative mutation in *Akt2* (Semple et al., 2009). However, as pointed out by Semple et al., the one patient studied in most detail might have had other, confounding metabolic abnormalities. Of note, like patients with an *Akt2* mutation, $Lep^{ob/ob}$ mice null for *Akt2* had elevated serum triglyceride levels, whereas $Lep^{ob/ob}$ mice with liver-specific deletion of Akt2 exhibit normal or reduced circulating triglyceride (Suppl. Tables 1 and 2). This emphasizes the role of non-hepatic tissues in determining lipid levels and the difficulties in interpreting metabolic data. Nonetheless, additional translational experiments will be required to establish whether the requirement for Akt in NAFLD is unique to rodents or does indeed recapitulate the pathophysiology in humans.

A longstanding paradox has been that people with T2DM and the metabolic syndrome or rodents with equivalent metabolic disorders have systemic insulin resistance in the face of increased hepatic lipogenesis, a classical insulin response (Petersen et al., 2007). Though a number of models could explain this, the concept of selective or partial insulin resistance has received increasing recent attention (Brown and Goldstein, 2008). Both humans with insulin resistance due to inherited mutations in the insulin receptor and mice with liver-specific deletion of the insulin receptor exhibit hyperglycemia and hyperinsulinemia but are protected against steatosis and hypertriglyceridemia (Biddinger et al., 2008; Semple et al., 2009). This finding is consistent with the idea that in classical “insulin-resistant” states, not all signaling is blunted, but rather some is preserved, in particular that to lipid synthesis. While it is likely that the pathways regulating glucose and lipid metabolism diverge somewhere downstream of the IRS proteins but upstream of FoxO1 and SREBP1c, respectively, the precise biochemical site is unknown (Dong et al., 2008; Kubota et al., 2008; Matsumoto et al., 2007). In a recent consideration of selective insulin resistance, Brown and Goldstein wrote that the “Identification of the branch point is a central question for future research” (Brown and Goldstein, 2008). In the studies presented in this paper, we have demonstrated that the point of selective insulin

resistance lies downstream of Akt. A major unresolved question is the nature of those pathways responsible for divergent signaling to glucose output and lipogenesis.

Experimental Procedures

Metabolic measurements and analytical procedures

Overnight fasted mice were used for measurements of blood glucose using a glucometer (OneTouch Ultra, Lifescan). Insulin assays were conducted on blood collected from mice after an overnight fast by tail bleed into heparinized tubes using an ELISA kit (Ultra Sensitive Rat Insulin ELISA kit, Crystal Chem, Inc.). Hepatic triglycerides were measured from animals sacrificed by CO_2 inhalation, snap-frozen in liquid nitrogen, and stored at −80°C until processed. Frozen livers were weighted and homogenized in lysis buffer (140mM NaCl, 50mM Tris, 0.1% Triton-X) using a Tissuelyser (Qiagen). Liver homogenates were then incubated at 37°C with 1% deoxycholate, and triglycerides measured colorimetrically using Infinity Triglyceride Reagent (ThermoDMA, Inc). Serum triglycerides, cholesterol, free fatty acids (NEFA), and ketone bodies were analyzed from blood collected after sacrifice by cardiac puncture using colorimetric assay kits (Infinity TG and CH reagents, ThermoDMA, Inc; NEFA-HR kit, Wako; β-hydroxybutyrate LiquiColor kit, Stanbio Laboratories, respectively). Hepatic long-chain fatty acyl-CoAs were isolated as previously described and measured by using an API 4000 tandem mass spectrometer (Applied Biosystems) in conjunction with 2 PerkinElmer 200 Series micro pumps and a 200 Series autosampler (PerkinElmer) (Neschen et al., 2005).

De novo lipogenesis assay

Male mice were fasted for 5 hours (8am to 1pm), injected with D_2O (400μl per 20g body wt), and sacrificed 3 hours later. Blood was collected by pipette after cutting the aorta/IVC using the diaphragm as a barrier to the peritoneal cavity. Liver was removed and snap-frozen in liquid nitrogen. Palmitate was analyzed as its trimethylsilyl derivative using gas chromatography-electron impact ionization mass spectrometry. The oven temperature was initially held for 1 min at 150° C, then increased by 20° C per min to 310° C and maintained for 8 min. The split ratio was 20:1 with helium flow 1 ml per min. The inlet temperature was set at 270° C and MS transfer line was set at 310° C. Under these conditions, palmitate elutes at ~ 5.7 min. The ^{2}H-enrichment was determined by using selective ion monitoring under electron impact ionization of m/z 313 and 314 (M+0 and M+1), 10 ms dwell time per ion. The concentration of palmitate was determined by comparing the corrected abundance of m/z 313 to 314 to that of heptadecanoate (17:0, m/z 327). To account for possible differences in the ionization efficiency of each fatty acid, the profile was compared against standards prepared by mixing known quantities of each fatty acid. Rate of lipid synthesis was determined as the percent contribution of newly made using the equation: % newly made palmitate = [total ^{2}H-labeling palmitate / (^{2}H-labeling body water × n)] × 100 where *n* is the number of exchangeable hydrogens, assumed to equal 22. The absolute amount of newly made palmitate was determined by multiplying the % newly made palmitate by the concentration of palmitate (Brunengraber et al., 2003; Diraison et al., 1997; Lee et al., 1994a; Lee et al., 1994b).

RNA isolation and gene expression studies

Total RNA was prepared from liver using Trizol reagent (Invitrogen), followed by chloroform extraction and DNAse treatment (DNA-free kit, Ambion). cDNA was synthesized with random decamers using the RetroScript Kit (Ambion), and mixed with Brilliant SYBR Green QPCR Master Mix (Stratagene) and primers as noted in Supplementary Table 5. Reactions were performed on an M×3000P Quantitative PCR System (Stratagene). The relative amounts of specific transcripts were calculated using TATA binding protein (*TBP*) mRNA as an invariant

control by the comparative C_T (ddC_T) method, with the control genotype set to 1.0 (either *Lep*$^{+/+}$ *Akt2*$^{lox/lox}$ or *Akt2*$^{lox/lox}$ chow-fed).

Statistics

Data are presented as mean ± SEM. Data were analyzed using one-way ANOVA using Newman-Keuls post-test, two-way ANOVA using Bonferroni post-test, or Student's t-test assuming unequal variance with 2-tailed analysis as described in the figure legends. Values of $p < 0.05$ were defined as statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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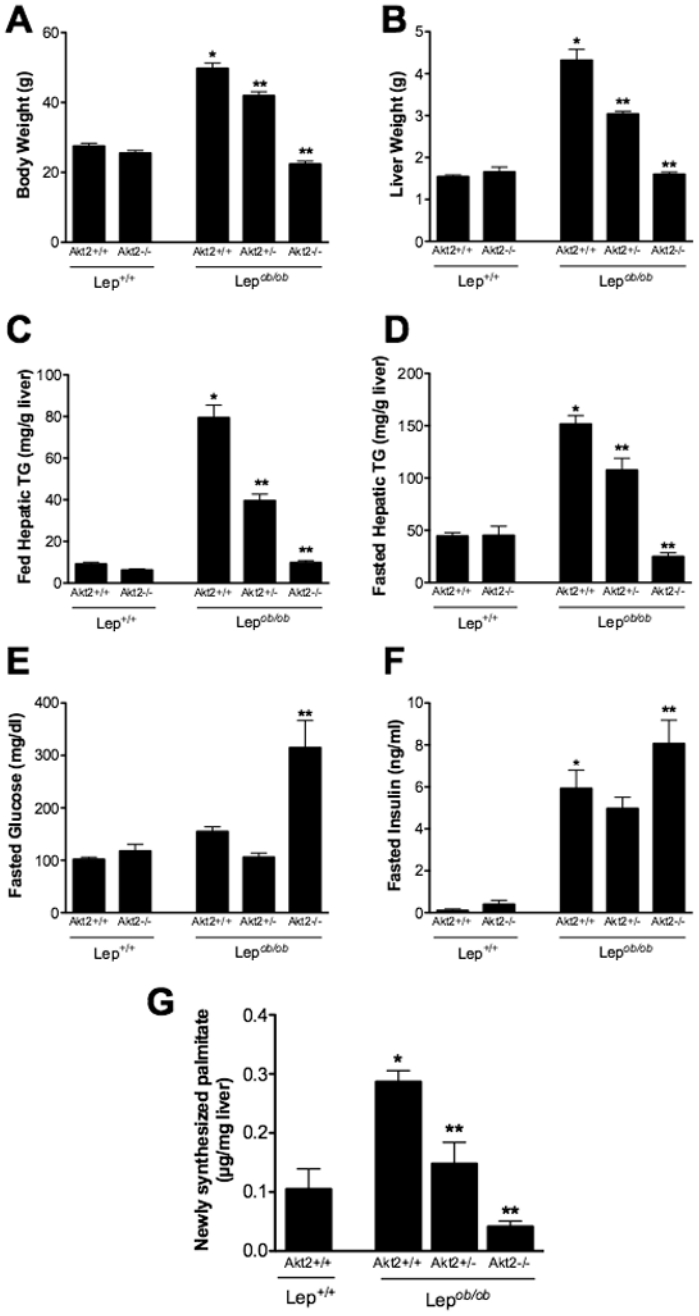

Figure 1. Deletion of Akt2 in *Lep*$^{ob/ob}$ mice results in decreased hepatic triglycerides and *de novo* lipogenesis

A,B. Body weight (A) or liver weight (B) of 12-week old fed male mice. C,D. Hepatic triglyceride levels of 12-week old fed (C) or overnight fasted (D) male mice. E,F. Blood glucose (E) and insulin (F) levels in 8-week old overnight fasted male mice. G. *De novo* lipogenesis: 12 week-old male mice were injected with D_2O after a 5 hour fast, sacrificed after 3 hours, and liver was removed and analyzed for palmitate by GC/MS. All values are expressed as mean ± SEM. n=5–6; *p<0.05 vs *Lep*$^{+/+}$;*Akt2*$^{+/+}$ and **p<0.05 vs *Lep*$^{ob/ob}$;*Akt2*$^{+/+}$ by one-way ANOVA using ewman-Keuls post-test.

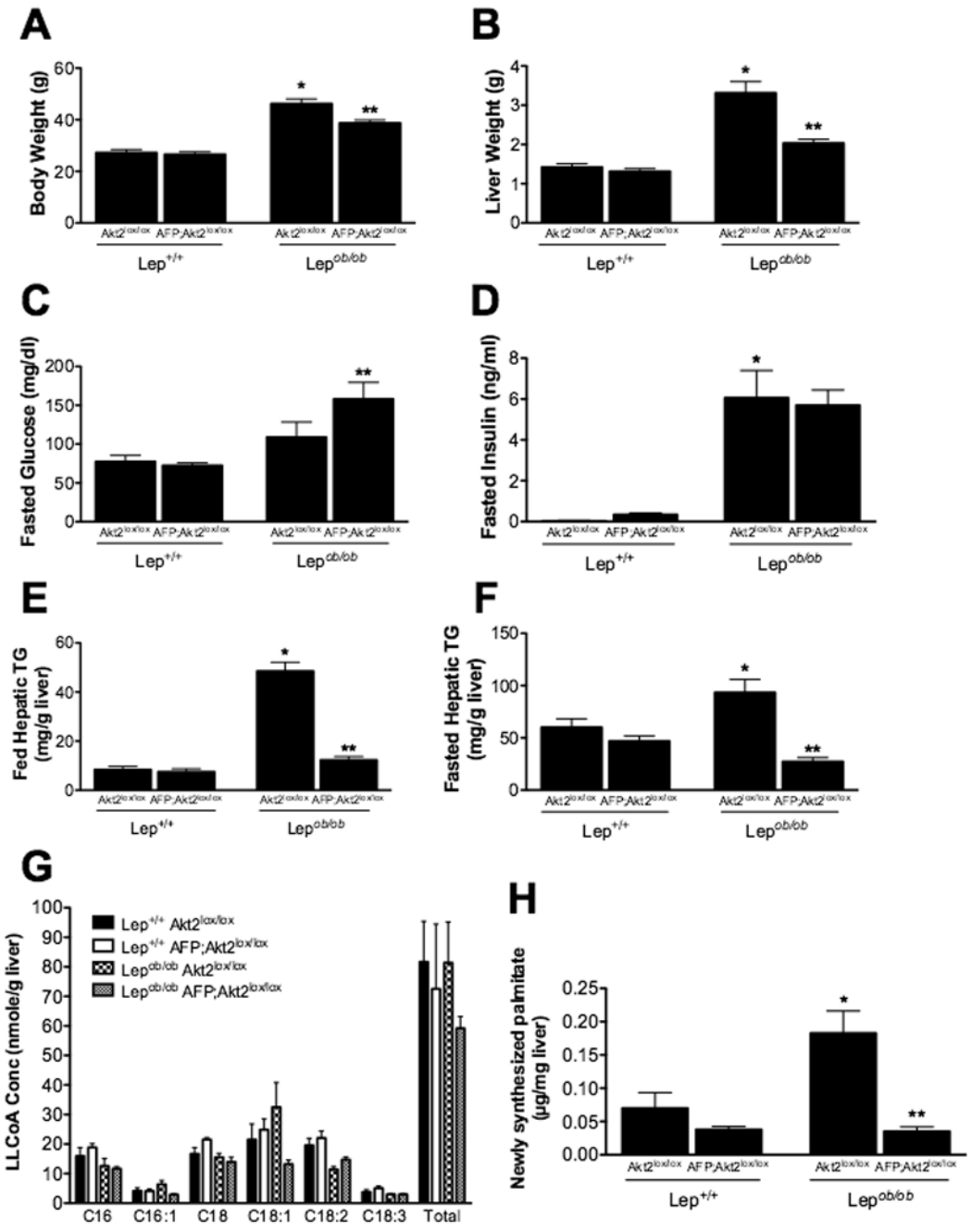

Figure 2. *Lep*$^{ob/ob}$ *AFP;Akt2*$^{lox/lox}$ mice have decreased hepatic triglyceride levels, which correlate with a decrease in *de novo* lipogenesis

A,B. Body weight (A) or liver weight (B) of 12-week old fed male mice. n=6–8. C,D. Blood glucose (C) and insulin (D) levels in 8-week old overnight fasted male mice. n=6–9. E,F. Hepatic triglyceride levels of 12-week old fed (E) or overnight fasted (F) male mice. n=6–9. G. Hepatic long-chain fatty acid CoA (LLCoA) concentrations from 12 week-old overnight fasted male mice. n=3–4. H. *De novo* lipogenesis measured as in Fig. 1. n=5–6. All values are expressed as mean ± SEM. *p<0.05 vs *Lep*$^{+/+}$*;Akt2*$^{lox/lox}$ and **p<0.05 vs *Lep*$^{ob/ob}$*;Akt2*$^{lox/lox}$ by one-way ANOVA using Newman-Keuls post-test.

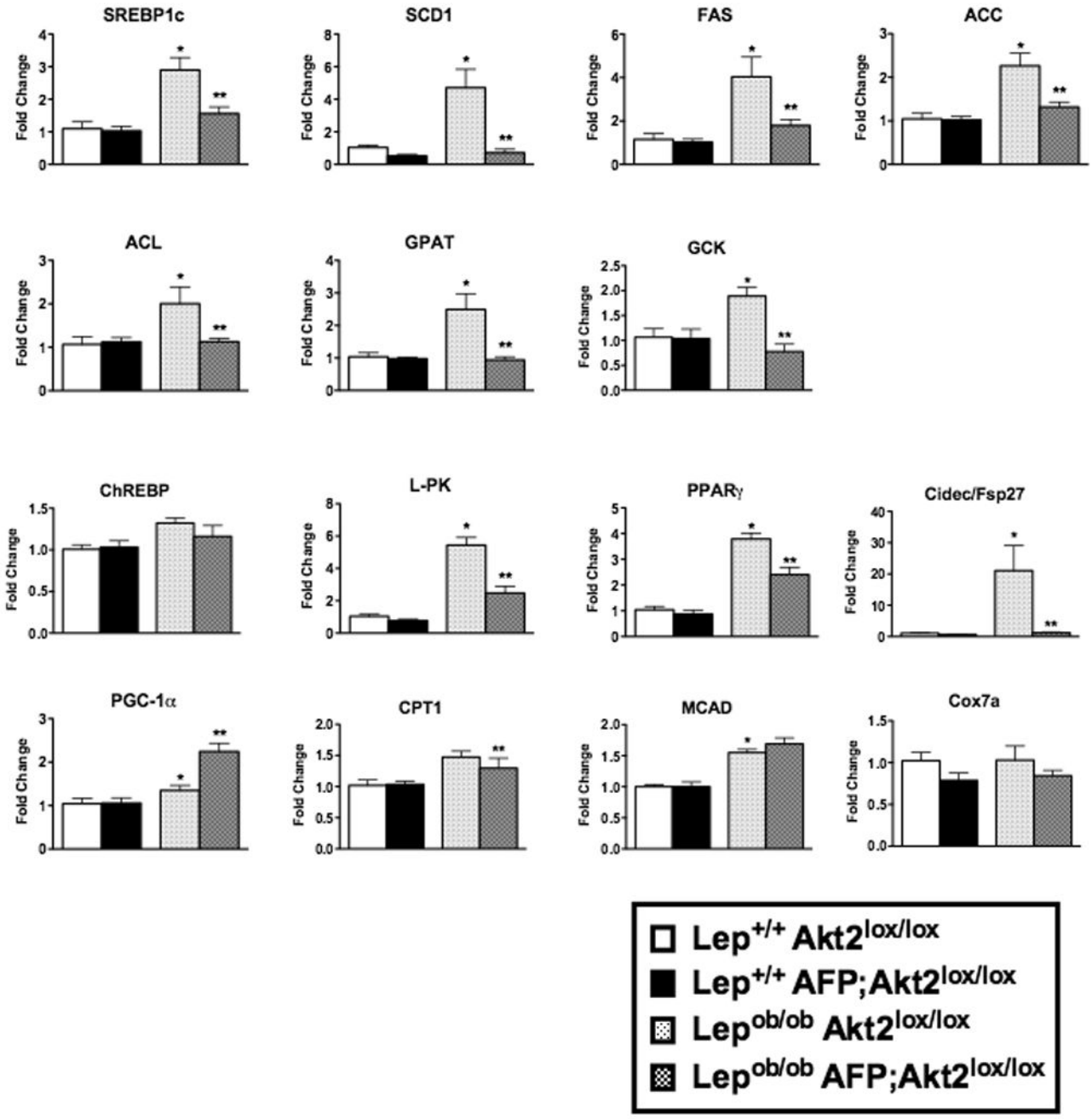

Figure 3. Hepatic gene expression in *Lep$^{ob/ob}$ AFP;Akt2$^{lox/lox}$* mice

Hepatic gene expression as measured by rtPCR of 12 week-old fed male mice. Data are presented as mRNA expression relative to that of TATA binding-protein (*TBP*) and normalized to expression in *Akt2$^{lox/lox}$*, which is set to 1.0 using the ddCT method. All values are expressed as mean ± SEM. n=6; *p<0.05 vs *Lep$^{+/+}$;Akt2$^{lox/lox}$* and **p<0.05 vs *Lep$^{ob/ob}$;Akt2$^{lox/lox}$* by one-way ANOVA using Newman-Keuls post-test.

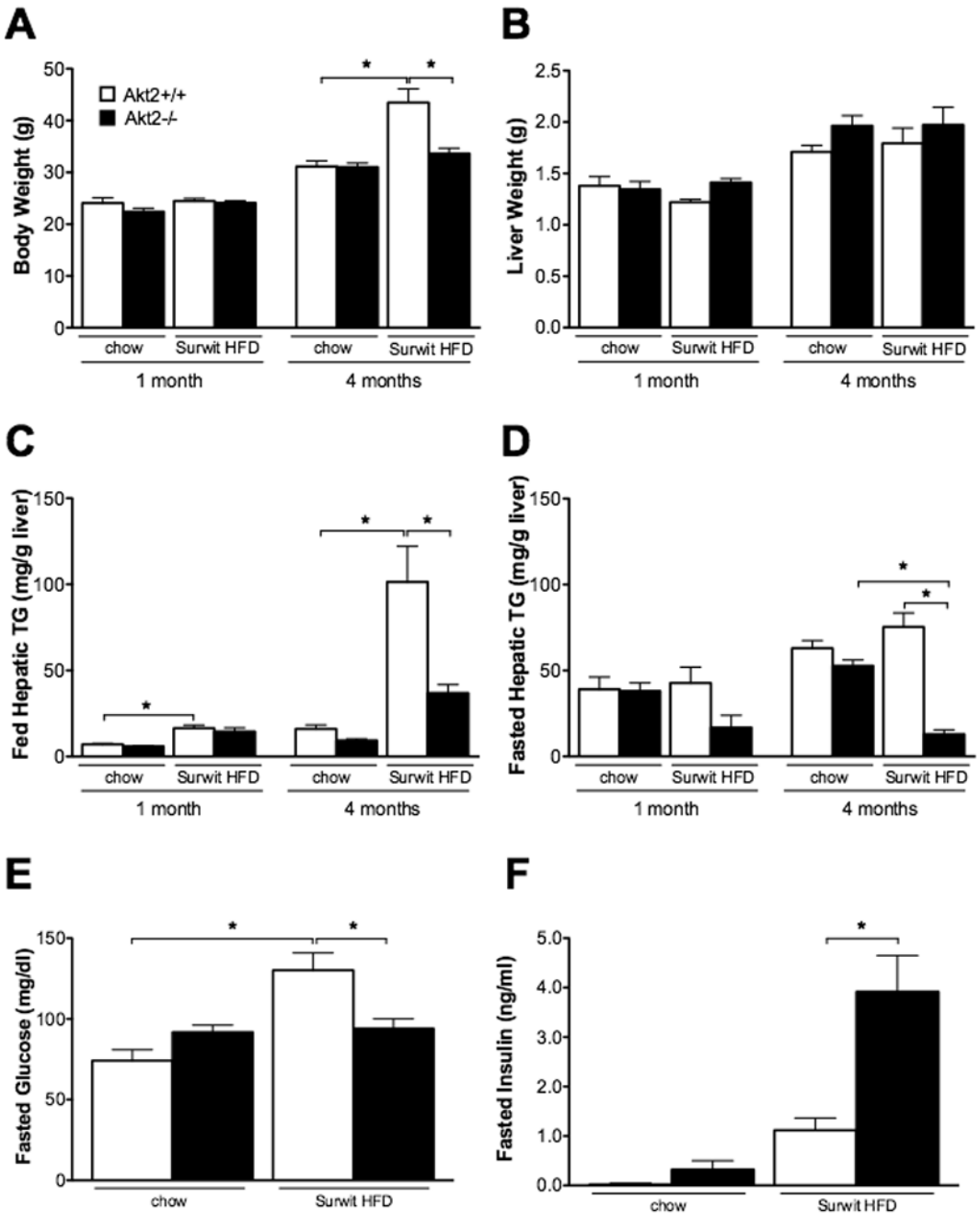

Figure 4. *Akt2*$^{-/-}$ mice have decreased hepatic triglyceride levels after 4 months on Surwit HFD

Male mice were started on Surwit HFD at approximately 5 weeks of age and sacrificed after either 1 or 4 months along with age-matched chow controls. Body weight (A), liver weight (B); hepatic triglyceride levels of fed (C) or overnight fasted (D); blood glucose (E) and insulin (F) levels from overnight fasted mice after 4 months on HFD. Values are expressed as mean ± SEM. n=5-9; *p<0.05 by one-way ANOVA using Newman-Keuls post-test.

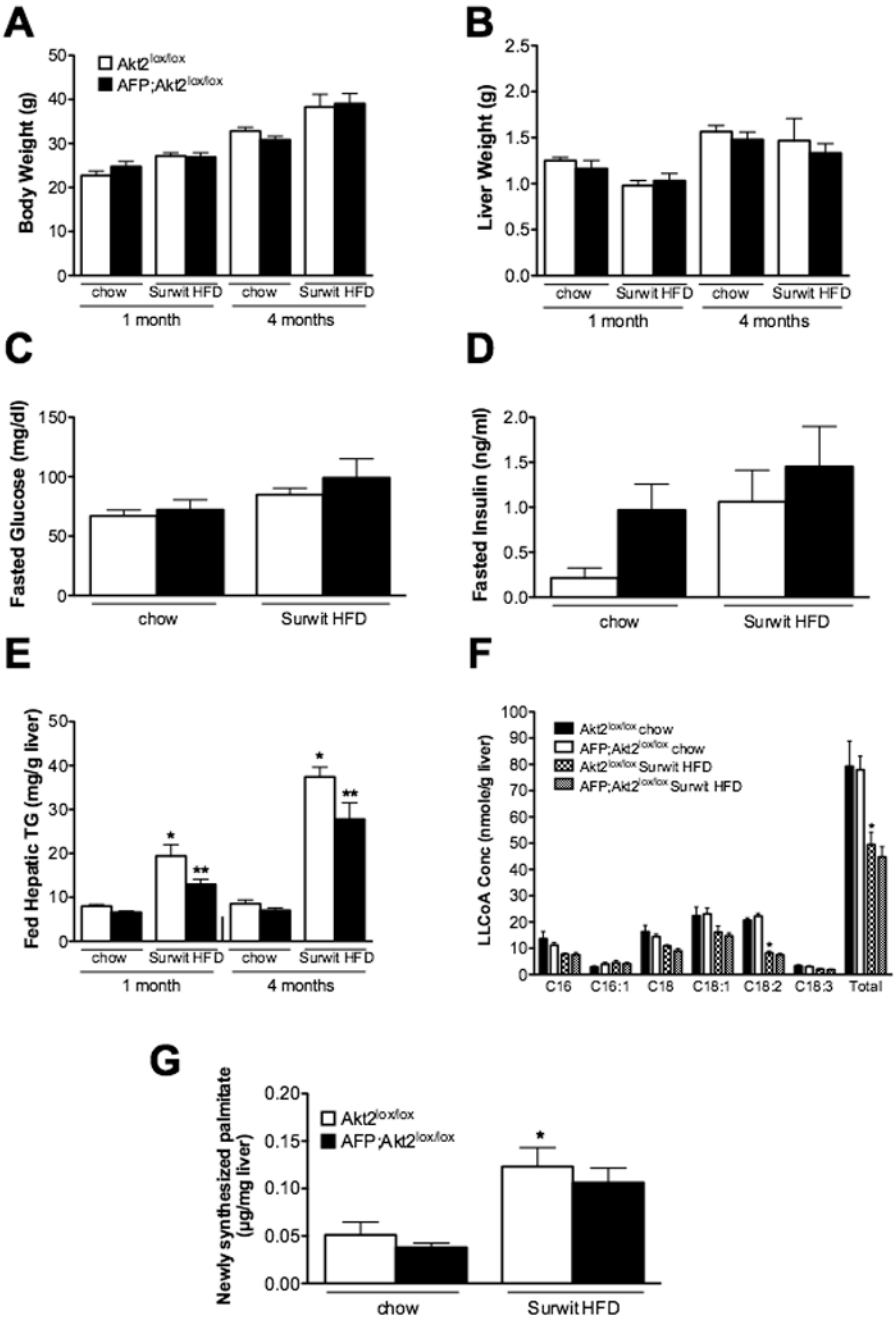

Figure 5. *AFP;Akt2*$^{lox/lox}$ mice on Surwit HFD have decreased hepatic triglyceride levels, but do not exhibit changes in *de novo* lipogenesis

Male mice were started on Surwit HFD at approximately 5 weeks of age and sacrificed after either 1 or 4 months along with age-matched chow controls. Body weight (A), liver weight (B); blood glucose (C) and insulin (D) levels from overnight fasted mice after 4 months on HFD; hepatic triglyceride levels of fed mice (E). n=5–9; *p<0.05 vs. *Akt2*$^{lox/lox}$ chow and **p<0.05 vs *Akt2*$^{lox/lox}$ HFD by one-way ANOVA using Newman-Keuls post-test. F. Hepatic long-chain fatty acyl CoA (LLCoA) concentrations from overnight fasted male mice after 1 month on Surwit HFD. n=4. *p<0.05 vs *Akt2*$^{lox/lox}$ chow by two-way ANOVA using Bonferroni post-test. G. *De novo* lipogenesis: Lipogenesis was assayed in male mice after 4

months on Surwit HFD as in Fig. 1. n=5; *p<0.05 vs *Akt2*$^{lox/lox}$ chow by one-way ANOVA using Newman-Keuls post-test. All values are expressed as mean ± SEM.

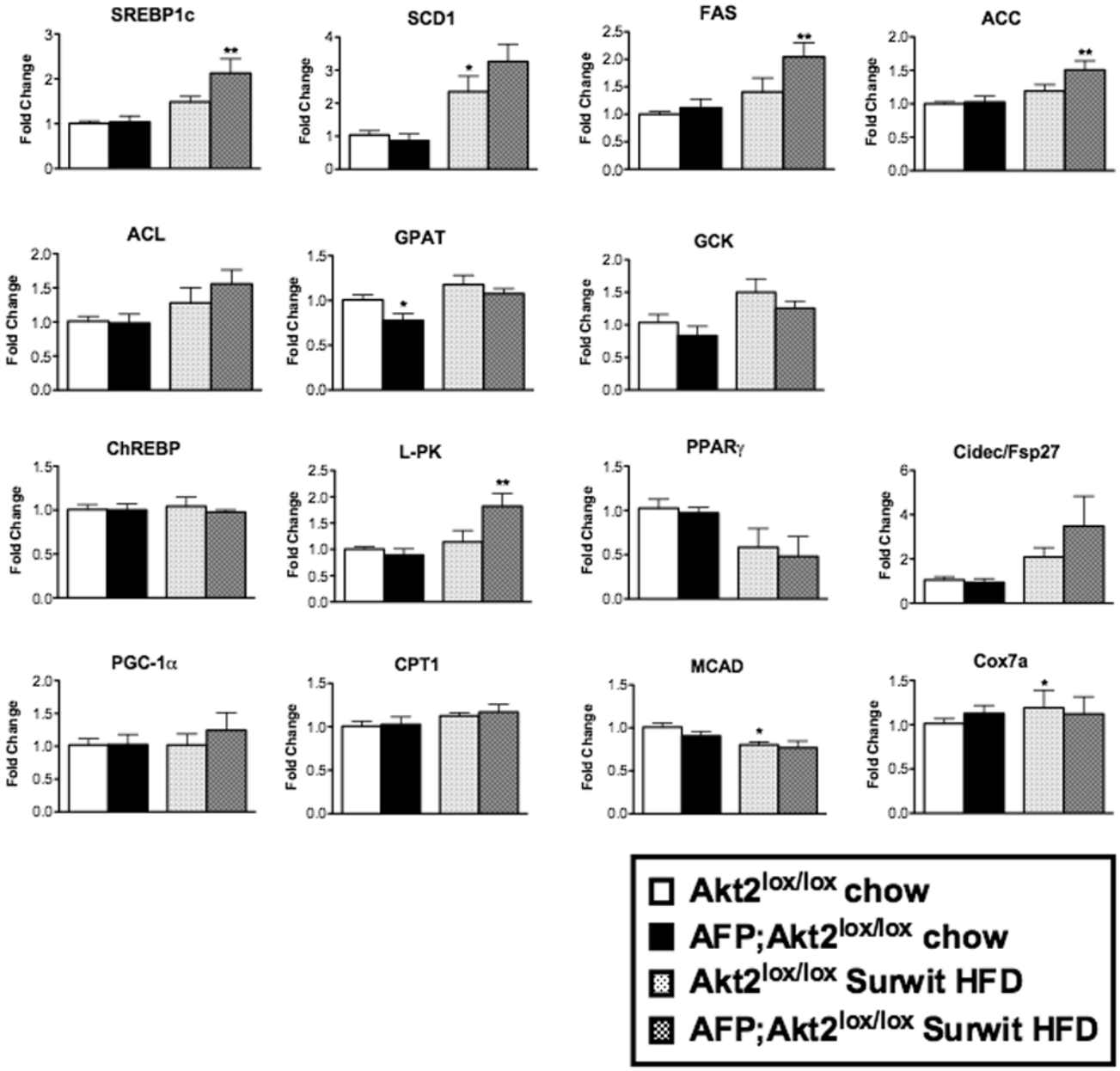

Figure 6. Hepatic gene expression in *AFP;Akt2$^{lox/lox}$* mice on Surwit HFD

Hepatic gene expression as measured by rtPCR of male mice after 1 month on Surwit HFD or age-matched chow-fed controls sacrificed under fed conditions. Data are presented as mRNA expression relative to that of *TBP* and normalized to expression in *Akt2$^{lox/lox}$* chow-fed, which is set to 1.0 using the ddC_T method. All values are expressed as mean ± SEM. n=6; *p<0.05 vs *Akt2$^{lox/lox}$* chow and **p<0.05 vs *Akt2$^{lox/lox}$* HFD by one-way ANOVA using Newman-Keuls post-test.

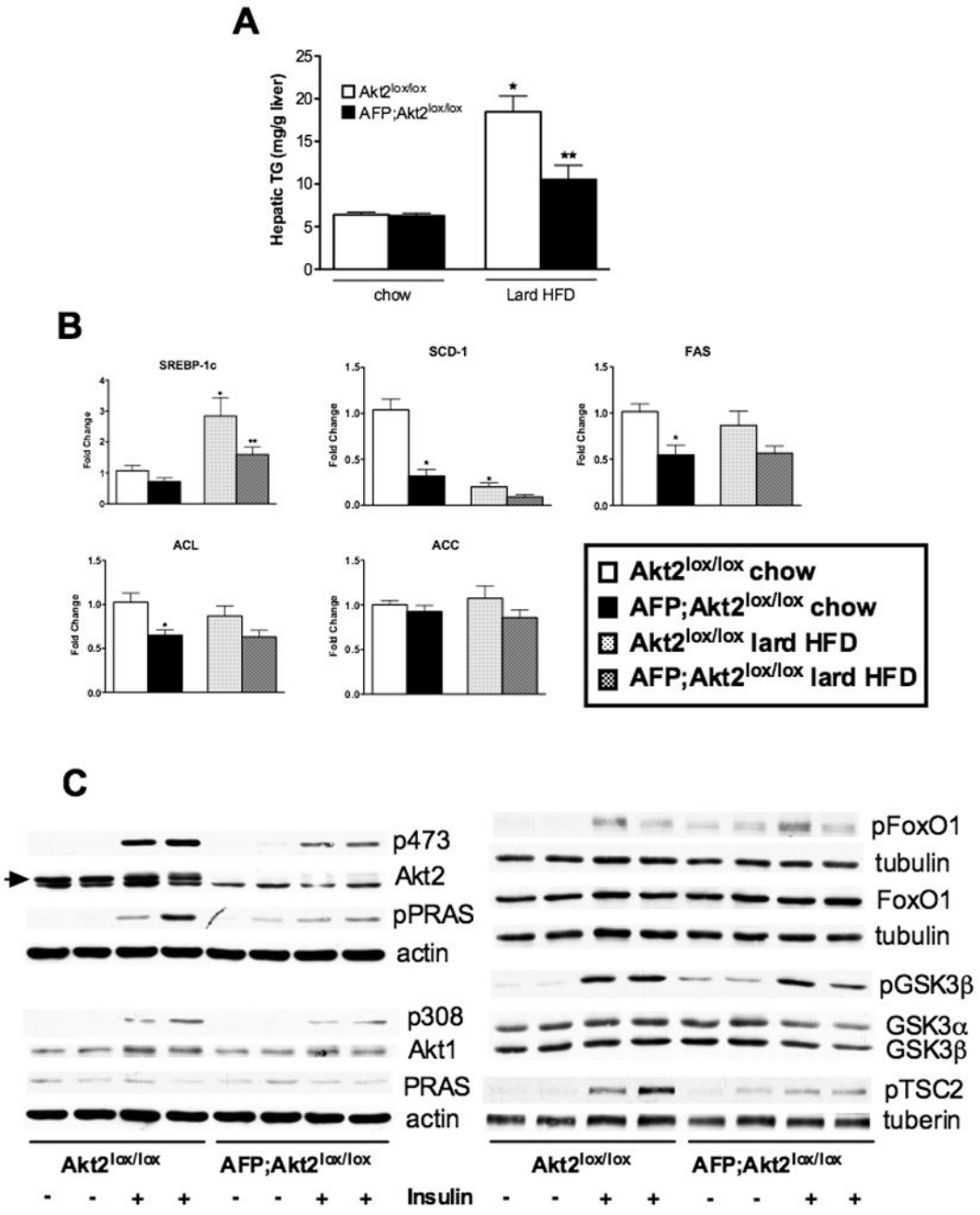

Figure 7. *AFP;Akt2*$^{lox/lox}$ mice on lard HFD have decreased hepatic triglyceride levels and *SREBP-1c* expression

Male mice were started on lard HFD at approximately 6 weeks of age and sacrificed after 1 month on diet under fed conditions along with age-matched chow-fed controls. A. Hepatic triglycerides. B. Hepatic gene expression as measured by rtPCR. Data are presented as mRNA expression relative to that of *TBP* and normalized to expression in *Akt2*$^{lox/lox}$ chow-fed, which is set to 1.0 using the ddC_T method. All values are expressed as mean ± SEM. n=5-6; *p<0.05 vs *Akt2*$^{lox/lox}$ chow and **p<0.05 vs *Akt2*$^{lox/lox}$ HFD by one-way ANOVA using Newman-Keuls post-test. C. Western blot of phospho-Akt (p473 and p308) and downstream signaling targets in hepatic lysates from *Akt2*$^{lox/lox}$ and *AFP;Akt2*$^{lox/lox}$ mice. 8-week old male mice were

fasted overnight and IP injected with either saline or 1U/kg insulin, then sacrificed after 20 minutes. Each lane represents an individual mouse. Loading controls are included when blots from different gels were used for phospho-protein and total protein, though the same protein extracts were used. The arrow indicates Akt2 as there is a slightly more mobile non-specific band.