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Increased Glycogen Synthase Kinase-3 β and Hexose-6-Phosphate Dehydrogenase Expression in Adipose Tissue May Contribute to Glucocorticoid-Induced Mouse Visceral Adiposity

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Abstract

BACKGROUND—Increased adiposity in visceral depots is a crucial feature associated with glucocorticoid (GC) excess. The action of GCs in target tissue is regulated by GC receptor (GR) and 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) coupled with hexose-6-phosphate dehydrogenase (H6pdh). Glycogen synthase kinase-3 β (GSK3 β) is known to be a crucial mediator of ligand-dependent gene transcription. We hypothesized that the major effects of corticosteroids on adipose fat accumulation are in part mediated by changes in GSK3 β and H6pdh.

METHODS—We characterized the alterations of GSK3 β and GC metabolic enzymes, and determined the impact of GR antagonist mifepristone on obesity-related genes and the expression

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Conflicts of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

of H6pdh and 11 β -HSD1 in adipose tissue of mice exposed to excess GC as well as in *in vitro* studies using 3T3-L1 adipocytes treated with GCs.

RESULTS—Corticosterone (CORT) exposure increased abdominal fat mass and induced expression of lipid synthase ACC and ACL with activation of GSK3 β phosphorylation in abdominal adipose tissue of C57BL/6J mice. Increased pSer⁹ GSK3 β was correlated with induction of H6pdh and 11 β -HSD1. Additionally, mifepristone treatment reversed the production of H6pdh and attenuated CORT-mediated production of 11 β -HSD1 and lipogenic gene expression with reduction of pSer⁹ GSK3 β , thereby leading to improvement of phenotype of adiposity within adipose tissue in mice treated with excess GCs. Suppression of pSer⁹ GSK3 β by mifepristone was accompanied by activation of pThr³⁰⁸ Akt and blockade of CORT-induced adipogenic transcription factor C/EBP α and PPAR γ . In addition, mifepristone also attenuated CORT-mediated activation of IRE1 α /XBP1. Additionally, reduction of H6pdh by shRNA showed comparable effects to mifepristone on attenuating CORT-induced expression of GC metabolic enzymes and improved lipid accumulation *in vitro* in 3T3-L1 adipocytes.

CONCLUSION—These findings suggest that elevated adipose GSK3 β and H6pdh expression contribute to 11 β -HSD1 mediating hypercortisolism associated with visceral adiposity.

Introduction

Patients with glucocorticoid (GCs) excess (Cushing's syndrome), acquire a prominent phenotype of central obesity and are at elevated risk for type 2 diabetes (T2DM), insulin resistance, hypertension and other cardiovascular diseases.^{1, 2} GCs are widely used as potent therapeutic agents but long-term use of higher doses of GCs often leads to visceral adiposity that initiates the processes leading to metabolic syndrome.^{3–7} More than 2% people in the US and UK are prescribed supraphysiological doses of GCs that potentially could cause central obesity and T2DM.^{8, 9} Given the potential pathological consequences of GCs, it is important to determine how they contribute to central obesity.

The actions of circulating GCs on target tissues are regulated by 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1), which converts inert 11-DHC (cortisone in humans) to the corticosterone (cortisol), the ligand for the GR receptor. Thus 11 β -HSD1 regulates the availability of GCs for binding and activating GR and determines the local GC action at prereceptor level in target tissues.^{10, 11} 11 β -HSD1 activity is dependent on its cofactor NADPH, which can be generated by an ER lumen-resident enzyme hexose-6-phosphate dehydrogenase (H6pdh),¹² which was originally named glucose dehydrogenase (GDH; EC 1.1.1.47). H6pdh is a microsomal enzyme that catalyzes the first two steps of pentose phosphate pathway using glucose- 6-phosphate (G6P), transported into the ER by the G6P transporter (G6PT), to produce NADPH from NADP within the ER.^{12–15} The exclusive subcellular localization of H6pdh within the ER distinguishes it from its cytosolic homologue, glucose-6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49).^{16–18} The ER is poorly permeable to NADPH and H6pdh has been considered as a major enzyme responsible for generating NADPH inside the ER lumen that is used for 11 β -HSD1 and steroid metabolism.^{19, 20} Reduced H6pdh prevents regeneration of cortisol from cortisone and improves lipid profiles and weight gain.^{21, 22} In contrast, H6pdh transgenic mice display increased 11 β -HSD1-mediated GC action related to dyslipidemia and adiposity.²³ Thus, an

H6pdh-driven increase in 11 β -HSD1 may contribute to GC-induced visceral obesity and T2DM, suggesting a potential therapeutic target.

GCs are essential for adipocyte differentiation and drive adipose tissue distribution, and are associated with visceral fat mass and adiposity.^{24, 25} GCs increase *de novo* lipid production in human pre-adipocyte cells through induction of fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and ATP-Citrate Lyase (ACL).^{26, 27} Furthermore, GCs activate adipose phosphoenolpyruvate carboxykinase (PEPCK), an enzyme that regulates the reesterification of fatty acids for triglyceride synthesis, which is linked to lipid deposition.²⁶ In addition, GCs alter ER stress by activation of the unfolded protein response (UPR) component X-box binding protein 1/inositolrequiring enzyme 1 alpha (XBP1/IRE1 α) that is indispensable for adipogenesis linked to obesity.^{28, 29}

The molecular mechanisms that control lipogenesis and the lipid metabolic profile are complex and variable. The Akt (protein kinase B, PKB) family of serine/threonine kinases and its downstream effectors have been shown to inhibit adipogenesis through various mechanisms including negative regulation of glycogen synthase kinase 3 (GSK3 β), the key activator of adipogenesis.^{30, 31} Serine/threonine phosphorylation of GSK3 β is required for ligand-dependent transcriptional activation.³² Increased adipose GSK3 β is positively correlated with lipogenesis and obesity in obese mice.^{33, 34} Importantly, the role of GSK3 β phosphorylation coupled with Akt signaling in visceral adiposity caused by GCs has not been explored.

The hypercortisolemia associated with Cushing's syndrome and other conditions can be treated with Mifepristone, a glucocorticoid receptor (GR) antagonist which blocks both GR and progesterone receptors (PR).^{35, 36} Pharmacological blockade of GR by mifepristone inhibits hypercortisolemia-related central obesity and metabolic disorders in animal models and in Cushing's syndrome patients.³⁷⁻⁴⁰ These observations validate the efficacy of mifepristone in the control of hypercortisolism associated with adiposity and insulin resistance. However, the contributions of tissue-specific alterations of GSK3 β and H6pdh to 11 β -HSD1 regulation of GC-induced obesity, and whether mifepristone would modulate the cross-talk between H6PDH and the lipogenic pathway, remain unclear.

Using mice exposed to exogenous GCs in the concentration found in patients with hypercortisolemia that mimics many phenotypes similar to that observed in the metabolic syndrome,⁴⁰⁻⁴³ we determined if corticosteroids mediate their effects on visceral fat adiposity in part by modulation of GC metabolic enzymes and GSK3 β signaling. The study also aimed to elucidate whether the molecular mechanism underlying effects of the GR antagonist mifepristone on GC-induced fat accumulation correlate with changes in H6pdh production and on the expression of genes related to adipogenesis.

Materials and Method

Animal treatment

Five-week-old male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and housed in a room maintained on a 12/12 h light/dark cycle with free access

to water and standard laboratory chow. The Jilin University of Medicine and the Charles R. Drew University Institutional Animal Care and Use Committees approved all animal experiments. Animals were given corticosterone (CORT; 100 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) in 1% ethanol, or 1% ethanol in tap water for 4 weeks, as previously described.^{42, 43} After 4 weeks, CORT-treated mice and their controls were divided into four groups: 1) Control mice treated with vehicle; 2) Control mice treated with mifepristone; 3) CORT-treated mice receiving vehicle; 4) CORT-treated mice receiving mifepristone. Mifepristone (25 mg/kg) or vehicle was given by intraperitoneal (i.p.) route of administration daily for 3 weeks (at 0700 and 0900 hours) as previously described.³⁸ Body weight was recorded before the initiation of the treatment and on the last day of treatment. At the end of the third week, adipose tissues and blood samples were collected and stored at -80 °C until metabolic assays. Blood samples were analyzed for corticosterone, insulin, and FFA levels using ELISA kit (Abcam, UK).

Cell culture and drug treatment

3T3-L1 cells were differentiated in defined differentiated medium with using the standard DMEM containing glucose (25 mM), which is required for maintaining this 3T3-L1 cell line growth according to the manufacturer's instruction (ATCC, Manassas, VA, USA). This concentration of glucose in cell cultures is similar that occurring in hypercortisolemia-related mice with diabetes³⁸ and is previously used for testing the direct effects of GCs on metabolism in 3T3-L1 adipocytes.^{10,43,46} On day 8 of differentiation, the medium was changed to 10% charcoal-stripped FBS-DMEM. After 12h, adipocytes were treated with CORT (10^{-6} M) in the absence or presence of mifepristone (10^{-8} – 10^{-6} M) for 48 h.

siRNA experiments in 3T3-L1 adipocytes

3T3-L1 cells were transfected with H6pdh shRNA (SA Biosciences, Frederick, USA) or scrambled control shRNA using lipofectamine 2000. Medium containing G418 (600 µg/ml) was used to select positive transfected cells. Stable transfected 3T3-L1 cells were established after selection with G418 and were induced to differentiate. On day 4 after differentiation, cells were treated with vehicle or CORT (10^{-6} M) for additional 48 h.

Microsomal enzymatic activity assays in adipose tissue—We obtained the adipose microsomal pellet and measured H6pdh enzyme activity by evaluating NADPH production in the presence of G6P and NADP by spectrophotometry, using absorbance 340 nm, according to our previous reports.²³ Protein (100 µg) from adipose microsomes was 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. The adipose microsomes were permeabilized with 1% Triton-100 to allow the free access of the cofactor to the intraluminal enzyme. Specific activities were calculated and expressed as micromoles of NADPH production per minute per milligram of protein.

11B-HSD1 activity was evaluated by adding of 1 mM NADPH and 250 nM 11-DHC with [³H]11-DHC as tracer to microsomes in KRB solution at 37 °C for 1- 2h, previously described.⁴³ Steroids were separated by TLC and the conversion of [³H]11DHC to

[³H]CORT was determined by scintillation counting of radioactivity. Adipose CORT concentrations were measured by using a CORT ELISA kit (Abcam).

GSK3 β activity assay—The adipose tissue was homogenized in lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na₃VO₄, 10 mM β -glycerophosphate, 50 mM NaF, 0.1% β -mercaptoethanol, 5 mM sodium pyrophosphate, 0.25 M sucrose, and protease inhibitor cocktail, as described previously.⁴⁴ The tissue homogenate was centrifuged at 14,000 *g* for 30 min at 4°C and the supernatant was applied to CM-Sepharose fast-flow resin column (Amersham) preequilibrated with 25 mM Tris-HCl, 2 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 10 mM Na- β -glycerophosphate, phosphatase inhibitor cocktails, 2.5% glycerol, and 5 mM NaF (buffer A). The column was washed with wash buffer (buffer A + 20 mM NaCl) and GSK3 was eluted using buffer A plus 250 mM NaCl. Fractions containing the highest concentrations of protein were pooled and used in a kinase assay to assess GSK3 activity by using an available GSK3 β kinase Assay kit (Promega, Madison, WI, USA).

RNA isolation and analysis of real-time quantitative PCR—Total RNA was isolated from adipose tissue (approximately 100 mg) by using RNeasy Lysis Buffer (Qiagen). The purity of RNA was assessed by the *A*₂₆₀/*A*₂₈₀ ratio and cDNA synthesis from 2.0 μ g RNA was performed using Kit (Applied Biosystems). The primers for mouse 11 β -HSD1 (F: 5'-CCTTGGCCTCATAGACACAGAAAC-3'; R: 5'-GGAGTCAAAGGC GATTTTCA-3'), H6pdh (F: 5'-TGGCTACGGTGTGTTTTGAA-3'; R: 5'-TATACAG GTACATCTCCTCTTCCT-3'), ACC (F: 5'-TGTAATCTGGCTGCATCC ATTAT-3'; R: 5'-TGGTAGACTGCCCGTGTGA-3'), ACL (F: 5'-ATGCCAAGACCATCCTCTCA CT-3'; R: 5'-TCTACAATGCCCTGAAGGT-3'), adiponectin (F: 5'-GAGACGCAGG TGTGTTTGGT-3'; R: 5'-CTTCCGCTCCTGTCAT TCCA-3'), FOXO1 (F: 5'-GGACAGC CGCGCAAGACCAG-3'; R: 5'-TTGAATTCTTCCAGCCCGCCGA-3'), SIRT1 (F: 5'-A ATCCCGACTTCAGATCCCC-3'; R: 5'-CAACATGAAAAGGGCTTGGG-3'), TXB 1 (F: 5'-CGCTGTGGGACGAGTTCAAT-3'; R: 5'-CGCCATGGGATCCATT-3'), CD1 37 (F: 5'-TTGGGAACATTTAATGACCAGA-3'; R: 5'-TCCCGGTCTTAAGCACAGA C-3'), IRE α (F: 5'-CCTACAAGAGTATGTGGAGC-3'; R: 5'-GGTCTCTGTGAACAAT GTTGAGAG-3'), and XBP1 (F: 5'-GGATTTGGAAGAAGAGAACCACAA-3'; R: 5'-CCG TGAGTTTTCTCCCGTAAAA-3') were designed with Primer express software 2.0. RT-PCR was performed using SYBR kits in the ABI Prism 7700 System. Results were expressed as a relative value after normalization to 18S rRNA. The data were analyzed using the comparative CT method and were confirmed by the standard curve method.

Protein extraction and Immunoblotting analysis (Western blot)—Adipose tissues were homogenized on ice for 1 min in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 100 mM sodium orthovanadate, and protease inhibitor) to obtain total proteins. Homogenates were centrifuged at 4°C at 12,000*g* for 10 min, and supernatants were collected. Protein concentrations were measured by Bradford assay. Proteins were separated on 10% SDS-PAGE gels (BioRad, CA, USA) for analysis of H6PDH, phospho-Ser⁷⁹ACC, ACL, phospho-Ser⁴⁵⁵ ACL, phospho-Thr³⁰⁸Akt, XBP1, IRE α , phospho-Ser⁷²⁴ IRE α , phospho-Ser⁹ GSK3 β , and β -actin (Cell Signaling, Tech, Danvers MA). Protein

signals were visualized using ECL (Biotechnology, Rockford, IL) by exposure to Amersham film and were quantified with the use of the Eagle Eye II Quantitation System (Stratagene, La Jolla, CA, USA).

Statistical analyses

All values are expressed as the mean \pm SEM. Data were analyzed using two-way analysis of variance (ANOVA). The post-hoc Tukey test was performed to reveal significant differences between groups. A $P < 0.05$ was considered as significant.

RESULTS

Chronic corticosterone (CORT) treatment in mice triggers visceral obesity and increased adipose H6PDH and lipogenic gene expression

As shown in Table 1, mice treated with CORT displayed increased weight gain and epididymal fat pad mass (visceral fat) compared to controls treated with vehicle. The levels of insulin, blood glucose, CORT, free fatty acid and triglycerides in plasma were also increased in CORT-treated mice relative to controls (Table 1). CORT-treated mice displayed increased H6pdh mRNA and protein expression in epididymal fat relative to controls ($P < 0.01$; Figure 1a and 1b), along with an approximately 1.5-fold increase in H6pdh activity ($P < 0.01$; Figure 1c). Similarly, the levels of CORT and of 11 β -HSD1 mRNA, protein and activity were elevated in adipose of CORT-treated mice relative to controls (Figure 1a, 1d, 1e, 1f). We also observed increased mRNA and total protein levels of the lipid synthases ACC and ACL, concomitant with increased phosphorylated Ser⁷⁹ACC and Ser⁴⁵⁵ACL, in CORT-treated mice relative to control mice (Figure 2a–c). CORT-treated mice also displayed 1.8- and 2.5-fold increases in PEPCCK mRNA and protein levels, respectively, compared to control mice ($P < 0.01$; Figure 3a and 3b). Moreover, CORT-treated mice displayed increased C/EBP α , PPAR γ , and leptin mRNA levels in their epididymal fat compared to controls ($P < 0.01$), but decreased adiponectin mRNA levels and similar SREBP expression (Figure 3c). Finally, compared to controls, CORT-treated mice had increased FOXO1 and SIRT1 mRNA levels, but decreased expression of browning gene TBX1 and CD137 mRNA in epididymal fat, as revealed by real-time PCR (Figure 3d).

Effects of GR antagonist mifepristone treatment on CORT-induced visceral obesity and metabolic phenotype

As expected, mifepristone treatment for 3 weeks reduced epididymal fat pad mass, weight gain, and plasma levels of glucose, insulin, CORT, and lipid in CORT-treated mice. However, mifepristone treatment had no significant effect on CORT or lipid level and had no effect on body weight in mice not receiving CORT. In parallel with the decrease in epididymal fat mass, mifepristone reduced epididymal fat H6pdh activity, mRNA and protein levels in CORT-treated mice (Figure 1a–c). Similarly, CORT-treated mice given mifepristone displayed lower CORT levels and lower 11 β -HSD1 activity, mRNA and protein levels in epididymal fat (Figure 1a, 1d, 1e and 1f). Mifepristone also attenuated the CORT-induced increase in 11 β -HSD1 and H6pdh mRNA levels in subcutaneous fat (data not shown). Moreover, mifepristone treatment of CORT-treated, but not control, mice reduced the levels of ACC and ACL mRNA and total protein, as well as of phosphorylated

Ser⁷⁹ACC and Ser⁴⁵⁵ ACL, in epididymal fat ($P < 0.01$ vs. respective vehicle-only controls; Figure 2a–c). In addition, PEPCK mRNA and protein expression were reduced in the epididymal fat of CORT-treated mice after mifepristone treatment to a level similar to their respective controls (Figure 3a and 3b). Additionally, mifepristone also reversed CORT-induced C/EBP α , PPAR γ , SREBP, leptin, FOXO1, and SIRT1 mRNA expression, and attenuated CORT-mediated decrease in adiponectin, TBX1 and CD137 mRNA levels in epididymal fat from CORT-treated mice (Figure 3c and 3d). However, mifepristone did not exert any significant effects on C/EBP α , PPAR γ , SREBP, leptin, adiponectin, FOXO1, SIRT1, as well as TBX1 and CD137 mRNA levels in vehicle-treated mice. In addition, mifepristone attenuated CORT-induced XBP1 and IRE mRNA levels in epididymal fat from CORT-treated mice (Figure 4a). Western blot analysis showed that the mifepristone reduced the increased protein expression of epididymal fat XBP1 and IRE induced by CORT (Figure 4b). These results support that mifepristone exerts anti-obesity effects in these mice.

Mifepristone attenuated CORT-induced adipogenesis via up-regulation of pThr³⁰⁸ Akt and down-regulation of pSer⁹ GSK3 β

It is known that phosphorylated Akt can reduce adipogenesis by inhibiting GSK3 β signaling.^{30, 31} Therefore, we determined if mifepristone might affect lipogenesis *in vivo* by affecting Akt phosphorylation. Western blot analysis displayed that pThr³⁰⁸ Akt was lower in the epididymal fat of CORT-treated mice, and this was restored to similar levels observed in control mice upon treatment with mifepristone (Figure 4c). Conversely, CORT-treated mice displayed increased levels of pSer⁹ GSK3 β and GSK3 β activity in epididymal fat, which was reversed by mifepristone treatment to similar levels observed in controls (Figure 4d and 4e). Mifepristone did not significantly affect pThr³⁰⁸ Akt expression level in epididymal fat of control mice, but decreased GSK3 β activity and phosphorylation levels.

The effect of GR antagonist mifepristone on key lipogenic genes and adipogenic signaling in 3T3-L1 adipocytes

Treatment of 3T3-L1 adipocytes with increasing concentrations of mifepristone led to dose dependent decrease in H6pdh and 11 β -HSD1 mRNA expression compared with those of controls (Figure 5a). RT-PCR analysis also revealed that treatment of 3T3-L1 adipocytes with mifepristone progressively decreased ACC, ACL, and PEPCK mRNA levels compared with those of controls (Figure 5a). In contrast, treatment of 3T3-L1 adipocytes with a concentration of CORT (10^{-6} mol/L), similar to concentrations in mice following CORT treatment, stimulated H6pdh and 11 β -HSD1 mRNA expression ($P < 0.01$) and 11 β -HSD1 activity (data not shown). These effects of CORT on 3T3-L1 cells were attenuated by mifepristone (Figure 5b). In addition, co-treatment of 3T3-L1 adipocytes with both CORT and mifepristone also blocked the CORT-induced increases in ACC, ACL, and PEPCK mRNA expression in these intact cells (Figure 5b). Consistent with the observations *in vivo*, treatment of 3T3-L1 adipocytes with CORT markedly decreased p-Thr³⁰⁸ Akt content, but increased pSer⁹ GSK3 β expression. These changes induced by CORT-treatment in 3T3-L1 cells were restored upon treatment with mifepristone (Figure 5c and 5d).

The functional impact of adipose H6PDH knockdown in 3T3-L1 adipocytes

Next, we generated H6PDH knockdown cells to further investigate how H6pdh suppression affects 11 β -HSD1 and adipogenic signaling. H6pdh shRNA-stable adipocytes cells showed a 55% reduction in H6pdh mRNA and a 68% decrease in 11 β -HSD1 mRNA expression compared with control LacZ cells expressing a scramble shRNA (Figure 5e). Consequently, the CORT-induced H6pdh and 11 β -HSD1 upregulation of mRNA expression and lipid droplet accumulation were attenuated in H6PDH knockdown cells relative to controls (Figure 5e and 5f). H6pdh knockdown cells also displayed reduced C/EBP and PPAR γ mRNA levels compared to control cells (Figure 5f). Thus, depletion of H6pdh by RNAi mimicked mifepristone-treatment in 3T3-L1 adipocyte cells, in that both reduced the CORT-induced stimulation of H6pdh and 11 β -HSD1.

Discussion

We found that GC-induced visceral obesity correlated with increased expression and activity of H6pdh and 11 β -HSD1 in abdominal adipose tissue and adipocytes in culture. High-CORT treatment also induced expression of the lipid synthases ACC and ACL, and GSK3 β phosphorylation in adipocytes. This increased production of H6pdh and GSK3 β corresponded with increased 11 β -HSD1, visceral fat mass, high lipid profile and weight gain. Thus, the visceral adiposity that develops in mice exposed to excess GCs may arise, in part, from elevated H6pdh and GSK3 β in abdominal fat. Induction of adipose H6pdh could increase the production of NADPH and thus stimulate 11 β -HSD1 upregulation of adipose GC action to drive lipogenesis linked to obesity.^{23, 45} These findings are consistent with recent reports that dexamethasone increased H6pdh mRNA levels and stimulated rat adipocyte 11 β -HSD1.⁴⁶ Importantly, we found that induction of H6pdh and lipogenic gene expression is closely correlated with the activation of p-GSK3 β in response to elevated circulating GC levels. Our findings suggest that exogenous GCs could exert positive effects on adipocyte H6pdh and 11 β -HSD1 expression through a GSK3 β -mediated mechanism. This interpretation is further supported by a recent report revealing that GSK3 β is required for GC-dependent activation of transcription.⁴⁷ Moreover, activation of GSK3 β itself is shown to trigger adipogenesis linked to the development of obesity in animal models.^{33, 34} Induction of GSK3 β could enhance GR-mediated CORT effects on the activation of H6pdh expression and thus promote 11 β -HSD1 driven intracellular GC action. These findings support our hypothesis that exogenous GC administration triggers increased H6pdh, 11 β -HSD1 and GSK3 β signaling, which in turn promote intracellular GC's ability to drive lipogenesis and adipogenesis. To our knowledge, how GC challenge modulates H6pdh and lipogenesis through alterations GSK3 β signaling has not been explored in humans or in animals.

It is well known that pharmacological inhibition of GR by mifepristone antagonizes hypercortisolemia-related adverse metabolic consequences in obese animal models and in Cushing's syndrome patients.^{48–50} In this work, we found that mifepristone treatment decreased CORT-induced production of adipose H6pdh and improved weight gain with reduction of epididymal fat mass. Moreover, we observed that mifepristone attenuated CORT-induced H6pdh gene expression and simultaneously prevented activation of 11 β -

HSD1 with concomitant reduction of adipose ACC, ACL, and PEPCK, which are stimulated by GCs that activate 11 β -HSD1 in adipocyte cell.^{26, 27} Furthermore, we found that knockdown of H6pdh by siRNA decreased 11 β -HSD1 expression and mimicked mifepristone-treatment with respect to blocking CORT-induced H6pdh production and lipid accumulation in 3T3-L1 adipocytes. Reduced expression of H6pdh in adipose by mifepristone treatment could decrease ER luminal NADPH availability. This would result in suppression of 11 β -HSD1 and consequently lipogenesis and adipose mass, potentially explaining the improvement of the obesity. This interpretation is supported to some extent by a recent publication reporting that mifepristone decreased GC-induced 11 β -HSD1 expression in mesenteric fat and improved the lipid profile in diet-induced obese mice.⁴⁸

In addition, the current study also observed that the suppression of adipose H6pdh by mifepristone paralleled the reduction of GSK3 β signaling that is activated by GCs.⁴⁷ Indeed, mifepristone is well known to be a pharmacological GR ligand and its effect is largely mediated by GR, a ligand-activated transcription factor that requires GSK3 β signaling.⁵¹ We observed that mifepristone attenuated GR ligand-CORT-mediated upregulation of H6pdh expression is associated with inhibition of GSK3 β in adipocytes. In contrast, increased adipose GSK3 β after CORT treatment induced H6pdh production with concomitant stimulation of 11 β -HSD1. Thus, the suppression of GSK3 β in adipose tissue may contribute to the mifepristone-mediated decrease in H6pdh and 11 β -HSD1. Additionally, mifepristone also reversed the CORT-induced the expression of adipose C/EBP α and PPAR γ mRNA, the major lipogenic transcriptional activators for terminal adipogenesis.^{52–54} Moreover, C/EBP α is the main transcriptional activator of 11 β -HSD1 in adipocytes,^{55, 56} suggesting that mifepristone-mediated inhibition of C/EBP α itself could limit GC-induced 11 β -HSD1 and lipogenesis in adipose tissue. Furthermore, C/EBP α is required for activation of SIRT1 that mediates stimulation of FOXO1 during adipogenesis.^{57, 58} Conversely, mifepristone attenuated CORT-induced SIRT1 and FOXO1 with reduction of C/EBP α . Reduction of SIRT1 and FOXO1 may also contribute to the improvement of GC-induced adipogenesis. Additionally, CORT-mediated reduction of CD137 and TBX, the key browning marker genes for browning of white adipocytes that is decreased in adipose tissue in obese individuals.^{59, 60} In contrast, mifepristone completely blocked GC-mediated inhibition of adipose CD137, although it did not change TBX expression in CORT-treated mice. These findings suggest that induction of TBX and CD137 may also limit GC-induced adipose adiposity.

Consistent with above observations, the current study also show that mifepristone inhibited the CORT-induced increases in expression of adipose XBP1 and IRE α , two key transcription factors of ER stress that is activated by GCs,⁶¹ and activation of the IRE/XBP1 pathway is indispensable for adipose adipogenesis.²⁸ Moreover, XBP1/IRE α can drive the C/EBP α promoter and activates its expression during adipogenesis.²⁸ Thus, mifepristone-mediated inhibition of adipose XBP1 and IRE α could decrease adipogenesis, which could limit GC-induced adiposity. Reduction of IRE/XBP1 may thus contribute to C/EBP α inhibition, which correlates with suppression of the GC-induced obesity.

A number of mechanisms may regulate the mifepristone-mediated suppression of GC-induced lipogenesis. Indeed, Akt has emerged as a negative regulator of adipogenesis and

exerting an anti-obesity effect through inhibition of GSK3 β .^{30, 31} Consistent with this concept, we found that mifepristone prevented the GC-mediated inhibition of p-Thr³⁰⁸Akt, which correlated with a reduction of lipogenic enzyme expression in adipose tissue. This is consistent with Akt effectively reducing fat accumulation.⁶² Conversely, the levels of p-Thr³⁰⁸Akt were reduced in response to the activation of H6pdh and 11 β -HSD1 with concomitant induction of ACC and ACL in adipose tissue. Our data support the possibility that induction of Akt in adipose tissue may be a potential metabolic signaling for mifepristone-mediated inhibition of hypercortisolism-related lipogenesis through reduction of H6pdh driven 11 β -HSD1. Additionally, mifepristone attenuated GC-induced p-GSK3 β , which corresponded to reduce lipogenic gene expression and increased p-Thr³⁰⁸Akt within adipose tissue, consistent with the idea that Akt activation causes inhibition of adipose GSK3 β and inhibits adipogenesis.^{33, 34} Thus, mifepristone prevention of GC-induced adipogenesis may be mediated at least, in part by stimulation of Akt-induced reduction of GSK3 β . Our findings provide the key mechanistic linking evidence for H6pdh mediating 11 β -HSD1 regulation of lipogenesis could be a key contributing factor to GC-induced visceral adiposity through modulation of GSK3 β coupled with Akt in adipose tissue.

To summarize, we showed that the GC-induced adiposity and adipogenesis observed in the epididymal adipose tissue of mice chronically treated with pharmacologically relevant doses of GCs may arise due to increased H6pdh-driven 11 β -HSD1 activity driving local GC action. Further, we showed that some of the benefits of the GR antagonist mifepristone may be associated with reduction of 11 β -HSD1 coupled with H6pdh amplifying local GC action through GSK3 β mediating lipogenic mechanisms in adipose tissue; it is likely that their efficacy in adipose tissue will contribute an added therapeutic benefit in treating hypercortisolism associated with adipose adiposity, a central feature of Cushing's syndrome. Our findings suggest that tissue-specific manipulation of adipose GSK3 β and H6pdh may be helpful for treatment of the adverse obese consequences associated with circulating corticosteroids excess.

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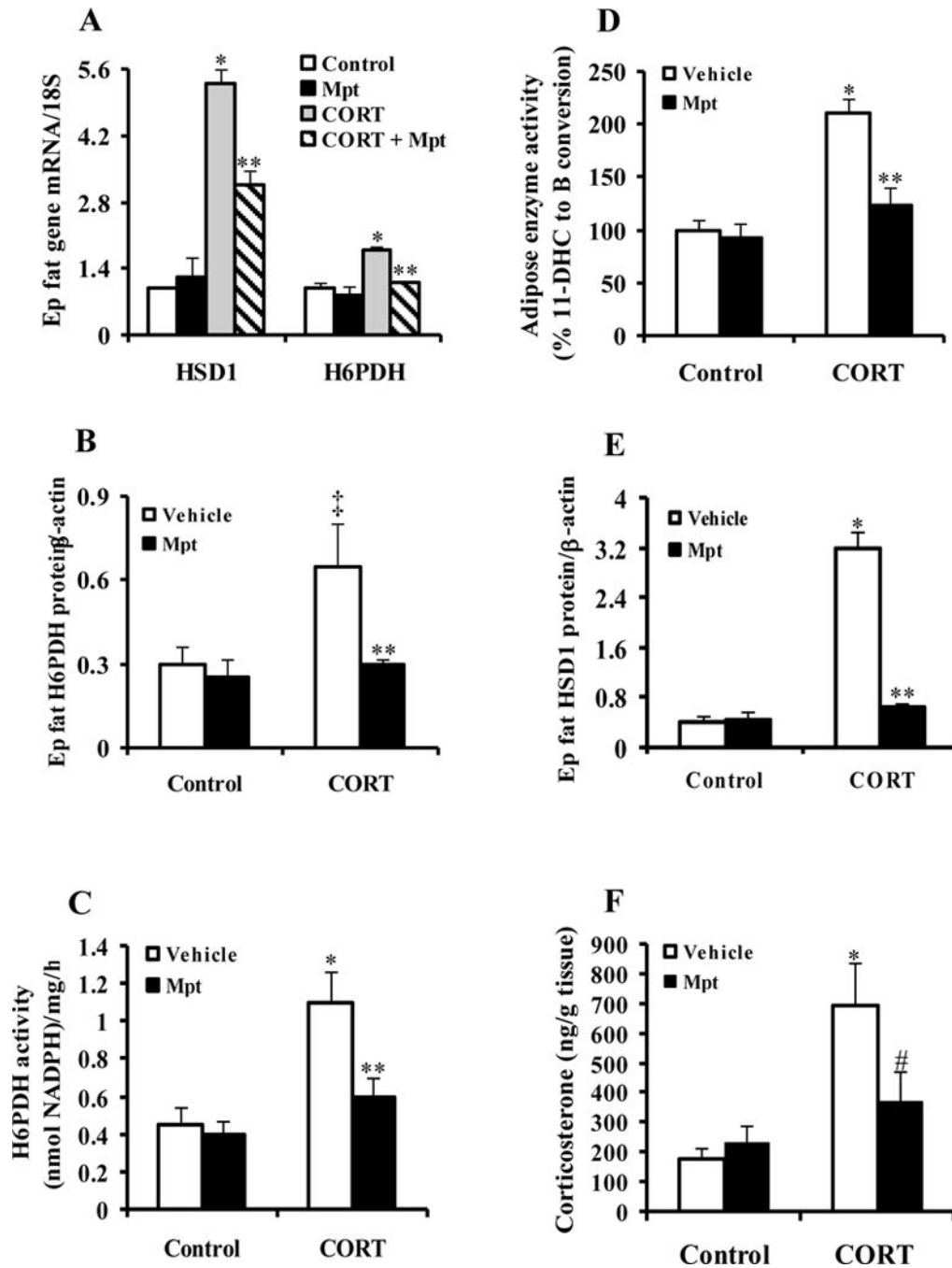


Figure 1. Effects of mifepristone on glucocorticoid metabolism in the epididymal (Ep) fat of vehicle- and CORT-exposed C57BL/6J mice. **a:** relative expression of adipose 11 β -HSD1 and *H6PDH* mRNA levels was measured by real-time PCR and normalized to 18S ($n=5-7$). Quantification of H6pdh (**b**) and 11 β -HSD1 (**e**) protein levels relative to the amount of β -actin ($n=6$). **c:** H6pdh activity was measured in Ep fat microsomes on the basis of NADPH formation using 2 mmol/l G6P as substrate in the presence of NADP. **d:** 11 β -HSD1 activity was measured in adipose microsomes using 11-dehydrocorticosterone (11-DHC) as

substrate in the presence of NADPH and data was expressed as % 11-DHC converted to corticosterone (B). **f**: Ep fat CORT concentrations. Data are means \pm SE. *P < 0.01 vs. vehicle-treated controls; **P < 0.01 vs. vehicle-treated CORT-exposed mice; ‡P < 0.05 vs. vehicle-treated controls; #P < 0.05 vs. vehicle-treated CORT-exposed mice.

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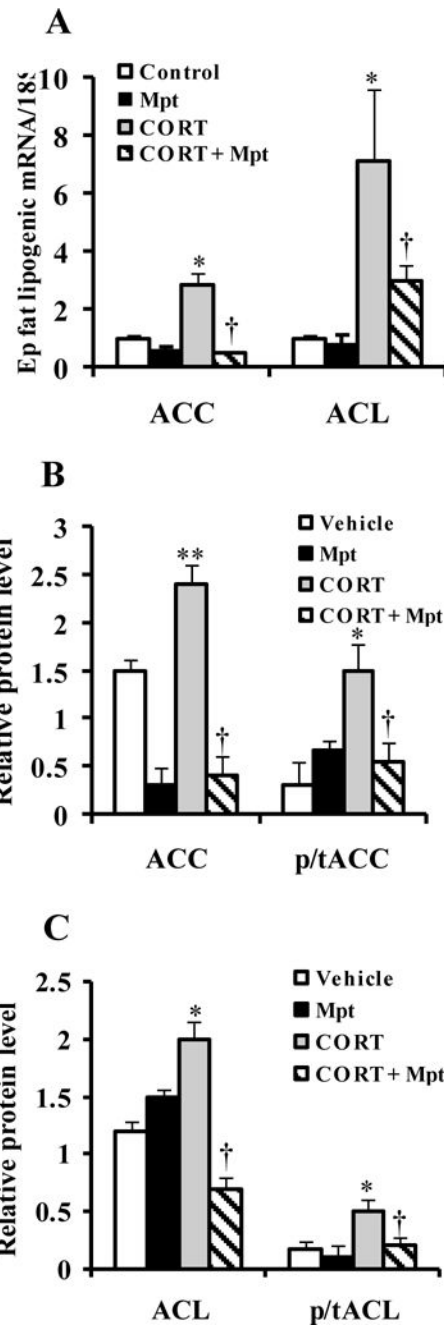


Figure 2. Comparison of lipid synthase ACC and ACL production in the Ep fat in vehicle- and CORT-exposed mice after mifepristone or vehicle treatment. **a:** Relative expression of ACC and ACL mRNA levels was measured by real-time RT-PCR and normalized to 18S ($n=6$). **b** and **c:** Relative levels of t-ACC, ACL, p-ACC and p-ACL were quantified and normalized to β -actin. Data are means \pm SE. * $P<0.01$ vs. vehicle-treated controls; ** $P<0.05$ vs. vehicle-treated CORT-exposed mice; † $P<0.01$ vs. vehicle-treated CORT-exposed mice.

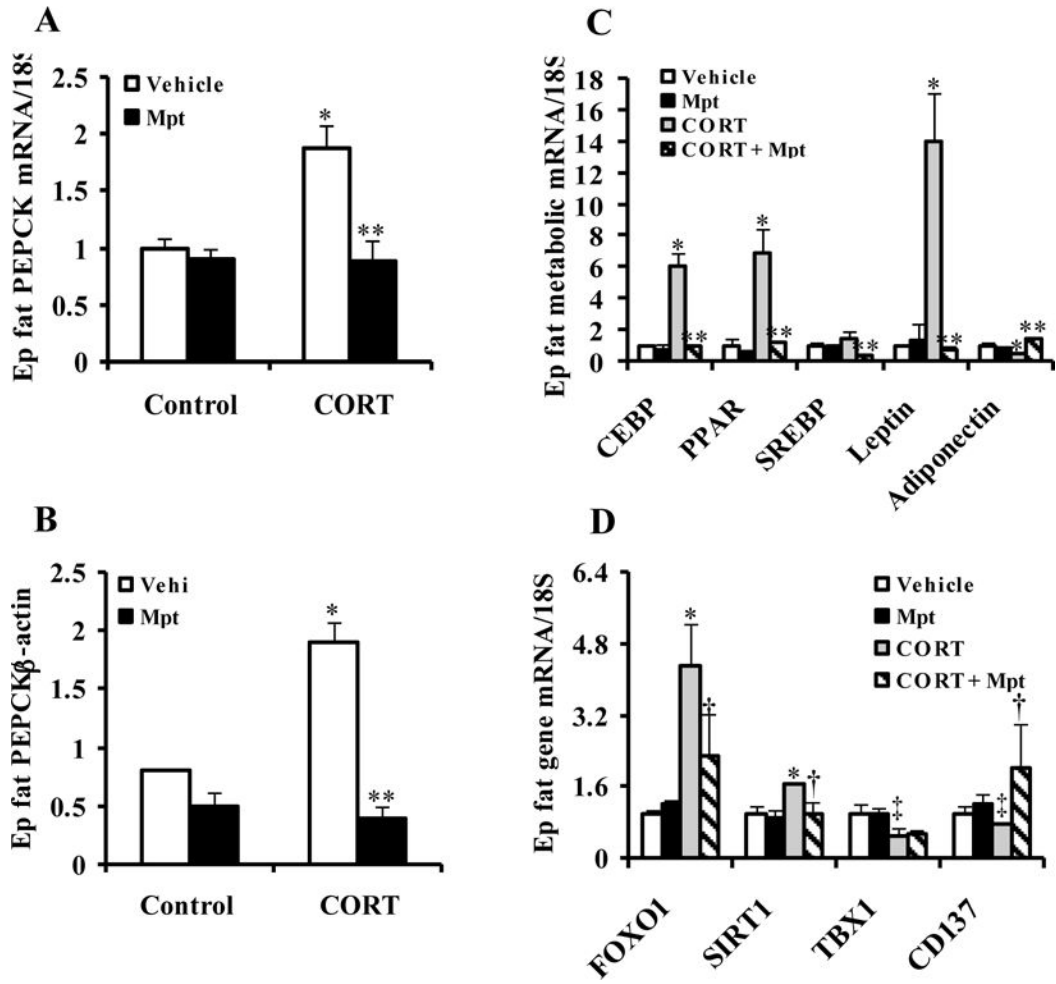


Figure 3. Alterations of PEPCK, C/EBP α , PPAR γ , SREBP, leptin, adiponectin, FOXO1, SIRT1, TBX1, and CD137 expression in the Ep fat in vehicle- and CORT-exposed mice after mifepristone or vehicle treatment. **a, c and d:** Relative expression of mRNA levels was measured by real-time RT-PCR and normalized to 18S ($n=6$). **b:** Relative epididymal fat PEPCK protein was standardized to the amount of β -actin ($n=6$). Data are mean \pm SE from six to eight mice per group. * $P < 0.01$ vs. vehicle-treated controls; ** $P < 0.01$ vs. CORT-exposed mice; † $P < 0.05$ vs. CORT-exposed mice; ‡ $P < 0.05$ vs. vehicle-treated controls.

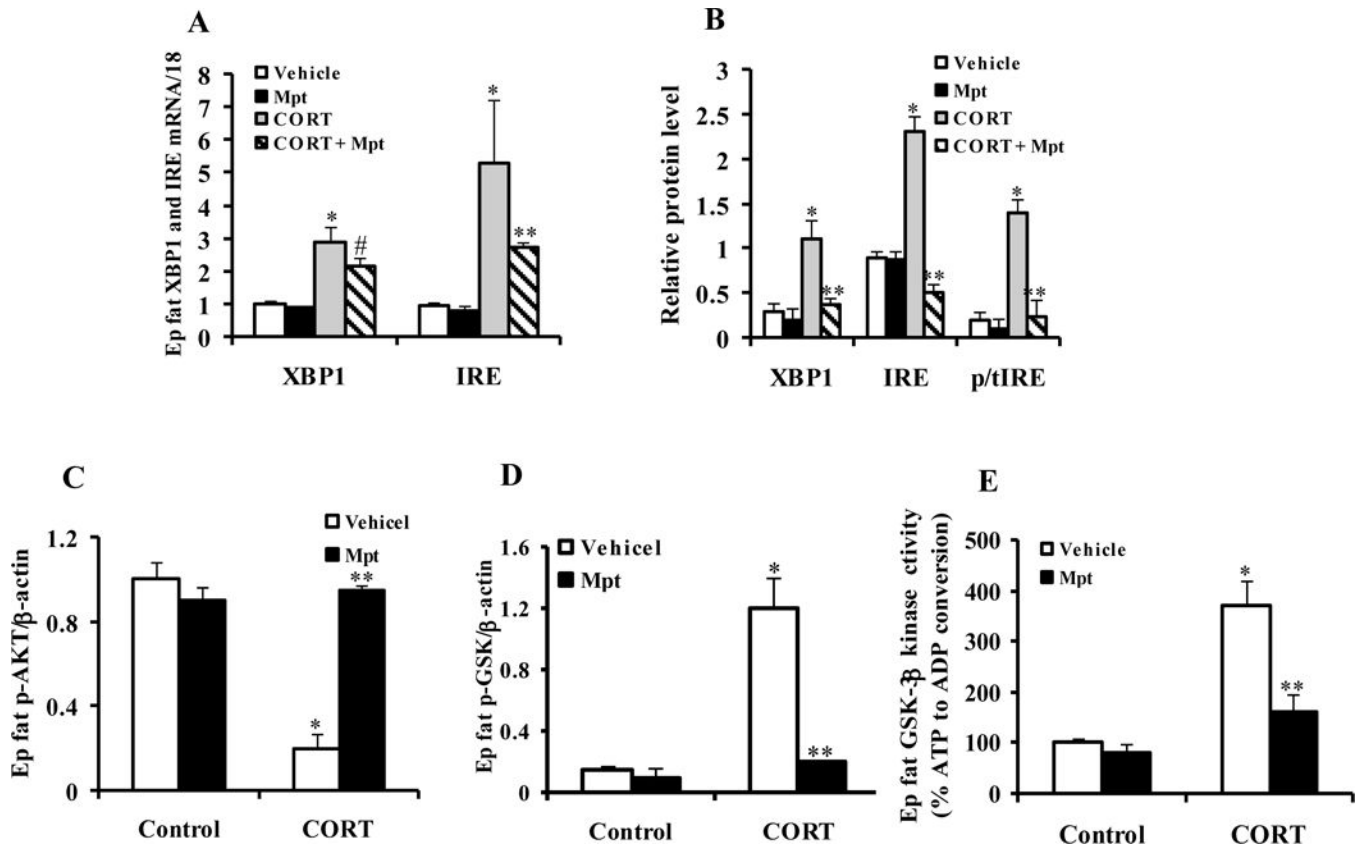


Figure 4.

ER stress response and the relative alterations of Th308 Akt/PKB and Ser9 GSK β phosphorylation content in the Ep fat in control animals and CORT-exposed mice treated with mifepristone or vehicle. **a:** Relative expression of XBP1 and IRE α mRNA levels was measured by real-time RT-PCR and normalized to 18S. **b:** Western blotting analyses were performed to compare changes in XBP1 protein expression and IRE phosphorylation. **c and d:** Relative adipose p-Th308 Akt and p-Ser9 GSK-3 β protein was standardized to the amount of β -actin. **e:** GSK-3 β kinase activity was determined using ATP as substrate and data was expressed as % conversion of ATP to ADP. Data are means \pm SEM. * P <0.01 vs. vehicle-treated controls; # P <0.05 vs. CORT-exposed mice; ** P <0.01 vs. CORT-exposed mice.

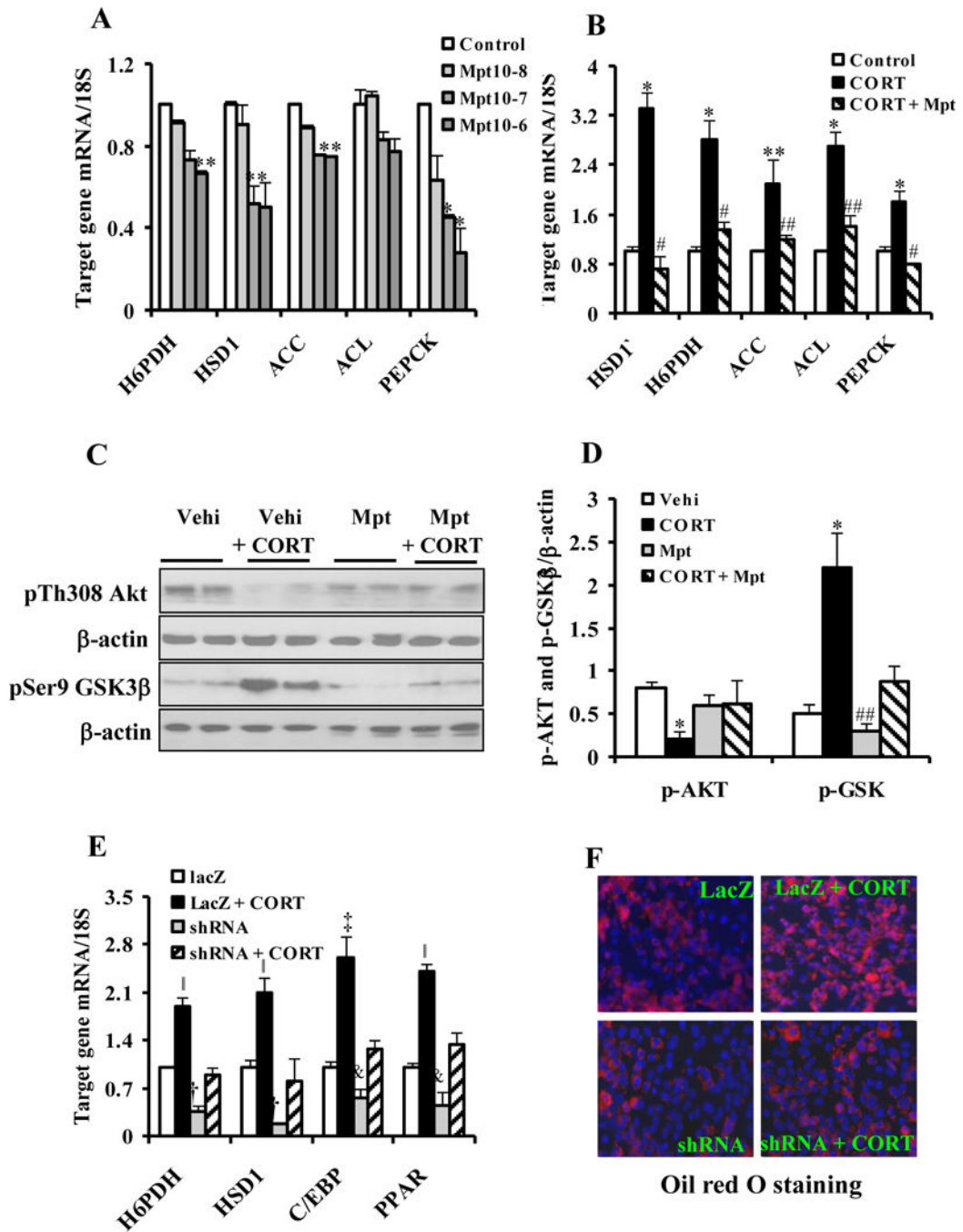


Figure 5. The effects of mifepristone and H6PDH shRNA in 3T3-L1 adipocytes. **a and b:** Cells were incubated with increased concentration of mifepristone (10^{-8} – 10^{-6} mol/L) and the levels of 11β -HSD1, H6pdh, ACC, ACL, and PEPCK mRNA expression were measured by real-time-PCR. **B:** Cells were treated with CORT (10^{-6} mol/L) in the absence or presence of mifepristone (10^{-6} mol/L) for 48h and relative expression of 11β -HSD1, H6pdh, ACC, ACL, and PEPCK mRNA levels were normalized to 18S rRNA. **C and D:** Western blots analysis showing p-Th³⁰⁸ Akt and p-Ser⁹ GSK expression levels in these cells treated with

CORT in the absence or presence of mifepristone for 48h. **c and d:** Relative adipose p-Th³⁰⁸ Akt and p-Ser⁹ GSK protein was standardized to the amount of β -actin. **e:** Suppression of H6PDH mRNA by H6pdh shRNA decreased 11 β -HSD1 and attenuated the effects of CORT on H6PDH, 11 β -HSD1, C/EBP, and PPAR γ expression as well as lipid content in H6pdh shRNA-stable 3T3-L1 adipocytes. **f:** Lipid droplet accumulation was monitored by fluorescent microscopy using Oil Red O staining with mounting medium DAPI (x20). Values are means \pm SEM from 3 separate culture preparations. * P < 0.01 vs. vehicle-treated control cells; ** P < 0.05 vs. vehicle-treated control cells; # P < 0.01 vs. CORT-treated cells; ## P < 0.05 vs. CORT-treated cells; || P < 0.01 vs. vehicle-treated LacZ control cells; ‡ P < 0.05 vs. vehicle-treated LacZ control cells; † P < 0.01 vs. CORT-treated H6PDH shRNA-stable cells; & P < 0.05 vs. CORT-treated H6PDH shRNA-stable cells.

Table 1

Body weight, fat mass, glucose, insulin, corticosterone, free fatty acids, and triglyceride

Metabolic parameter	Vehicle-treated mice		CORT-treated mice	
	Vehicle	Mifepristone	Vehicle	Mifepristone
Body weight (g)	27.6 ± 0.25	28 ± 0.2	29.8 ± 0.4 [‡]	28 ± 0.5 ^{**}
Epi fat pad weight (g)	0.79 ± 0.05	0.77 ± 0.04	1.24 ± 0.07 [*]	0.86 ± 0.02
Blood glucose (mM)	7.6 ± 0.6	7.3 ± 0.6 [*]	12.5 ± 1.2 [*]	8.8 ± 0.9 [‡]
Insulin (μU/mL)	5.2 ± 1.1	5.1 ± 0.8	67 ± 10 [*]	9.4 ± 2.3 [‡]
Corticosterone (nM)	145 ± 20	191 ± 37	463 ± 68 [*]	440 ± 55
FFA (nM)	4.7 ± 0.6	4.6 ± 0.7	10 ± 0.7 [*]	7.3 ± 1.14 [#]
Triglyceride (nM)	10.7 ± 2.3	9.1 ± 1.2	26 ± 2.7 [*]	20.7 ± 2.1 [#]

Data are mean ± SEM of six to eight mice per group.

^{*} P < 0.01 vs. Vehicle-treated control mice;[‡] P < 0.05 vs. Vehicle-treated control mice;^{**} P < 0.05 vs. Vehicle-treated CORT- exposed mice;[‡] P < 0.01 vs. Vehicle-treated CORT- exposed mice;[#] P < 0.05 vs. Vehicle-treated CORT- exposed mice;