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Lack of adipose-specific hexose-6-phosphate dehydrogenase causes inactivation of adipose glucocorticoids and improves metabolic phenotype in mice

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Abstract

Excessive glucocorticoid (GC) production in adipose tissue promotes the development of visceral obesity and metabolic syndrome (MS). 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) is critical for controlling intracellular GC production, and this process is tightly regulated by hexose-6-phosphate dehydrogenase (H6PDH). To better understand the integrated molecular physiological effects of adipose H6PDH, we created a tissue-specific knockout of the H6PDH gene mouse model in adipocytes (adipocyte-specific conditional knockout of H6PDH (H6PDH^{AcKO}) mice). H6PDH^{AcKO} mice exhibited almost complete absence of H6PDH expression and decreased intra-adipose corticosterone production with a reduction in 11 β -HSD1 activity in adipose tissue. These mice also had decreased abdominal fat mass, which was

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Author Contribution

J.W., Y.W., L.L., M.J., and Y.j.L. contributed to the design and performance of experiments. L.L., Y.W., J.W., Y.L., and Y.j.L. acquired and analyzed the data. Y.j.L. drafted and wrote the manuscript and supervised this project. M.J., Y.L., K.L., T.C.F., and Y.j.L. reviewed this manuscript. All authors discussed and agreed on the results, and gave final approval.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

paralleled by decreased adipose lipogenic acetyl-CoA carboxylase (ACC) and ATP-citrate lyase (ACL) gene expression and reduction in their transcription factor C/EBP α mRNA levels. Moreover, H6PDH^{AcKO} mice also had reduced fasting blood glucose levels, increased glucose tolerance, and increased insulin sensitivity. In addition, plasma free fatty acid (FFA) levels were decreased with a concomitant decrease in the expression of lipase adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) in adipose tissue. These results indicate that inactivation of adipocyte H6PDH expression is sufficient to cause intra-adipose GC inactivation that leads to a favorable pattern of metabolic phenotypes. These data suggest that H6PDH^{AcKO} mice may provide a good model for studying the potential contributions of fat-specific H6PDH inhibition to improve the metabolic phenotype *in vivo*. Our study suggests that suppression or inactivation of H6PDH expression in adipocytes could be an effective intervention for treating obesity and diabetes.

Introduction

Obesity is a major public health threat that dramatically increases the risk of developing metabolic syndrome (MS) and type 2 diabetes (T2DM) [1–3]. Glucocorticoid (GC) plays a critical role in regulating both energy metabolism and glucose homeostasis. It also contributes to the pathogenesis of MS [4]. Patients with excessive GCs (Cushing's syndrome) develop MS (e.g. visceral fat deposition, lipid abnormalities, insulin resistance, and hypertension) [5,6]. GCs promote adipocyte proliferation and differentiation [7], but evidence also indicates that GCs can induce the expression of hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), two key enzymes for adipose release of free fatty acid (FFA) that is linked to hyperlipidemia and systemic insulin resistance [8,9]. However, circulating cortisol levels are not elevated in the vast majority of patients with obesity and MS. Indeed, many adverse phenotypes of obesity and MS can be induced by enhancing local GC levels *within* adipose tissue by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1), an intracellular endoplasmic reticulum (ER) lumen-resident enzyme. 11 β -HSD1 converts inactive cortisone (in humans), 11-dehydrocorticosterone (11-DHC) into cortisol/corticosterone (in rodents) and thus enhances intracellular GC activity. 11 β -HSD1 is largely expressed in metabolic tissues such as liver and adipose; 11 β -HSD1 also regulates glucose homeostasis, adipose differentiation and fat metabolism through increased local tissue GC production. In humans that are obese and have MS, adipocyte 11 β -HSD1 expression is increased [10]. Transgenic mice, overexpressing 11 β -HSD1 in adipose, recapitulate MS phenotypes [11]. Additionally, 11 β -HSD1 KO mice are resistant to obesity and MS [12]. In addition, pharmacologic inhibition of endogenous 11 β -HSD1 has been shown to reduce weight gain and improve lipid metabolic profile and insulin sensitivity in obese animal models [13,14]. These results support the hypothesis that 11 β -HSD1 plays a pathogenic role in the development of obesity and insulin resistance in adipose tissue through elevated active GC levels. These elevated active GC levels may promote adipogenesis and increase the lipid profile through enhancing lipogenesis and lipolysis in fat tissue.

At the molecular levels, 11 β -HSD1 activation of GCs requires hexose-6-phosphate dehydrogenase (H6PDH) to provide its cofactor NADPH [15,16]. H6PDH is an ER-luminal

enzyme that utilizes glucose 6-phosphate (G6P), provided by the G6P transporter (G6PT), to generate NADPH from NADH in the ER that can be used by NADPH-dependent 11 β -HSD1 reductase activity. Thus, H6PDH is required for 11 β -HSD1 amplification of intracellular GC activation [17,18]. Transgenic overexpression of H6PDH in adipose tissue increases 11 β -HSD1-driven intra-adipose GC production and exhibits many typical features of abdominal obesity and MS [19]. Similarly, in patients with MS and T2DM, as well as in obese animals, adipose cortisol production is increased with elevated local H6PDH mRNA levels [20–23]. These studies highlight the central role of elevated adipose H6PDH in the pathogenesis of MS and obesity.

In contrast, mice with inactivation of H6PDH have reduced 11 β -HSD1 amplification of active GCs in the metabolic tissues of liver, adipose, and muscle. In addition, the mice also have reduced fasting glucose levels and gonadal fat mass, although this did not alter their metabolic phenotype in a non-fasting state [24]. In contrast, inhibition of adipocyte 11 β -HSD1 improves visceral obesity and MS associated with a reduction in adipose H6PDH in animal obesity models [25]. Moreover, patients with target gene mutations in H6PDH have impaired cortisol production [26,27]. Taken together, these studies suggest that adipose tissue H6PDH is able to couple the regulation of cellular GC metabolism and energy homeostasis relative to MS and visceral obesity. However, the contribution of H6PDH in adipocytes to the development of MS could not be elucidated using conventional global knockout mouse models. In addition, the potential contribution of selective H6PDH inhibitors or lack of H6PDH specifically in any specific tissue including adipose tissue to pre-receptor inactivation of fat GCs remains unknown. Importantly, the phenotypic consequences of reduction in fat-specific GC action caused by inactivation of H6PDH specifically in adipose tissue has not been explored.

To test the hypothesis that decreasing the H6PDH expression or activity exclusively in adipose tissue would reduce 11 β -HSD1-driven GCs production and that this reduction would lead to a favorable metabolic phenotype, we generated a tissue-specific animal model of adipocyte-specific H6PDH deletion under the control of the murine adipocyte-specific adiponectin promoter (adipocyte-specific conditional knockout of H6PDH (H6PDH^{AcKO}) mice). Here, we show that H6PDH^{AcKO} mice have decreased intra-adipose-specific GC production and exhibited a favorable metabolic phenotype of abdominal fat distribution and insulin sensitivity associated with improved fat lipid homeostasis.

Methods

Generation of adipose specific knockout mice of H6PDH

Tissue-specific deletion of H6PDH in murine fat cells were achieved by breeding a floxed H6PDH allele mice with an adipocyte-specific expression Cre line. Briefly, conditional targeted H6PDH ES clones (EM: 07643) were obtained from the European Conditional Mouse Mutagenesis Program. ES cells were then microinjected into C57blk/6 blastocysts to generate chimeric mice. Germline transmitted chimeric mice were bred to a Flp recombinase line to obtain a Neomycin cassette-free H6PDH floxed line. The Neo-free H6PDH floxed mice were further crossed with an adipocyte-specific Cre (adipoQ-Cre mouse line, JAX mice 010803) BAC transgenic line to generate H6PDH^{AcKO} mice.

Animal study and biochemical assays

H6PDH^{AcKO} mice and their floxed allele controls (H6PDH^{fl/fl}, lacking the *Cre* gene) were housed in a room maintained on a 12:12-h light–dark cycle with free access to water and standard laboratory chow. All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee of Charles R. Drew University. All animal experiments were performed at Charles R. Drew University. Body weight, food intake, blood glucose levels and blood pressure (BP) were determined weekly. BP was measured by non-invasive tail-cuff plethysmography in mice using a Visitech 2000 automated device (Visitech Systems, Apex, NC). Animals were killed via 5% isoflurane inhalation. Blood samples were collected between 09:00 and 10:00, and adipose tissues were weighed, dissected, and frozen rapidly in liquid nitrogen and stored at -80°C for metabolic assays. Blood samples were analyzed for the level of plasma corticosterone, insulin, and FFA using commercially available kits (Abcam, Cambridge, MA, U.S.A.).

Intraperitoneal glucose and insulin tolerance tests

For glucose tolerance tests (GTTs), H6PDH^{AcKO} mice and floxed/floxed controls (Floxed controls) were fasted overnight (16 h) at 14 weeks age and then given an intraperitoneal injection of glucose (2 g/kg, body weight) [25]. Blood samples were collected from tail tips before, and at different times after, the glucose load. For the insulin tolerance test (ITT), mice were fasted for 8 h, and blood samples were collected at indicated time points following insulin injection (0.75 U/kg, ip; Humulin) for glucose measurement [9].

Histological analysis

At the end of the experiments, the epididymal fat pad from both the control and H6PDH^{AcKO} mice were collected and fixed in 10% formalin, embedded in paraffin, and sectioned. Adipose morphometry was visualized by Hematoxylin and Eosin staining (H&E) and three representative images per section were taken from each mouse (three mice/group). Adipocyte size in adipose tissue was analyzed using the NIH ImageJ software as described previously [19].

Microsomal H6PDH activity assays in adipose tissue

The adipose microsomal pellet was obtained by centrifugation of the supernatant for 1 h at $100\,000\times g$. The adipose microsomal H6PDH activity was measured as previously reported [19]. Briefly, protein (100 μg) from adipose microsomes were incubated with 0.5–5 mM G-6-P, 1–5 mM NADP, and 100 mM glycine buffer solution at 22°C for 0–10 min. H6PDH activities were calculated and expressed as micromoles of NADPH production per minute per milligram of protein using absorbance at 340 nm by spectrophotometry [28].

Adipose tissue 11 β -HSD1 activity and corticosterone assays

Adipose 11 β -HSD1 activity was measured by the addition of 1 mM NADPH and 250 nM 11-DHC with [^3H]11-DHC as the tracer to microsomes in a KRB solution at 37°C for 1 h as previously described [25]. Steroids were extracted with ethyl acetate and separated by a thin layer chromatography system. Radioactivity for each fraction was determined by scintillation counting and enzyme activity was determined. Corticosterone concentrations

were measured in adipose tissue as previously described [29]. Briefly, epididymal fat pads were weighed and homogenized in PBS buffer (PH 7.4) by a tissue solubilizer. Steroids in adipose tissue were extracted with ethyl acetate and fat corticosterone levels were measured using a corticosterone EIA kit [30,31].

RNA extraction and gene expression analysis

Total RNA was extracted from mouse tissues using TRIzol reagent (Thermo Fisher Scientific Inc. Waltham, MA) and the first-strand cDNA was synthesized from 2.0 µg mRNA using High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific Inc. Waltham, MA). Real-time PCR primers for mouse H6PDH (F: 5'-TGGCTACGGGTTGTTTTT GAA-3'; R: 5'-TATACACGGTACATCTCCTCTTCCT-3'), 11β-HSD1 (F: 5'-CCTTGGCC-TCATAGACACAGA AA C-3'; R: 5'-GGAGTCAAAGGCGATTTGTCAT-3'), ACC (F: 5'-TGT AAATCTGGCTGCATCCATTAT-3'; R: 5'-TGGTAGACTGCCCGTGTGA-3'), ATP-citrate lyase (ACL) (F: 5'-ATGCCAAGACCATCCTCTCACT-3'; R: 5'-TCTCACAAATGCCCTTGAAGGT-3'), HSL (F: 5'-GGGCAAAGAAGGATCGAAGAA-3'; R: 5'-GCGTAAATC CATGCTGTGTGA-3'), ATGL (F: 5'-TCGTGGATGTTGGTGGAGCT-3'; R: 5'-TGTGGCCTCATTCTCCTA-3'), C/EBPα (F: 5'-TGGACAAG AACAGCAACGAGTAC-3'; R: 5'-CGGTCATTGTCCTGGTCAACT-3'), and C/EBPβ (R: 5'-CTGCGGGGTTGTTGATGT-3'; R: 5'-ATGCTCGAAACGGAAA GGT-3') were designed by Primer express software 2.0 (Thermo Fisher Scientific Inc. Waltham, MA). Quantitative Real-time PCR analysis was performed with SYBER green kit following manufacturer's protocol in the ABI StepOne Plus System (Thermo Fisher Scientific Inc. Waltham, MA). Threshold cycle (C_t) readings for each of the unknown samples were then used to calculate the amount of target genes. The expression for target genes was normalized to the 18S rRNA values.

Western immunoblotting analysis

Adipose tissues were homogenized and the protein concentration was determined by Bradford assay as previously described [20]. The proteins were separated on 4–12% acrylamide SDS/PAGE gels (Bio-Rad, Hercules, CA) to evaluate the expression of H6PDH (Cat# sc-67394, Santa Cruz, Dallas, Texas), 11β-HSD1 (Cat# sc-20175, Santa Cruz, Dallas, Texas), ACC (Cat# C83B10, Cell Signaling, Danvers, MA), ACL (Cat# 4332, Cell Signaling, Danvers, MA), pSer⁷⁹ ACC (Cat# D7D11, Cell Signaling, Danvers, MA), pSer⁴⁵⁵ ACL (Cat# 4331, Cell Signaling, Danvers, MA), total HSL (Cat# 4107S, Cell Signaling, Danvers, MA), phospho-Ser⁶⁶⁰ HSL (Cat# 4126S Cell Signaling, Danvers, MA), ATGL (Cat# 2138S, Cell Signaling, Danvers, MA), and β-actin (Cat# 3700S, Cell Signaling, Danvers, MA). Adipose proteins were transferred to nitrocellulose membranes and incubated with the appropriate primary and secondary antibodies. Protein bands were visualized using ECL (Thermo Fisher Scientific Inc. Waltham, MA) and quantified by Eagle Eye II Quantitation System (Stratagene, CA, U.S.A.).

Statistical analysis

All values are expressed as the mean \pm SEM. Data were analyzed using an unpaired Student's *t*-test. To compare multiple groups, we conducted one-way ANOVA at first. When ANOVA revealed significant differences, then group comparisons were performed using Newman–Keul's post hoc test. A *P*-value <0.05 was considered statistically significant.

Results

H6PDH gene expression is impaired in adipose tissue of H6PDH^{AcKO} mice

To evaluate the expression levels of H6PDH in adipose tissue, both H6PDH mRNA and protein expression were measured by RT-PCR and Western blot analysis. Figure 1A shows that H6PDH mRNA expression was reduced by 71% in epididymal fat and 74.1% in subcutaneous fat of the H6PDH^{AcKO} mice, compared with flox controls ($P<0.01$). Western blot analysis further demonstrated that H6PDH protein expression was extremely low in epididymal fat as well as in the subcutaneous fat of H6PDH^{AcKO} mice, compared with flox controls (Figure 1B–D). Consistent with these observations, both epididymal and subcutaneous fat microsomal H6PDH activity, as determined by NADPH production, was barely detectable in the H6PDH^{AcKO} mice compared with controls (Figure 1E). In contrast, endogenous H6PDH mRNA levels in the liver (Figure 1F) and skeletal muscle, as well as in kidney, were not different between H6PDH^{AcKO} mice and their age-matched controls. Similarly, no significant differences in H6PDH protein expression were observed between two genotypes (data not shown). These results demonstrate that our conditional KO of H6PDH deletes H6PDH expression effectively in adipocytes, but leaves H6PDH expression unaffected in other tissues.

Metabolic phenotype characterization of H6PDH^{AcKO} mice

To determine the physiological effects of adipose H6PDH deletion, we then assessed the differences in body weight, food intake, and white adipose fat pad mass between H6PDH^{AcKO} and their age-matched controls at 18 weeks old. As shown in Figure 2A, adipose tissue for H6PDH^{AcKO} and flox control mice showed similar changes in weight gain. Similarly, there was no significant difference in food intake between the H6PDH^{AcKO} mice and flox controls (data not shown). In addition, no significance was observed in the value of mean systolic blood pressure between H6PDH^{AcKO} mice (102 ± 16 mmHg) and flox controls (108 ± 18 mmHg). In contrast, adipose fat pad weight was significantly reduced (by 53%) in epididymal fat, 34% in subcutaneous fat, and 62% in mesenteric fat pad ($P<0.05$) in H6PDH^{AcKO} mice compared with controls (Figure 2B). Adipocyte size for H6PDH^{AcKO} mice was decreased by 20% than controls ($P<0.001$, Figure 2C,D). H6PDH^{AcKO} mice also showed decreased epididymal fat pad mass (0.33 ± 0.12 g; $n=7$) compared with controls (0.59 ± 0.14 g; $n=7$, $P<0.01$) in fasting conditions. These data indicate that H6PDH^{AcKO} mice exhibited a favorable abdominal fat distribution to matched controls.

Decreased adipose lipogenesis in H6PDH^{AcKO} mice

To determine if the reduced fat content in H6PDH^{AcKO} adipocytes occurred in conjunction with changes in lipogenesis, we analyzed the expression of lipid synthase ACC and ACL, the major markers of lipid content in adipose tissue. Real-time RT-PCR revealed that epididymal fat ACC and ACL mRNA levels were decreased (61 and 65%, respectively) in H6PDH^{AcKO} mice compared with controls (Figure 3A). Western blot analysis also confirmed that epididymal fat ACC and ACL protein levels were decreased with a reduction in phosphorylated levels of pSer⁷⁹ ACC and pSer⁴⁵⁵ ACL in H6PDH^{AcKO} mice (Figure 3C,E). Similarly, lower ACC and ACL mRNA levels (Figure 3B) with decreased ACC, pSer⁷⁹ ACC, ACL, and pSer⁴⁵⁵ ACL protein expression were observed in subcutaneous fat of H6PDH^{AcKO} mice (Figure 3D,F). These data indicate that reduction in adipose lipogenesis may contribute to abdominal fat loss observed in H6PDH^{AcKO} mice.

H6PDH^{AcKO} mice had impaired adipose lipolysis and reduced plasma FFA levels

To study if adipose H6PDH inactivation could influence lipolysis, we next measured the plasma FFA levels and the expression of HSL and ATGL, two key lipolytic enzymes in adipose tissue. Figure 4A shows that H6PDH^{AcKO} mice had significantly decreased plasma FFA levels compared with that of flox controls under both fasting and fed states ($P < 0.01$ and $P < 0.05$, respectively). Consistent with decreased FFA levels, epididymal fat mRNA levels of HSL were decreased (50 and 36%, in fasting and fed states) in H6PDH^{AcKO} mice compared with that of controls (Figure 4B). This was confirmed by the results of Western blot analysis which demonstrated that the epididymal fat total for HSL protein level was decreased with strongly reduced phosphorylated levels of Ser⁶⁶⁰ HSL in H6PDH^{AcKO} mice compared with controls (Figure 4C,D). Similarly, lower ATGL mRNA and protein levels were observed in epididymal fat from H6PDH^{AcKO} mice compared with controls under both fasting and fed conditions (Figure 4B–D). These results indicate that the reduction in adipose lipolysis may contribute to decreased circulating FFA levels.

Adipose H6PDH^{AcKO} mice showed decreased fasting glucose levels and increased tolerance to glucose and insulin

To further explore the impact of adipose H6PDH deletions on glucose homeostasis and insulin sensitivity, we assessed glucose handling and insulin sensitivity in H6PDH^{AcKO} mice and flox controls by performing glucose and insulin tolerance tests. As shown in Figure 5A, the fasting glucose levels were decreased in H6PDH^{AcKO} mice (71 ± 12 mg/dl) compared with controls (102 ± 11 mg/dl; $P = 0.002$). In parallel with fasting hypoglycemia, H6PDH^{AcKO} mice showed decreased glucose levels during GTT. In addition, the area under the curve (AUC) levels of glucose tolerance in H6PDH^{AcKO} mice were reduced compared with controls (Figure 5B). Moreover, decreased plasma insulin levels were detected in H6PDH^{AcKO} mice in comparison with controls during GTT (Figure 5C). This is supported by the results of the ITT which showed that H6PDH^{AcKO} mice had improved glucose disposal in response to insulin (Figure 5D). Consistent with these observations, fasting plasma insulin levels were decreased in H6PDH^{AcKO} mice compared with that of controls ($P < 0.01$; Figure 5E). These data indicate that the H6PDH^{AcKO} mice exhibited an improved glucose handling with increased insulin sensitivity.

H6PDH^{AcKO} mice exhibit decreased endogenous 11 β -HSD1 and corticosterone levels in adipose tissue

Because H6PDH is required for 11 β -HSD1 activity, we next examined the impact of adipose H6PDH deletion on 11 β -HSD1 expression and GC metabolism in adipose tissue. 11 β -HSD1 mRNA level was reduced by 59% in epididymal fat ($P<0.01$) and 45% in subcutaneous fat ($P<0.01$) of H6PDH^{AcKO} mice compared with their controls (Figure 6A). Western blot analysis indicated that epididymal and subcutaneous adipose 11 β -HSD1 protein expression was substantially down-regulated in H6PDH^{AcKO} mice compared with controls (Figure 6B–D). Likewise, 11 β -HSD1 reductase activity was decreased by 2.4-fold in epididymal fat and 1.85-fold in subcutaneous fat ($P<0.01$; Figure 6E) in H6PDH^{AcKO} mice compared with controls. In addition, real-time RT-PCR analysis showed that H6PDH^{AcKO} mice also markedly reduced C/EBP α mRNA levels in epididymal fat (40%; $P<0.01$) and subcutaneous fat (37%; Figure 6F), a key transcription factor for 11 β -HSD1. However, H6PDH^{AcKO} mice maintained C/EBP α mRNA expression in adipose tissue. Moreover, there was a positive correlation between adipose 11 β -HSD1 and C/EBP α mRNA expression in H6PDH^{AcKO} mice and controls ($R^2 = 0.8752$, $P<0.001$). In support of our findings, adipose corticosterone levels were significantly decreased by 2.3-fold in epididymal fat and 4.1-fold in subcutaneous fat in H6PDH^{AcKO} mice (Figure 6G; $P<0.001$). In spite of this, H6PDH^{AcKO} mice did not have altered plasma corticosterone levels compared with their controls (Figure 6H).

Discussion

We have established a mouse model for exclusive adipocyte-specific knockdown of H6PDH under the control of the murine adiponectin promoter (H6PDH^{AcKO}). We confirmed that the expression of H6PDH in H6PDH^{AcKO} mice was diminished in adipocytes, but unaffected in other tissues. We demonstrated that H6PDH^{AcKO} mice had reduced expression and activity of 11 β -HSD1 and exhibited a favorable metabolic phenotype. We observed that corticosterone levels were decreased in the adipose tissue of H6PDH^{AcKO} mice that was associated with a reduction in adipose 11 β -HSD1 activity. These findings indicate that the conversion of corticosterone from 11-DHC is impaired in response to inactivation of H6PDH expression in the adipose tissue of H6PDH^{AcKO} mice. Since 11 β -HSD1 activity is dependent on the production of H6PDH-driven NADPH in target tissues, reduced adipose H6PDH expression decreased ER luminal NADPH availability to 11 β -HSD1-driven GC production in adipose tissue. These data suggest that H6PDH^{AcKO} mice effectively decrease adipose GC production through the reduction in the GC-amplifying effects of endogenous 11 β -HSD1 in adipose tissue. This interaction is supported by recent studies reporting that intracellular GC reactivation is impaired in the metabolic tissues of adipose, liver and muscle in H6PDH knockout mice [24,32,33]. However, H6PDH^{AcKO} mice did not have altered endogenous H6PDH expression in non-adipocyte tissues for liver, muscle, kidney, and brain. Thus, reduced adipose H6PDH expression can account for the phenotype observed in H6PDH^{AcKO} mice, independent of non-adipocyte H6PDH expression.

Importantly, we observed that H6PDH^{AcKO} mice exhibited fasting hypoglycemia, decreased insulin levels, and decreased plasma FFA levels suggesting that H6PDH^{AcKO} mice exhibit

improved insulin sensitivity and decreased fatty acid flux. The major difference between H6PDH^{AcKO} and global H6PDH knockout mice is that H6PDH^{AcKO} mice showed both improved glucose and insulin tolerance in response to decreased adipose GCs production while maintaining normal corticosterone levels, whereas global H6PDH knockout mice had normal glucose tolerance and insulin sensitivity with increased plasma GC levels [24,32,34]. Similarly, 11 β -HSD1 KO mice showed no changes in these glucose metabolic parameters and plasma FFA levels [35,36], but elevated plasma GC levels [36,37]. Different circulating GC levels might contribute to the difference observed in glucose tolerance and systemic insulin sensitivity between H6PDH^{AcKO} and global H6PDH knockout mice. Our data indicate that adipose-specific reduction in H6PDH is sufficient to account for the fasting hypoglycemia and insulin-sensitive phenotype, as observed in H6PDH^{AcKO} mice, independent of circulating and non-adipocyte GC effects.

In adipocytes, GCs promote fat lipolysis by stimulation of both HSL and ATGL, two key enzymes for the hydrolysis of triacylglycerol as well as for the release of FFA into the circulation that leads to hyperlipidemia linked to systemic insulin resistance [38,39]. Increased adipose 11 β -HSD1 and H6PDH have been shown to be important mediators for enhancing the contribution of GCs to elevated plasma FFA levels and insulin resistance [19]. In contrast, pharmacological inhibition or target knockout of 11 β -HSD1 what shown to reduce lipemia and improve insulin sensitivity in rodents as well as in humans [40,41]. In accord with this concept, H6PDH^{AcKO} mice exhibited lower levels of both HSL and ATGL expression in response to reduced local GC production in the abdominal adipose tissue and decreased levels of plasma FFA, suggesting an impaired lipolytic activity in adipose tissue in H6PDH^{AcKO} mice. This could explain the decreased plasma FFA levels and may thus provide an additional mechanism for maintaining the phenotype of glucose homeostasis and insulin sensitivity observed in H6PDH^{AcKO} mice. Our findings are supported by a recent study reporting that global H6PDH knockout mice showed decreased fat HSL mRNA levels [24]. In contrast, adipose-specific activation of H6PDH induced adipose lipase and elevated plasma FFA levels. This resulted in fasting hyperglycemia and glucose intolerance in response to elevated GC production in adipose tissue [19]. These findings support the notion that the reduction in fat lipolysis most likely occurs via reduced intra-adipose GC production-induced fat lipase expression.

In addition, H6PDH^{AcKO} mice develop a lean fat metabolic phenotype, which is similar to global H6PDH knockout mice that also show reduced gonadal fat weight in the fed state. The difference is that H6PDH^{AcKO} mice pronounced decreased epididymal fat mass in both fed and fasted states, while global H6PDH knockout mice did not have altered fasted gonadal fat mass [24]. Moreover, fat tissue histology revealed that H6PDH^{AcKO} mice had small adipose cell size that differs from the normal fat cell size of H6PDH knockout mice [24]. These data could explain the difference in phenotype of reduced abdominal fat-depot mass between mice who lack global and adipose-specific H6PDH. More importantly, we observed that H6PDH^{AcKO} mice have less intra-abdominal mesenteric fat and epididymal fat mass than seen in subcutaneous fat. However, in H6PDH^{AcKO} mice, this did not affect blood pressure, despite the relationship between adipocytes and blood pressure [42,43]. Similarly, 11 β -HSD1 KO mice had normal blood pressure but did not change mesenteric fat weight [35,44]; this is different from what is seen in H6PDH^{AcKO} mice.

In fact, intra-abdominal fat accumulation is thought to be a leading cause of adverse metabolic consequence relative to the development of visceral obesity and MS [45,46]. Clinical studies have revealed that patients with obesity and MS have increased H6PDH expression and elevated local activation of cortisol from cortisone with induction of 11 β -HSD1 in visceral fat [21,22]. Additionally, the key lipogenic transcription factor, mRNA encoding C/EBP α , is decreased with concomitantly reduced lipogenic enzymes ACC and ACL with reduction in 11 β -HSD1 in adipose tissue of H6PDH^{AcKO} mice. Decreased C/EBP α could attenuate the progress of lipogenesis and thus contribute to reduced abdominal fat mass. This interpretation is supported by studies reporting that enhanced adipose H6PDH increased fat lipogenesis and induced visceral fat accumulation with elevated C/EBP α , which itself is activated by GCs [11,19,47]. Our present data are consistent with a recent study reporting that inhibition of adipose 11 β -HSD1 coupled with suppression of H6PDH, improved the phenotype of visceral obesity [25]. Moreover, C/EBP α is also a key transcriptional activator of 11 β -HSD1 in adipose and liver [48,49]. Adipose H6PDH knockdown-mediated reduction in C/EBP α may thus represent an additional mechanism to promote adipose 11 β -HSD1 reduction that is linked to decreased lipogenesis and fat loss in H6PDH^{AcKO} mice. Additionally, adipocyte 11 β -HSD1 can also be stimulated by insulin [50,51] and H6PDH^{AcKO} mice had decreased insulin levels in response to the favorable metabolic phenotype with reduction of adipose 11 β -HSD1 activity and gene expression observed in H6PDH^{AcKO} mice. These findings imply that H6PDH^{AcKO} mice-mediated induction of hypoinsulinemia may contribute to reduced adipose 11 β -HSD1 activity. This interpretation is supported by earlier observations that insulin directly increases 11 β -HSD1 production, though did not affect H6PDH in 3T3-L1 adipocytes [51]. Decreased insulin level itself may thus provide another possible mechanism to account for reduced adipose 11 β -HSD1 production linked to the phenotype of H6PDH^{AcKO} mice. In addition, reduced fasting plasma insulin levels are associated with increased insulin sensitivity and decreased plasma FFA levels in H6PDH^{AcKO} mice. This interpretation is supported by earlier observations that reduction in plasma FFA levels contribute to low basal insulin levels [52,53].

One of the major strength of our study is the use of adipose-specific conditional KO. This enabled us to delineate the crucial contribution of H6PDH in adipocyte from other tissues (including liver or muscle) related to adipose function and glucose homeostasis in rodents. However, considering H6PDH^{AcKO} mice are conditional (not inducible) knockout mice, compensatory changes that develop in knockout mice cannot be ruled out, even in the adipose-specific KO mice, which could have contributed to the current results. Importantly, it is unanswered yet whether the absence of adipose H6PDH could prevent or reduce the adverse effects of high-calorie food. This represents a caveat of the current study, as we did not assess if these mice are protected against the metabolic changes that occur after exposure to high-fat diet in mice.

In summary, the present study demonstrates that reduction in adipose-specific H6PDH leads to reduced fat cell-specific production of GCs, and decreased adipose tissue lipolysis with resultant decreased plasma FFA level. This is known to be responsible for both global insulin sensitivity and glucose homeostasis. In addition, the mice exhibited a favorable metabolic phenotype. These findings also imply that inhibition of H6PDH in adipose tissue

may represent a novel and potential therapeutic approach to prevent or delay the progress of MS and obesity.

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Abbreviations

ACC	acetyl-CoA carboxylase
ACL	ATP-citrate lyase
ATGL	adipose triglyceride lipase
C/EBPs	CCAAT-enhancer-binding proteins
ER	endoplasmic reticulum
FFA	free fatty acid
GC	glucocorticoid
G6P	glucose 6-phosphate
GTT	glucose tolerance test
HSL	hormone-sensitive lipase
H6PDH	hexose-6-phosphate dehydrogenase
H6PDH^{AcKO}	adipocyte-specific conditional knockout of H6PDH
ITT	insulin tolerance test
MS	metabolic syndrome
NADPH	nicotinamide adenine dinucleotide phosphate
11-DHC	11-dehydrocorticosterone
11β-HSD1	11 β -hydroxysteroid dehydrogenase type 1.

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Clinical perspectives

- H6PDH is able to couple the regulation of cellular GC activity and plays an important role in the etiology of MS and obesity. However, the functional consequences of selective H6PDH inhibition, or lack in adipose tissue, remains unclear.
- In the present study, we established an adipocyte-specific knockdown mouse model of H6PDH. This specific model showed decreased adipose-specific GC production and exhibited a favorable metabolic phenotype of insulin sensitivity and abdominal fat distribution with reduced abdominal adipose mass independent of circulating and nonadipocyte GC effects.
- Adipose-specific inhibition of H6PDH may have potential as a novel and effective pharmaco-therapeutic approach to target visceral fat GC action for prevent or delay the progress of MS and obesity.

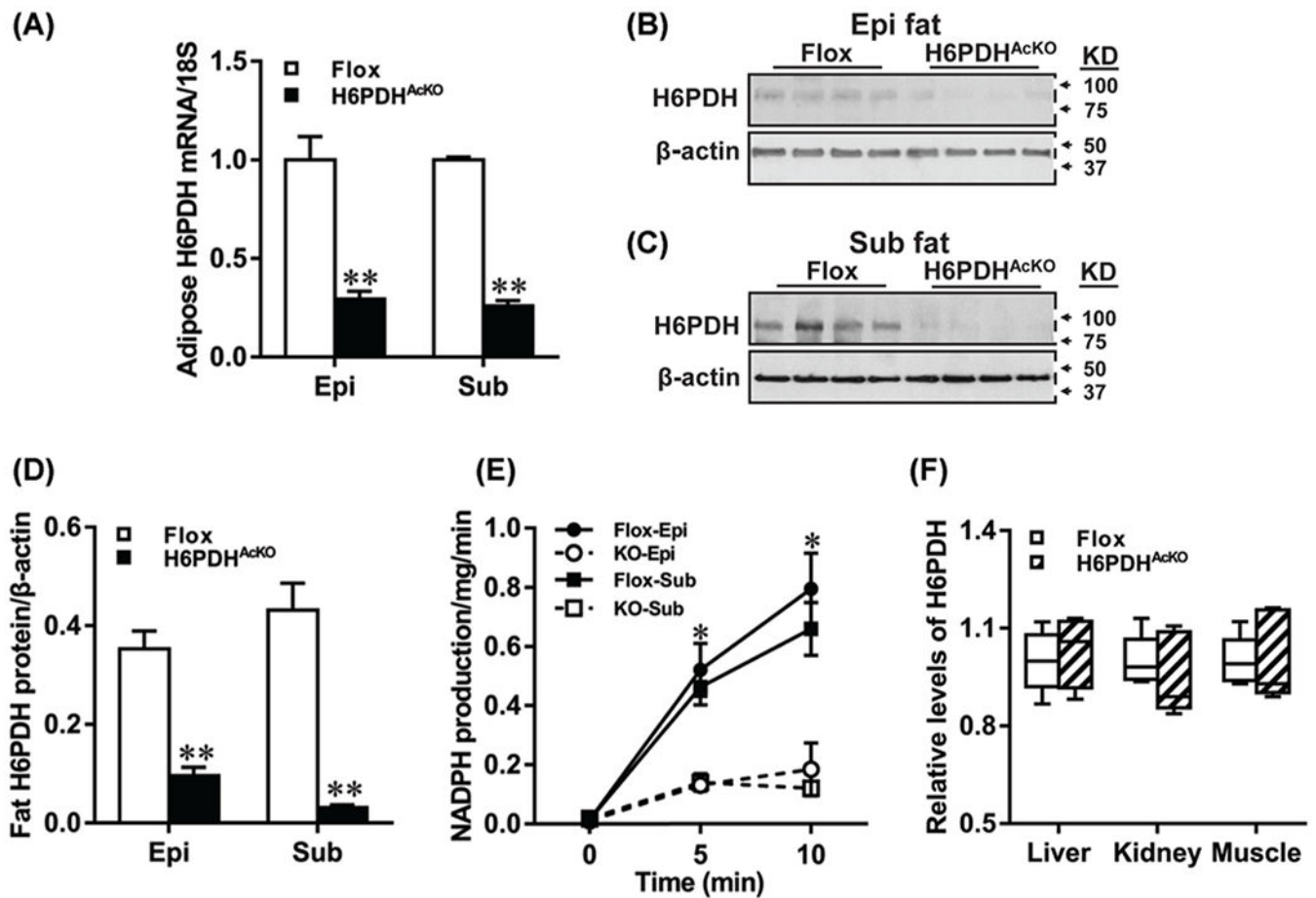


Figure 1. H6PDH mRNA and protein expression in adipose and other tissues of H6PDH^{AcKO} and control mice

(A) Quantitative real-time PCR analysis exhibiting the mRNA expression of H6PDH in epididymal (Epi) and subcutaneous (Sub) fat of H6PDH^{AcKO} mice and flox controls were normalized to 18S. (B–D) Western blotting images for H6PDH in Epi fat (B) and Sub fat (C) of the two genotypes and the quantification was relative to the amount of β-actin with NIH ImageJ (D). (E) H6PDH activity was measured in the adipose tissues using 2 mM G6P as substrate with the presence of NADP. (F) mRNA expression in liver, kidney and muscle of H6PDH^{AcKO} mice and control littermates. Data are means ± SE of eight mice/group.

* $P < 0.05$ and ** $P < 0.01$ vs. Flox control mice.

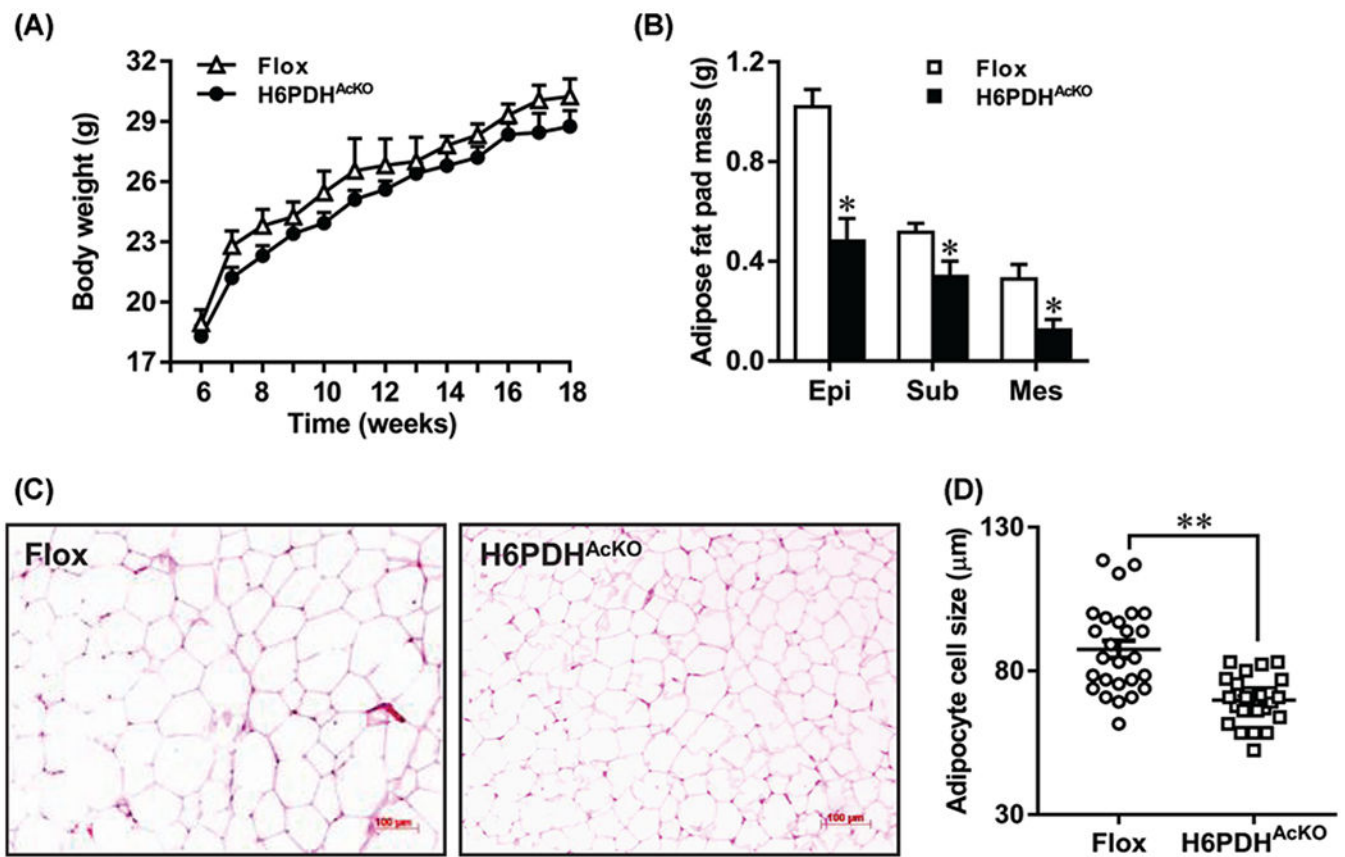


Figure 2. Body weight and fat pad mass of adipose-specific H6PDH knockout mice
(A) Unchanged body weight gain of H6PDH^{AcKO} mice on a chow diet ($n=8-10$ mice/group). **(B)** Weight of fat mass of H6PDH^{AcKO} mice in fed state. Epi, epididymal fat; Sub, subcutaneous fat; Mes, mesenteric fat. Mice ($n=8-10$ /group) were killed at the age of 18 weeks. **(C,D)** Average adipocytes size of epididymal fats on histological slides was measured with NIH ImageJ. * $P<0.05$ and ** $P<0.01$ vs. Flox controls.

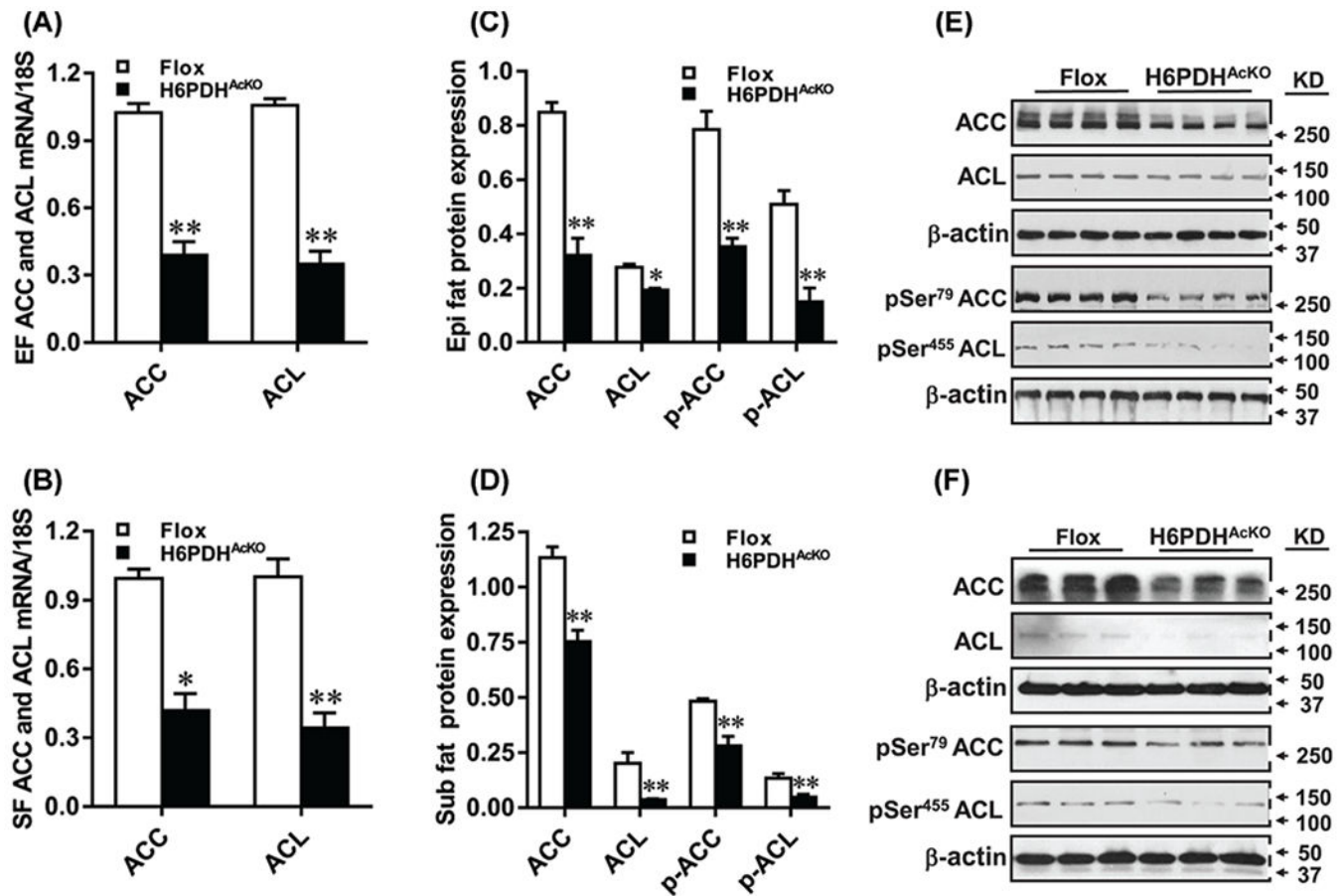


Figure 3. Alterations of lipogenic gene mRNA and protein expression in epididymal fat (EF) and subcutaneous fat (SF) of H6PDH^{AcKO} (■) or Flox controls (□) (A,B) Relative mRNA expression levels of ACC and ACL in EF (A) and SF (B). (C–F) The expression and quantification of ACC, ACL, pSer⁷⁹ ACC, and pSer⁴⁵⁵ ACL protein levels in EF (C,E) and SF (D,F) of H6PDH^{AcKO} and Non-KO controls. Data are means ± SE of 6–8 mice/group. **P*<0.05 and ***P*<0.01 vs. controls.

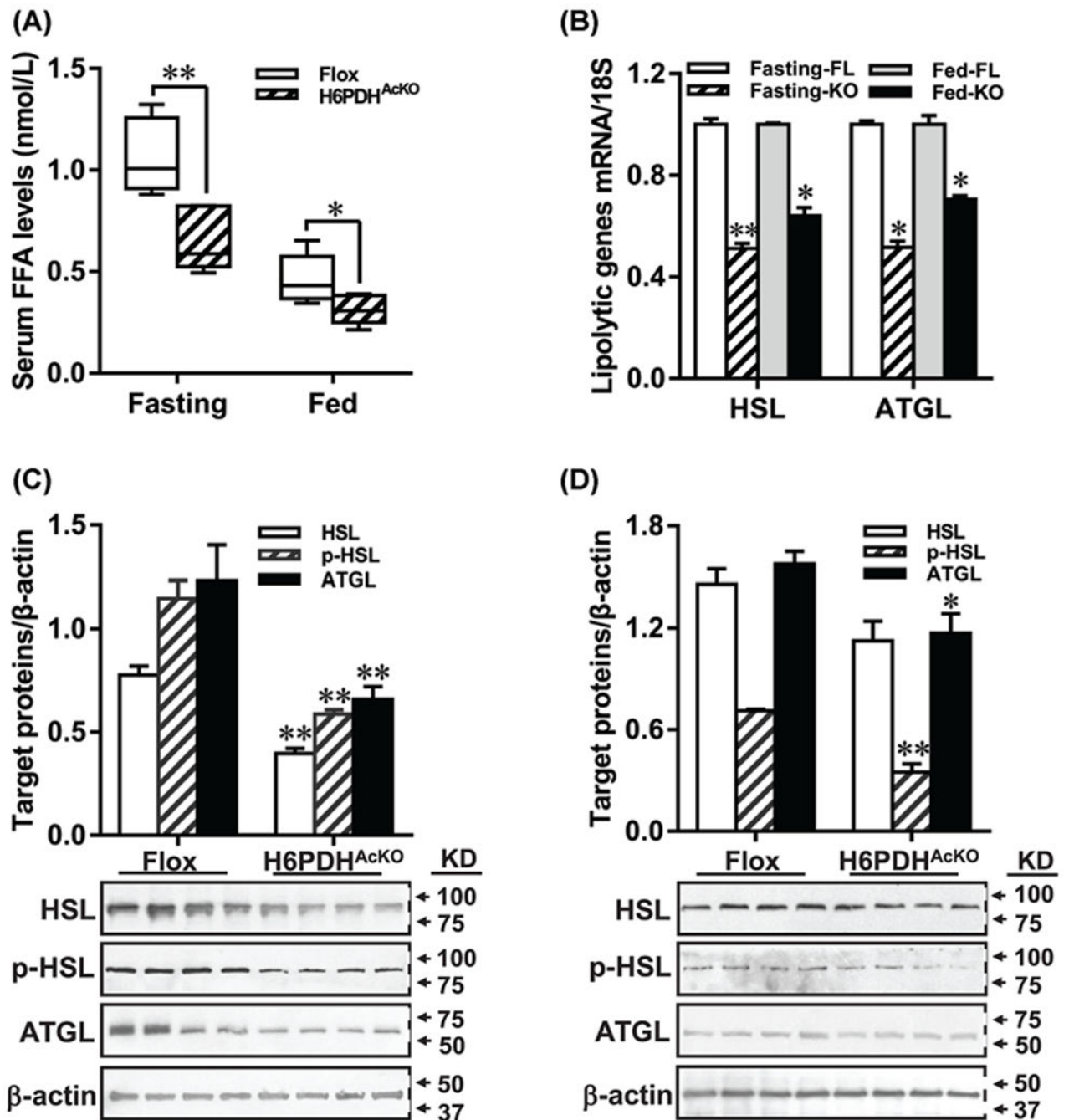


Figure 4. Adipose lipolytic enzymes mRNA and protein expression of H6PDH^{AcKO} and Flox controls in fasted and fed condition

(A) Serum FFA concentrations in H6PDH^{AcKO} and Non-KO controls under both fasted and fed states. (B) Relatively mRNA expression changes of HSL and ATGL in Epi fat of H6PDH^{AcKO} mice in both fasting and fed states. 18s rRNA was used as the internal normalizer. (C,D) Western blot analysis showed the alterations of ATGL, total and Ser⁶⁶⁰ phosphorylation (p) of HSL in Epi fat of H6PDH^{AcKO} and their respective controls mice in

fasted (**C**) and fed (**D**) states. Data are means \pm SE of eight mice/group. * $P < 0.05$ and ** $P < 0.01$ vs. Non-KO control mice.

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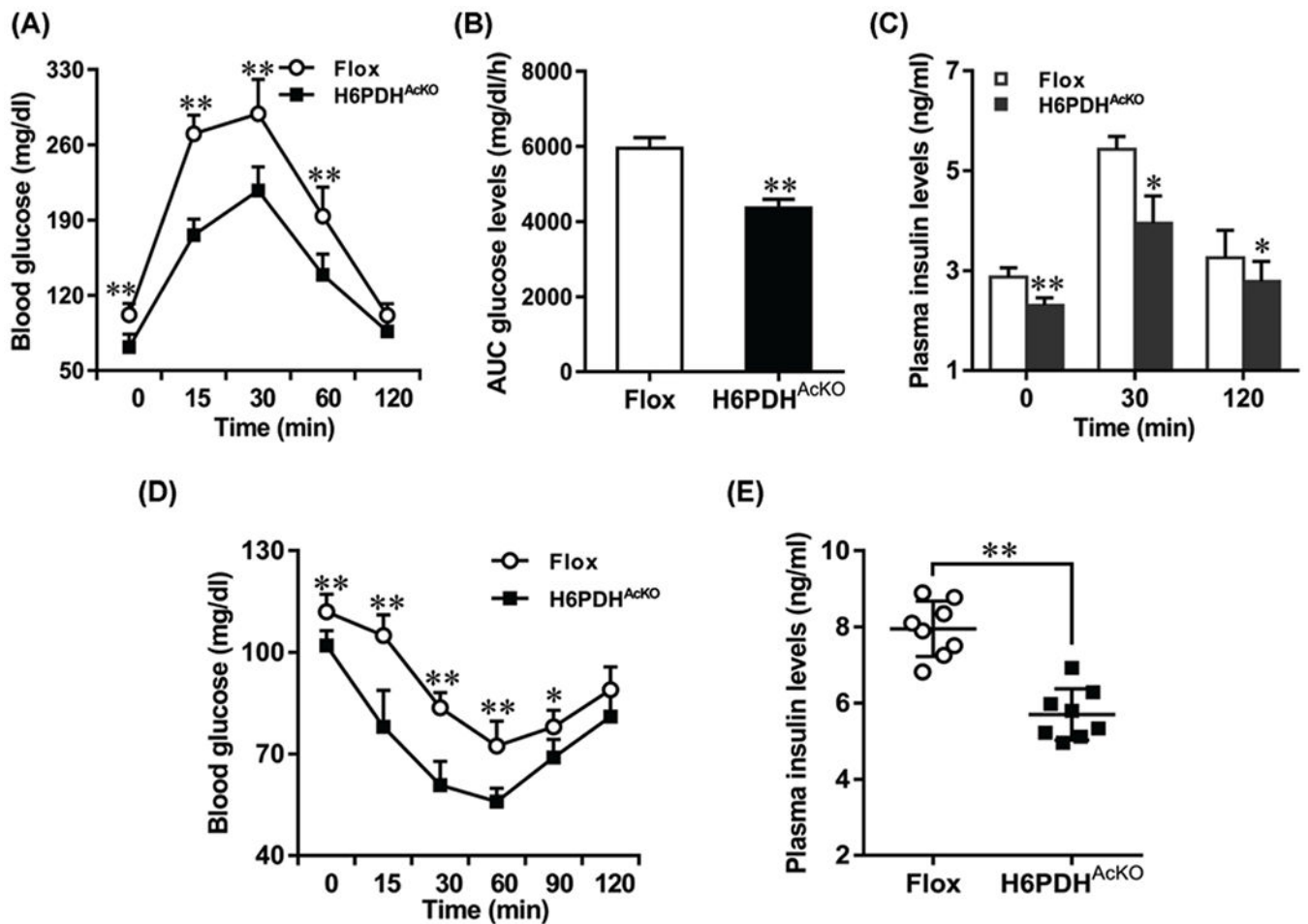


Figure 5. Glucose metabolism in H6PDH^{AcKO} and Flox mice

(A) GTT of H6PDH^{AcKO} and control mice aged 14 weeks. (B) The glucose AUC during GTT. (C) Insulin concentrations at 0, 30, and 120 min during the GTT. (D) ITT in knockout and control mice. ITT was performed on mice after an 8-h fast, and 0.75 U/kg insulin was used. (E) Plasma insulin level in H6PDH^{AcKO} and Flox mice. Data are means \pm SE of eight mice/group. * P <0.05 and ** P <0.01 vs. Flox controls.

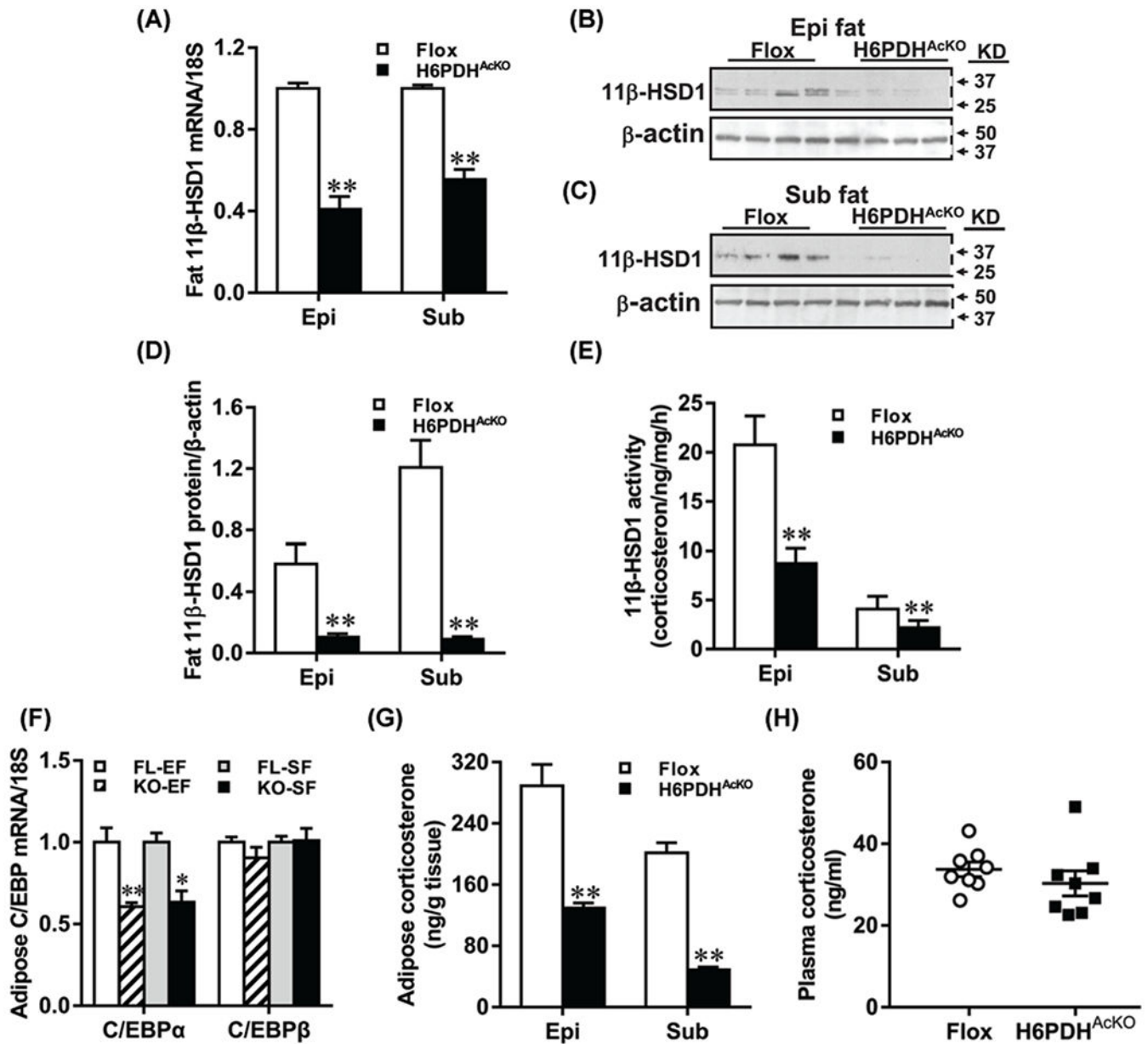


Figure 6. 11β-HSD1 and corticosterone levels in white adipose tissue of H6PDH^{AcKO} mice and non-KO controls

(A) mRNA expression of 11 β-HSD1 in epididymal (Epi) and subcutaneous (Sub) of H6PDH^{AcKO} mice and Flox controls were normalized to 18S. (B–D) Western blot analysis of the protein expression of 11β-HSD1 in Epi fat (B) and Sub fat (C) between the two groups and the quantification was determined relative to the amount of β-actin (D). (E) Adipose 11β-HSD1 reductase activity was measured in the adipose microsomes using 11-DHC as substrate in the presence of NADPH. (F) mRNA expression of C/EBPα and C/EBPβ in Epi fat (EF) and Sub fat (SF) were determined by real-time PCR and normalized to 18S. (G,H) Adipose (G) and plasma (H) corticosterone level in H6PDH^{AcKO} and Flox mice. Data are means ± SE of 7–8 mice/group. **P*<0.05 and ***P*<0.01 vs. Flox controls.