THE ROLE OF CONSTITUTIVE ANDROSTANE RECEPTOR AND ESTROGEN SULFOTRANSFERASE IN ENERGY HOMEOSTASIS

by

Jie Gao

Bachelor of Engineering, China Pharmaceutical University, 1999
Master of Science, China Pharmaceutical University, 2002

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School of Pharmacy

This dissertation was presented

by

Jie Gao

It was defended on

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and approved by

Billy W. Day, Professor, Pharmaceutical Sciences

Donald B. DeFranco, Professor, Pharmacology & Chemical Biology

Samuel M. Poloyac, Associate Professor, Pharmaceutical Sciences

Song Li, Associate Professor, Pharmaceutical Sciences

Dissertation Advisor: Wen Xie, Professor, Pharmaceutical Sciences

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Obesity and type 2 diabetes are related metabolic disorders of high prevalence. The constitutive androstane receptor (CAR) was initially characterized as a xenobiotic receptor regulating the responses of mammals to xenotoxicants. In this study, I have uncovered an unexpected role of CAR in preventing obesity and alleviating type 2 diabetes. Activation of CAR prevented obesity and improved insulin sensitivity in both the HFD-induced type 2 diabetic model and the *ob/ob* mice. In contrast, CAR null mice maintained on a chow diet showed spontaneous insulin insensitivity. The metabolic benefits of CAR activation may have resulted from inhibition of hepatic lipogenesis and gluconeogenesis. The molecular mechanism through which CAR activation suppressed hepatic gluconeogenesis might be mediated *via* peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α). CAR can interact with PGC-1α and sequestrate it into promyelocytic leukemia (PML) nuclear bodies, thus preventing the PGC-1α from binding to the promoter region of gluconeogenesis genes.

Estrogen sulfotransferase (EST), the enzyme responsible for the sulfonation and inactivation of estrogens, plays an important role in estrogen homeostasis. Here, I showed that induction of hepatic *Est* is a common feature of type 2 diabetes. Loss of *Est* in female mice improved metabolic function in *ob/ob*, dexamethasone- and high-fat diet-induced mouse models of type 2 diabetes. The metabolic benefit of *Est* ablation included improved body composition, increased energy expenditure and insulin sensitivity, and decreased hepatic gluconeogenesis and

lipogenesis. This metabolic benefit appeared to have resulted from decreased estrogen deprivation and increased estrogenic activity in the liver. Interestingly, the effect of *Est* was gender specific, as *Est* ablation in *ob/ob* males exacerbated the diabetic phenotype, which was accounted for by the decreased islet β cell mass and failure of glucose-stimulated insulin secretion *in vivo*. The loss of β cell mass in *obe* males was associated with increased macrophage infiltration and inflammation in white adipose tissue.

In summary, I had uncovered critical roles of CAR and EST in energy metabolism and the pathogenesis of type 2 diabetes. Both of CAR and EST may represent novel therapeutic targets in the management type 2 diabetes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I would like to dedicate my dissertation to my dear wife Da Lin, who took good care of me and inspired me in every aspect of my life. And to my parents who supported me all the way along. Without them, I have neither capability nor courage to accomplish my dissertation work.

ABBREVIATION

ABCA1, ATP binding cassette transporter 1**; ACC-1**, acetyl CoA carboxylase 1; **Adam8,** ADAM metallopeptidase domain 8; **AF-1,** activation function 1; **AF-2,** activation function 2; **AICAR,** 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, Acadesine, N1-(β-D-Ribofuranosyl)-5-aminoimidazole-4-carboxamide; **AMPK,** AMP-activated protein kinase; **ASM,** acid-soluble metabolite**; BAT**, brown adipose tissue; **BSEP,** bile salt export pump**; CAR,** constitutive androstane receptor; **CITCO,** 6-(4-Chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime; **CLAMS,** comprehensive lab animal-monitoring system; **CPT1**, carnitine palmitoyltransferase 1; **CREB**, cAMP-responsive-element-binding protein; **CYP**, Cytochrome P450; **DEX**, Dexamethasone; **DMSO**, Dimethyl sulfoxide; **DR-1**, direct repeat spaced by one nucleotide; **E2**, 17β-estradiol; **ER**, endoplasmic reticulum; **EST**, estrogen sulfotransferase; **FABP**, fatty acid binding protein; **FATP**, fatty acid transporter protein; **MTP**, microsomal triglyceride transport protein; **FAS**, fatty acid synthase; **FBS**, fetal bovine serum; **FFA**, free fatty acid; **FoxA2**, forkhead factor A2; **FoxO1**, forkhead transcription factor O1; **FSK,** forskolin**; FXR,** farnesoid X receptor**; G6Pase**, glucose-6-phosphatase; **GPR**, G protein-coupled receptor; **GR**, glucocorticoid receptor; **GRP78**, Glucose-regulated protein 78; **GSIS,** glucose stimulated insulin secretion**; GTT**, glucose tolerance test; **H&E**, Haematoxylin Eosin; **HFD,** high-fat diet; **HMGcoR**, HMG-CoA reductase; **HMGCS2**, hydroxymethylglutaryl

CoA synthase 2; **HNF4α**, hepatocyte nuclear factor 4α; **IBABP,** Ileal bile acid binding protein**;** **IGF-1**, insulin-like growth factor 1; **IRS**, insulinresponse sequence (FoxO1-binding sites); **ITT**, insulin tolerance test; **LDLR**, low density lipoprotein receptor; **LXR**, liver X receptor; **Mac1,** macrophage 1 antigen**;** **MCP1,** monocyte chemotactic protein-1**;** **MIP1**, macrophage inflammatory protein-1; **NASH,** non-alcoholic related steatohepatitis**;** **PAPS**, 3-phosphoadenosine-5-phosposulfate; **PB,** phenobarbital**;** **PCR,** Polymerase chain reaction**;** **PEPCK**, phosphoenolpyruvate carboxykinase; **PGC-1a**, PPARg coactivator-1a; **PML**, promyelocytic leukemia; **PPAR**, peroxisome proliferator-activated receptor; **PPRE**, peroxisome proliferator response element ; **PXR**, pregnane X receptor; **RXR**, retinoid X receptor; **SCAP**,SREBP cleavage-activating protein; SREBP, sterol-regulatory-element-binding protein; **TCA**, citric acid cycle. **SCD-1**, stearoyl CoA desaturase-1; **SHP**, short heterodimer partner; **SOCS3**, suppressor of cytokine signaling 3; **SREBP**, Sterol Regulatory Element-Binding Proteins; **STS**, steroid sulfatase; **SULT,** sulfotransferase**;** **T2D,** type 2 diabetes; **T3,** tri-iodothyronine**;** **T4,** thyroxine; **TCPOBOP**, 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene, 3,3′,5,5′-Tetrachloro-1,4-bis(pyridyloxy)benzene; **TNF-α,** tumor necrosis factor-alpha; **TORC2,** transducer of regulated CREB activity 2; **TUNEL**, Terminal deoxynucleotidyl transferase dUTP nick end labeling; **UGT**, UDP-glucuronosyltransferase; **VLDL**, very low density lipoprotein; **VLDLR**, very low density lipoprotein receptor; **WAT**, white adipose tissue; **WT**, wild type; **XBP1**, X-box binding protein 1;

1.0 CHAPTER I: NUCLEAR RECEPTORS AND METABOLIC REGULATIONS

Nuclear receptors are ligand activated transcription factors that regulate the expression of target genes to affect physiological processes such as reproduction, development, and energy metabolism. Followed by the cloning of several members of steroid receptors in the early-1980s, these proteins are first recognized as the sensors and mediators of steroid hormone signaling. The subsequent cloning of other nuclear receptor genes based on sequence similarity unexpected revealed a large family of nuclear receptor-like genes. There are a total of 48 members discovered within this transcriptional factor family, which included not only classic endocrine receptors mediating steroid hormones signaling (Evans 1988), but also many so-called orphan receptors whose ligands, target genes and physiological function are initially unknown (Giguere 1999).

The organization of functional domains is largely conserved among all nuclear receptors with few exceptions (Fig.1). These proteins contain an NH2-terminal region that harbors a ligand-independent transcriptional activation function (AF-1), which contains two highly conserved zinc finger motifs that target the receptor to specific DNA sequences known as hormone response elements; a hinge region that permits protein flexibility to allow for simultaneous receptor dimerization and DNA binding; and a large COOH-terminal region that harbors the ligand-binding domain, dimerization surface, and a ligand-dependent activation function (AF-2). Upon ligand binding, nuclear receptors undergo a conformational change that

coordinately dissociates corepressors and facilitates recruitment of coactivator proteins to enable transcriptional activation of their target genes (McKenna et al. 1999).

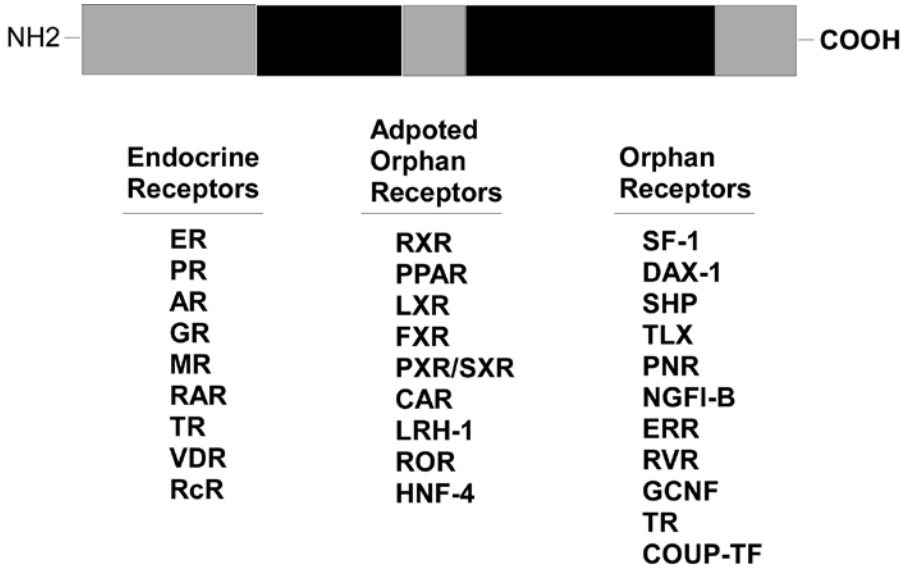

Figure 1. Schematic structure of typical nuclear receptors and their classification based on type of ligand.

Classic nuclear steroid hormone receptors included the glucocorticoid (GR), mineralocorticoid (MR), estrogen (ER), androgen (AR), and progesterone (PR) receptors. Steroid hormones are synthesized mainly from endogenous endocrine sources that are regulated by negative-feedback control of the hypothalamic-pituitary axis. Steroid hormones are circulated in the body to their target tissues where they bind to their receptors with high affinity (within nM range). Upon hormone binding, steroid receptors bind to DNA as homodimers, and regulate its target genes expression. Steroid receptors are well documented to regulate a variety of metabolic and developmental process including sexual differentiation, reproduction, energy metabolism, and electrolyte balance (Carson-Jurica et al. 1990).

Orphan nuclear receptors include receptors for fatty acids (peroxisome proliferator-activated receptors; PPARs), oxysterols (Liver X receptors; LXRs), bile acids (farnesoid X receptor; FXR), and xenobiotics (pregnane X receptor; PXR and constitutive androstane receptor; CAR). Receptors in this group bind their hydrophobic lipid ligands with relatively lower affinities comparable to physiological concentrations that can be affected by dietary intake (within μM range). In the past decades, the importance of these nuclear receptors is illustrated in many physiological states such as reproductive biology, inflammation, cancer, diabetes, cardiovascular disease, and obesity. The emerging evidence suggested these receptors functions as lipid/nutrients sensors to maintain energy homeostasis through regulating the transcription of a family of genes involved in nutrients/lipid metabolism, storage, transport, and elimination (Chawla et al. 2001). In this introduction section, I will briefly review several members of nuclear receptors serving as sensors for nutrients/stress to regulate energy homeostasis in both physiological and pathological conditions.

1.1 ENDOCRINE NUCLEAR RECEPTORS: HORMONAL/NUTRIENTS SENSOR UPON ENERGY STRESS

Steroid hormones such as glucocorticoid, sex steroids, and thyroid hormone had profound effects on energy metabolism. Glucocorticoid levels in serum are elevated during starvation or other stress conditions, and exert a variety of metabolic functions through GR. During fasting, glucocorticoids promote hepatic gluconeogenesis and glycogen synthesis in liver, and promotes amino acid catabolism, adipogenesis and insulin resistance in peripheral tissues. Adipose-specific glucocorticoid activation in mice results in visceral obesity, hyperphagia,

hyperlipidemia, and insulin resistance; whereas its inactivation increases energy expenditure and protects the animal from diet-induced obesity and diabetes (Walker et al. 2006). Liver specific inactivation of GR in mice also ameliorates hyperglycemia in streptozotocin-induced diabetes mellitus (Opherk et al. 2004).

Thyroid hormone signaling mediated through thyroid hormone receptors (TRα and TRβ) also have profound effect on energy metabolism. Thyroid hormones are known to increase in metabolic rate and result in weight loss. TRβ activation has beneficial effect on serum LDL cholesterol, triglyceride and proatherogenic lipoprotein (Baxter et al. 2004).

1.2 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS: FATTY ACID SENSOR

The peroxisome proliferators-activated receptors (PPAR α, β, γ) can be activated by endogenous polyunsaturated fatty acids, eicosanoids, and various synthetic ligands (17). Global and tissue specific gene-knockout mice generated in past decade highlight the critical while distinct function of each member in fatty acid homeostasis.

Through the analyses of PPARα null mice, it has been well established that PPARα is a key regulator of fatty acid catabolism. PPARα regulated a panel of target genes that function together to coordinate metabolic adaptation for energy conservation purpose during fasting/starvation condition. PPARα activation up-regulates expression of genes to buffer intracellular fatty acids and delivers PPARα ligands to the nucleus such as liver fatty acid–binding protein (Wolfrum et al. 2001); to promote transport of fatty acids into peroxisomes for β-oxidation such as the ABC transporters (ABCD2 and ABCD3) (Fourcade et al. 2001); and to

catalyze ω-oxidation in the final catabolic step in the clearance of PPARα ligands such as CYP4A enzymes (Lee et al. 1995).

PPARγ has been well established as a key regulator of adipogenesis, and its activation offers beneficial effects to various pathological conditions such as insulin resistance, atherosclerosis, and cancer. The ligands for PPARγ include fatty acids, arachidonic acid metabolites, and anti-diabetic drugs thiazolidinediones. Different from PPARα, activation of PPARγ promotes fat storage by increasing adipocyte differentiation and expression of a number of important lipogenic proteins, such as fatty acid–binding protein 4 (FABP/aP2), fatty acid translocase CD36, and lipid transporter ABCA1 (Rosen et al. 2001).

PPARβ is ubiquitously expressed in many tissues, and broadly involved in fatty-acid metabolism, mitochondrial respiration, thermogenesis and programming of muscle fiber type. The naturally occurring ligands for PPARδ include polyunsaturated fatty acids and eicosanoids (Bensinger et al. 2008). Highly potent and selective synthetic PPARδ agonists have also been developed. Administration of the synthetic ligand GW501516 in animal model lead to significant increase in HDL cholesterol, a decrease in LDL cholesterol and a decrease in fasting triglyceride level. These results indicate the critical role of PPARδ signaling in lipoprotein metabolism (Oliver et al. 2001).

1.3 LIVER X RECEPTOR: THE STEROL SENSORS

LXRα is abundantly expressed in tissues associated with lipid metabolism such as liver, adipose, kidney, intestine, lung, adrenals, and macrophages, whereas LXRβ is ubiquitously expressed. The LXRs are activated by naturally occurring oxysterols including 24(S)-

hydroxycholesterol, 22(*R*)-hydroxycholesterol, 24(*S*), 25-epoxycholesterol, and 27-hydroxycholesterol (Lu et al. 2001).

LXRs act as cholesterol sensors that respond to elevated sterol concentrations, and induced a panel of genes that govern transport, catabolism, and elimination of cholesterols. LXR activation in liver up-regulates the expression of cholesterol transporters ABCG5 and ABCG8 to increase secretion of cholesterol into bile, and the expression of CYP7A1 which is the rate-limiting enzyme for bile-acid synthesis and cholesterol elimination (Peet et al. 1998). In the intestine, LXRs regulate the expression of ABCG5 and ABCG8 which is responsible for cholesterol re-absorption (Repa et al. 2002). In peripheral cells such as macrophages, LXRs control the expression of genes involved in the return of peripheral cholesterol to the liver by a process known as reverse cholesterol transport. In addition, LXRs induce expression of the cholesterol-efflux transporters ABCA1 and ABCG1 in the condition of cholesterol over-loading (Castrillo et al. 2004).

LXRs also regulate many genes such as fatty acid synthase (FAS), stearoyl-coA desaturase 1 (Scd1) involved in fatty acid synthesis through modulating the expression of sterol regulatory element binding protein-1c (SREBP-1c) (Repa et al. 2000).

1.4 FARNESOID X RECEPTOR: THE BILE ACID SENSOR

FXR is highly expressed in the enterohepatic system including liver and intestine, where it acts as a bile acid sensor that protects the body from elevated bile acid concentrations. The endogenous ligands for FXR are bile acids including chenodeoxycholic acid, cholic acid, and their respective conjugated metabolites (Russell 1999). In liver, FXR activation results in the up-

regulation of bile salt efflux genes such as BSEP to increases the flow and secretion of bile acid into bile. FXR activation also suppresses the expression of the key CYP genes involved in bile acid synthesis through up-regulation of SHP (small heterodimer partner), an atypical orphan nuclear receptor that functions as a transcriptional repressor. In ileal enterocytes, FXR activation induce the expression of ileal bile acid binding protein IBABP to buffer intracellular bile acids and promote their translocation into the portal circulation (Sinal et al. 2000).

Recent studies demonstrated that activation of FXR can suppress hepatic TG and fatty acid (FA) synthesis as a result of SHP-dependent inhibition of SREBP-1c. Activation of FXR also results in increased hepatic expression of receptors (VLDL receptor and syndecan-1) that are involved in lipoprotein clearance and increased apoC-II that co-activates lipoprotein lipase (LPL) (Watanabe et al. 2004). Administration of bile acids (CDCA or CA) to humans or animals results in reduced plasma triglyceride and HDL levels and increased LDL, which is consistent with a key role for bile acids in controlling plasma lipids (Modica et al. 2006).

FXR null mice show impaired glucose tolerance and insulin sensitivity compared to wild-type mice. Consistent with this observation, insulin signaling was found to be impaired in the liver, muscle and white adipose tissue of FXR null mice (Cariou et al. 2006). Administration of FXR agonist GW4064 or infected with adenovirus expressing FXR-VP16 significantly improved glucose tolerance and insulin sensitivity in diabetic *db/db* mice, which suggests that FXR agonists might be useful in the treatment of hyperglycemia and hyperlipidemia in patients with type 2 diabetes (Zhang et al. 2006).

1.5 PXR AND CAR: THE XENOBIOTIC SENSORS

To protect the body against the accumulation of xenobiotics and toxic endogenous lipids, two nuclear receptors the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) regulate a panel of conjugating enzymes responsible for transforming and eliminating many prescription drugs, environmental contaminants, steroids, and toxic bile acids. Consistent with their role as xenobiotic sensors, both receptors are expressed primarily in liver and small intestine. PXR null mice have abolished CYP3A inducibility and the protection of liver from the effects of toxic compounds, while CAR null mice also showed a lack of induction of Cyp2b10 and many other conjugation enzymes by TCPOBOP and PB in the liver and small intestine (Xie et al. 2001).

Recently, an increasing body of evidence suggests that PXR and CAR also have an endobiotic function that impacts energy homeostasis through the regulation of glucose and lipids metabolism. Interestingly, disruptions of energy homeostasis, such as those observed in obesity and diabetes, also have a major impact on drug metabolism. The detailed information regarding the integral role of PXR and CAR in drug metabolism and energy homeostasis will be reviewed in Chapter 2.

1.6 OTHER ORPHAN RECEPTORS

In additional to the above discussed nuclear receptors, many other nuclear receptor family members have also been demonstrated to play critical roles in regulating energy homeostasis. ERRs have been showed to induce mitochondrial fatty acid β-oxidation and

oxidative coupled respiration along with peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) (Schreiber et al. 2004). HNF4α has also been shown a key regulator of hepatic gluconeogenesis, and its single gene mutation cause Maturity Onset Diabetes of the Young (MODY1) in humans (Gupta et al. 2004). The three members of the NR4A subfamily (Nur77, Nurr1 and NOR-1) have also recently been shown to act as important regulators of gluconeogenesis in fasting condition, and elevated expression of NR4A is observed in diabetic animal models and also associated with insulin resistance and diabetes in some human studies (Pei et al. 2006). SHP is an atypical nuclear receptor and acts by inhibiting other NR pathways. It has been shown that SHP can interact with many other NR and control broad aspects of nutrient metabolism. In humans, mutations in the SHP gene are associated with mild obesity (Bavner et al. 2005), whereas SHP deficiency in mice causes increased energy expenditure, improved pancreatic β cell function and improved glucose homeostasis (Wang et al. 2006).

In addition to HNF4α, NR4A and SHP, some less characterized orphan receptors such as Rev-erbβ and RORs are also likely to be involved in metabolic regulation. Further study on the physiological function of these orphan NRs may reveal the importance of these proteins in metabolic regulation and potential to serve as therapeutic targets.

2.0 CHAPTER II: CAR IS AN ANTI-OBESITY NUCLEAR RECEPTOR THAT IMPROVES INSULIN SENSITIVITY

2.1 PXR AND CAR: AT THE CROSSROADS OF DRUG METABOLISM AND ENERGY METABOLISM

The pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) are two closely related and liver-enriched nuclear hormone receptors originally defined as xenobiotic receptors. PXR and CAR regulate the transcription of drug-metabolizing enzymes and transporters, which are essential in protecting our bodies from the accumulation of harmful chemicals. An increasing body of evidence suggests that PXR and CAR also have an endobiotic function that impacts energy homeostasis through the regulation of glucose and lipids metabolism. Of note and in contrast, disruptions of energy homeostasis, such as those observed in obesity and diabetes, also have a major impact on drug metabolism. This review will focus on recent progress in our understanding of the integral role of PXR and CAR in drug metabolism and energy homeostasis.

2.1.1 PXR and CAR: Master Regulators of Drug Metabolism and Drug Transporter

Over the long period of evolution, every organism has developed a complex defense system to prevent the accumulation of toxic xenobiotics and endogenous metabolites. Although

many of the water-soluble chemicals are readily eliminated by transporter proteins, lipophilic compounds often require biotransformation to become more water-soluble before being excreted. The enzymes responsible for biotransformation include Phase I and Phase II enzymes. The cytochrome P450 (P450) enzymes belong to a superfamily of heme-containing Phase I enzymes that catalyze monooxygenase reactions of lipophilic compounds facilitated by the reducing power of the NADPH P450 oxidoreductase. Phase II enzymes catalyze the conjugation of water-soluble groups to xeno- and endobiotics. Conjugation reactions include glucuronidation, sulfation, methylation, and N-acetylation. In many cases, biotransformation leads to metabolic inactivation of chemicals. However, biotransformation may also activate the so-called prodrugs to pharmacologically active products or even to toxic metabolites (Handschin et al. 2003; Pascussi et al. 2008).

Most drug-metabolizing enzymes are inducible in response to xenobiotics, which represent an adaptive response of our bodies to chemical insults. The molecular basis for this inducible defense system remained largely unknown until 1998, when pregnane X receptor [(PXR) alternatively termed steroid and xenobiotic receptor, or SXR, in humans] was discovered (Blumberg et al. 1998; Kliewer et al. 1998). PXR is expressed predominantly in the liver and intestine, and it is activated by a wide variety of natural and synthetic compounds. Upon activation, PXR forms a heterodimer with retinoid X receptor and activates the transcription of drug-metabolizing enzyme and transporter genes. Examples of PXR target genes include Phase I CYP3As and CYP2Cs, Phase II UDP-glucuronosyltransferase 1A1 (UGT1A1), and sulfotransferases (SULTs), and drug transporters MDR1 and MRP2. PXR has since been established as a xenosensor and master regulator of xenobiotic responses. The essential role of PXR in xenobiotic regulation and in dictating the species specificity of xenobiotic responses was

confirmed through the creation and characterization of PXR null mice (Xie et al., 2000; Staudinger et al., 2001) as well as humanized PXR transgenic mice (Xie et al. 2000; Ma et al. 2007).

The constitutive androstane receptor (CAR) is a sister xenobiotic receptor of PXR. Purified first from hepatocytes as a protein bound to the phenobarbital responsive element in the CYP2B gene promoter, CAR was subsequently shown to bind to the CYP2B gene promoter as a heterodimer with retinoid X receptor. In general, it is believed that endogenous CAR resides in the cytoplasm of hepatocytes. Upon exposure to its agonist phenobarbital (PB) or 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), CAR translocates from the cytoplasm to the nucleus and triggers the transcription of its target genes. Transfected CAR exhibited a high basal activity and was once termed a "constitutively active receptor." The name of constitutive androstane receptor was conceived due to the binding and inhibition of CAR activity by androstanes (Forman et al. 1998). CAR null mice showed a lack of induction of Cyp2b10 and many other Phase I and Phase II enzymes and drug transporters by TCPOBOP and PB in the liver and small intestine (Wei et al. 2000).

2.1.2 Drug Metabolism Capacity Influenced by Energy Metabolism

Drug metabolism can be affected by various pathophysiological factors, including diabetes and liver diseases that may affect the expression or activity of drug-metabolizing enzymes and transporters. Nuclear receptors, including the xenobiotic receptors PXR and CAR and sterol sensor liver X receptor (LXR), may function as the links between drug metabolism and energy metabolism.

P450 enzyme down-regulation has been well documented in animal models of obesity, steatosis, and nonalcoholic steatohepatitis (NASH) (Su et al. 1999). Down-regulation of constitutive P450 expression in rat liver was proportional to the extent of hepatic lipid accumulation. Moreover, the capacity of drug metabolism was reported to be impaired in human patients with obesity, hepatic steatosis, and NASH (Fiatarone et al. 1991; Blouin et al. 1999; Cheymol 2000). Liver lipid accumulation, especially in the early stage of steatosis, can significantly down-regulate several important P450s (Zhang et al. 2007), which is consistent with the decreased P450 expression in liver microsomes derived from patients with steatosis and NASH (Donato et al. 2006; Donato et al. 2007; Fisher et al. 2009). A recent report showed that the polyunsaturated fatty acids can down-regulate PB-induced CYP2B expression in a CAR-dependent manner in rat primary hepatocytes (Finn et al. 2009), which was consistent with an earlier report that polyunsaturated fatty acids can attenuate PB-induced nuclear accumulation of CAR (Li et al. 2007). Because hepatic steatosis often leads to increased free fatty acid levels, the inhibitory effect of free fatty acids on CAR provides a plausible explanation for the negative effect of steatosis on drug metabolism. Hepatic steatosis is often associated with increased lipogenesis, in which the sterol regulatory element-binding protein 1 (SREBP-1) is a key lipogenic transcription factor. It has been reported that SREBP-1 inhibited the transcriptional activities of PXR and CAR by functioning as a non-DNA binding inhibitor and blocking the interaction of PXR and CAR with nuclear receptor cofactors (Roth et al. 2008).

The effect of hepatic steatosis on drug metabolism can also be mediated by the lipogenic nuclear receptor LXR through the cross-talk between LXR and CAR. LXRs, both the α and β isoforms, were defined as sterol sensors. In rodents, LXR activation increases hepatic cholesterol catabolism and formation of bile acids by inducing cholesterol 7α-hydroxylase (Cyp7a1) (Peet et

al. 1998). LXRs were later found to be an important regulator of SREBP-1c and lipogenesis (Repa et al. 2000). It has been reported that LXR-deficient mice fed with a high-cholesterol diet showed significantly higher induction of Cyp2b10 mRNA level upon PB treatment compared with wild-type mice, suggesting that LXR may repress PB-mediated CAR activation and Cyp2b10 induction in vivo (Gnerre et al. 2005). Our laboratory recently showed that LXRα and CAR are mutually suppressive and functionally related in vivo. In particular, loss of CAR increased the expression of lipogenic LXR target genes, leading to increased hepatic triglyceride accumulation; whereas activation of CAR inhibited the expression of LXR target genes and LXR ligand-induced lipogenesis. In contrast, a combined loss of LXR α and β increased the basal expression of xenobiotic CAR target genes; whereas activation of LXR inhibited the expression of CAR target genes and sensitized mice to xenobiotic toxicants. The mutual suppression between LXRα and CAR was also observed in cell culture and reporter gene assays (Zhai et al. 2010).

The AMP-activated protein kinase (AMPK) can also mediate the effect of energy metabolism on drug metabolism. As an important energy sensor, AMPK is activated in response to stresses, such as starvation, exercise, and hypoxia, which deplete cellular ATP supplies. Upon activation, AMPK increases energy production such as lipid oxidation, whereas it decreases energy-consuming processes such as gluconeogenesis and lipogenesis, to restore energy balance (Long et al. 2006). Recent reports suggest that activation of AMPK is essential for PB induction of drug-metabolizing enzymes in mouse and human livers (Rencurel et al. 2005; Rencurel et al. 2006; Shindo et al. 2007). The induction of Cyp2b10 by PB in primary mouse hepatocytes was associated with an increased AMPK activity, and the treatment of AMPK agonist 5-amino-1-β-D-ribofuranosyl-1H-imidazole-4-carboxamide (AICAR) triggered the nuclear accumulation of

CAR in mouse livers (Shindo et al. 2007). In primary human hepatocytes, treatment with AICAR or metformin, another AMPK agonist, alone can induce CYP2B6 and/or CYP3A4 expression as efficient as PB, whereas overexpression of the dominant-negative mutant AMPKα1 or use of AMPK inhibitor completely blocked the PB induction of CYP2B6 and CYP3A4 (Rencurel et al. 2006). The essential role of AMPK in the induction of Cyp2b10 was further demonstrated in AMPKα1/α2 liver-specific knockout mice, in which the PB and AICAR induction of Cyp2b10 was blunted (Rencurel et al. 2006). Note that liver tissue from AMPKα1/α2 liver-specific knockout mice had markedly increased basal mRNA expression of Cyp210/3a11, which were not observed in isolated primary mouse hepatocytes. In an independent report, AICAR was shown to prevent the nuclear translocation of CAR in isolated primary rat hepatocytes (Konno et al. 2008). It is unclear whether the discrepancies between the studies were due to the differences in animal species or experimental conditions. The link between PB treatment and AMPK activation remains to be firmly established. It was proposed that PB may alter mitochondrial function and trigger the generation of reactive oxygen species, which subsequently activate LKB1 kinase and AMPK phosphorylation (Blattler et al. 2007). The expression of CAR and its target genes has been reported to be regulated by the circadian clock-controlled PAR-domain basic leucine zipper (PAR bZip) family of transcription factors. Mice deficient of three PAR bZip proteins were hypersensitive to xenobiotic compounds, and the deficiency in detoxification may contribute to their early aging (Gachon et al. 2006). AMPK can also regulate the circadian clock by phosphorylating and destabilizing the clock component cryptochrome 1 (Lamia et al. 2009). Together, these reports suggest the connections between molecular clocks, energy metabolism, and nuclear receptor signaling pathways.

Diabetes, a major manifestation of the metabolic syndrome, also has an impact on drug metabolism. Drug clearance was significantly increased in untreated patients of type 1 diabetes, which can be reversed by insulin treatment (Zysset et al. 1988; Goldstein et al. 1990). This result was consistent with the observation in a streptozotocin-induced type 1 diabetic mouse model, in which the mice exhibited an increased Cyp2b10 basal expression that can be corrected by insulin treatment (Sakuma et al. 2001). The increased Cyp2b expression in type 1 diabetic mice seemed to be CAR-dependent and was associated with increased activities of peroxisome-proliferator-activated-receptor-γ-coactivator-1α (PGC-1α) and AMPK (Dong et al. 2009). The increased expression of Cyp2b in type 1 diabetes is reminiscent of starvation conditions during which the insulin level is decreased, and the expression of both Cyp2b10 and CAR is induced (Maglich et al. 2004). Consistent with the observations in type 1 diabetes, insulin deprivation enhanced both dexamethasone- and β-naphthoflavone-induced expression of Cyp3a and Cyp1a in rat primary hepatocytes (Sidhu et al. 1999). In contrast, the expression of Cyp2b and 2e was suppressed by insulin in rat hepatoma cells (De Waziers et al. 1995). The molecular mechanism for the interaction between the insulin pathway and PXR/CAR activity was suggested by a recent study showing that Forkhead box O1 protein (FOXO1), a member of the insulin-sensitive transcription factor family, can interact with and function as a coactivator to CAR- and PXR-mediated transcription (Kodama et al. 2004). It was believed that in conditions of insulin deficiency, such as starvation and type 1 diabetes, FOXO1 translocates into the nucleus, becomes activated, and increases the transcriptional activity of CAR and PXR.

2.1.3 Energy Metabolism Regulated by Xenobiotic Receptors PXR/CAR and P450s

Effects of P450s and PXR/CAR on Hepatic Lipid Metabolism.

P450 enzymes are known to be involved in the biotransformation of both xenobiotics, such as drugs, as well as endobiotics, such as cholesterol, steroid hormone, bile acids, and prostanoids. Although the role of P450s in drug metabolism has been well recognized and extensively studied, the endobiotic functions of P450s and to what extent they will affect systemic or tissue-specific homeostasis are less understood. Because all P450s receive electrons from a single donor, cytochrome P450 reductase [(CPR) NADPH:ferrihemoprotein reductase) (Smith et al. 1994), deletion of CPR will in principle inactivate all P450s. Mouse models with liver-specific deletion of CPR were generated by two independent groups (Gu et al. 2003; Henderson et al. 2003). Liver CRP null mice, in which the Cre expression was under the control of the albumin promoter, showed dramatically decreased liver microsomal P450 and heme oxygenase activities as expected (Pass et al. 2005). It was surprised to find that the liver of CPR null mice exhibited hepatomegaly and massive hepatic steatosis by two months of age, accompanied by severely reduced circulating cholesterol and triglyceride levels. These results have clearly implicated P450 activity in hepatic lipid homeostasis. Given the fact that the liver CPR null mice have their CPR gene deletion occur neonatally, as controlled by the albumin promoter, a subsequent study was designed to investigate the effect of P450 inactivation in adult mice by using a conditional knockout strategy. The conditional knockout of CRP was achieved by crossing the CRP floxed mice with the CYP1A1-Cre transgenic mice, in which the expression of Cre recombinase is controlled by the rat CYP1A1 gene promoter. The CYP1A1-Cre transgene has a low basal expression, but its expression can be markedly induced by a pharmacological administration of 3-methlycholanthrene, an aryl hydrocarbon receptor agonist, leading to a time-dependent and hepatic-specific deletion of CRP (Finn et al. 2007). Associated with the time-dependent reduction of CPR expression in the liver after the treatment of 3-methlycholanthrene

in adult mice, these animals developed fatty liver and had reduced non-fasting plasma cholesterol and triglyceride levels. The hepatic steatosis is mainly accounted by the accumulation of triglycerides. The source of accumulated triglycerides was believed to be dietary fatty acids, because a fat-deficient diet can reverse the steatotic phenotype (Finn et al. 2009).

The xenobiotic receptor CAR also plays an important role in hepatic lipid homeostasis. Activation of CAR by its agonist TCPOBOP has been reported to inhibit the expression of hepatic lipogenic genes and alleviate hepatic steatosis in high-fat diet fed mice and ob/ob mice (Dong et al. 2009; Gao et al. 2009). Several possible but not mutually exclusive molecular mechanisms have been proposed to explain the inhibitory effect of CAR on lipogenesis. In one study, it was suggested that activation of CAR or PXR reduced the level of SREBP-1 by inducing Insig-1, a protein blocking the proteolytic activation of SREBPs (Roth et al. 2008). Our own study suggested that CAR may inhibit lipogenesis by inhibiting the LXR agonist responsive recruitment of LXRα to the Srebp-1c gene promoter (Zhai et al. 2010).

In contrast to the inhibitory effect of CAR on lipogenesis, activation of PXR seemed to promote lipogenesis. Transgenic mice expressing a constitutively activated PXR showed hepatomegaly and marked hepatic steatosis, and treatment of mice with a PXR agonist elicited a similar effect. PXR-induced lipogenesis was independent of the activation of SREBP-1c and was associated with the induction of fatty acid translocase (FAT/CD36), peroxisome proliferator-activated receptor γ2 (PPARγ2), and stearoyl-CoA desaturase-1 (Zhou et al. 2006). Further promoter analyses showed that both CD36 and PPARγ2 are direct transcriptional targets of PXR (Zhou et al. 2008). A recent report showed that the thyroid hormone (TH)-responsive spot 14 protein, the expression of which correlates with lipogenesis, was a PXR target gene (Breuker et al. 2010). Therefore, the induction of spot 14 may have also contributed to the lipogenic effect of

PXR. Treatment of mice with the PXR agonist pregnenolone-16α-carbonitrile (PCN) is known to alleviate the lithocholic acid-induced hepatotoxicity (Staudinger et al. 2001; Xie et al. 2001). It was recently reported that the PCN-mediated stimulation of lipogenesis contributes to the protection from lithocholic acid-induced hepatotoxicity (Miyata et al. 2010).

The steatotic effect of PXR was also associated with suppression of several genes involved in fatty acid β-oxidation. Activation of PXR by its agonist PCN can inhibit lipid oxidation by down-regulating the mRNA expression of carnitine palmitoyltransferase 1α (CPT1α) and mitochondrial 3-hydroxy-3-methylglutarate-CoA synthase 2 (HMGCS2), two key enzymes involved in β-oxidation and ketogenesis, in a PXR-dependent manner. Mechanistically, it has been shown that PXR can directly interact with Forkhead box A2 (FoxA2) and prevent FoxA2 binding to the Cpt1a and Hmgcs2 gene promoters (Nakamura et al. 2007). Finally, unlike CAR, activation of PXR had little effect on the lipogenic effect of LXR (Zhai et al. 2010).

Effects of PXR and CAR on Hepatic Glucose Metabolism

Chronic treatment with PB has been reported to decrease plasma glucose levels and improve insulin sensitivity in diabetic patients (Lahtela et al. 1985). Treatment with PB suppressed the expression of gluconeogenic enzymes PEPCK1 and G6Pase in mouse liver (Ueda et al. 2002) and in primary rat hepatocytes (Argaud et al. 1991). The regulation of the gluconeogenic pathway by PXR was initially suggested by the suppression of PEPCK and G6Pase in VP-hPXR transgenic mice (Zhou et al. 2006). Treatment of wild-type mice with the PXR agonist PCN also suppressed cAMP-dependent induction of G6Pase in a PXR-dependent manner (Kodama et al. 2004). Several possible but not mutually exclusive molecular mechanisms have been proposed to explain the inhibitory effect of PXR and CAR on

gluconeogenesis. It was reported that PXR can form a complex with phosphorylated cAMP response element-binding protein (CREB) in a ligand-dependent manner to prevent CREB binding to the cAMP response element and inhibit CREB-mediated transcription of G6Pase (Kodama et al. 2007). PXR and CAR can also physically bind to FoxO1 and suppress its transcriptional activity by preventing its binding to the insulin response sequence in the gluconeogenic enzyme gene promoters (Kodama et al. 2004). PXR and CAR may also inhibit hepatocyte nuclear factor-4α activity by competing for the DR1 (direct repeat spaced by one nucleotide) binding motif in the gluconeogenic enzyme gene promoters (Miao et al. 2006). The in vivo significance of CAR-mediated suppression of gluconeogenesis was supported by two recent reports that activation of CAR ameliorated hyperglycemia and improved insulin sensitivity in *ob/ob* mice and high-fat diet fed wild-type mice (Dong et al. 2009; Gao et al. 2009).

Potential link between CAR and Thyroid Hormones signaling

It has been reported that the expression of CAR was inducted during long-term fasting, possibly due to the up-regulation of PGC-1α (Ding et al. 2006). Moreover, CAR-deficient mice were defective in fasting adaptation and lost more weight during calorie restriction (Maglich et al. 2004). One possible mechanism by which CAR affects fasting response is through the effect of CAR on TH metabolism. Treatment of wild-type mice with a CAR agonist decreased serum T4 level (Qatanani et al. 2005). The decrease in T4 level was associated with a concomitant increase in the serum thyroid-stimulating hormone level in wild-type but not in CAR(−/−) mice. The effect of CAR on TH homeostasis was believed to be achieved through the regulation of TH-metabolizing Phase II enzymes. Activation of CAR induced the expression of glucuronosyltransferases UGT1A1 and 2B1 and sulfotransferases SULT2A1, 1C1, and 1E1 in

wild-type mice but not in CAR(−/−) mice. UGT1A1 and UGT2B1 are responsible for T4 and T 3 glucuronidation, respectively (Visser et al. 1993). SULTs are important for the inactivation of THs by blocking outer ring deiodination and causing irreversible inactivation of THs (Visser 1994). However, the net effect of CAR on TH homeostasis remains controversial. It was reported that activation of CAR decreased the serum reverse T3 level, resulting in the de-suppression of thyroid hormone target genes during partial hepatoectomy (Tien et al. 2007).

2.1.4 Conclusions and Perspectives

Figure 2 summarizes the major interactions between drug metabolism and energy metabolism and the central roles of PXR and CAR in these cross-talks. Induction or suppression of P450s and other drug-metabolizing enzymes can alter drug metabolism and clearance. Our understanding of the regulation of drug metabolism and activities of PXR and CAR by liver energy status may offer possible explanations for the variation of drug responses associated with metabolic diseases and provide rationale for appropriate dose adjustment. On the other hand, activation of PXR and CAR could have a significant impact on energy homeostasis by affecting lipogenesis, gluconeogenesis, and fatty acid oxidation. It is tempting to speculate that pharmacological modulation of PXR and CAR may be beneficial in managing metabolic diseases.

Among the remaining challenges, although PXR has been shown to affect lipid and glucose metabolism, the *in vivo* role of PXR in obesity and type II diabetes remains to be demonstrated. The endogenous ligands for PXR and CAR that elicit the metabolic functions of these two receptors also remain to be identified. The molecular mechanisms underlying the beneficial effects of CAR activation still need further investigation.

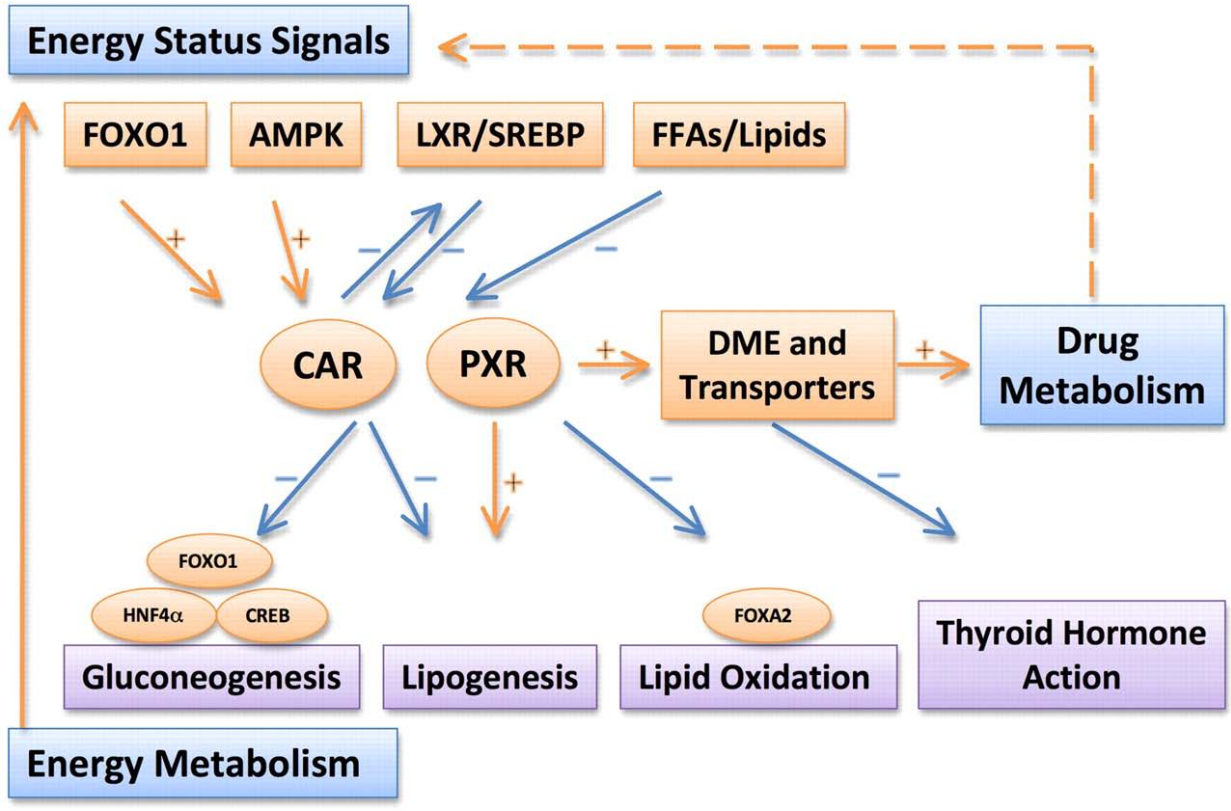

Figure 2. Summary of the major interactions between drug metabolism and energy metabolism and the central roles of PXR and CAR in these cross-talks.

Note that the drug metabolism and energy status can be affected by pathophysiological conditions, such as obesity, diabetes, and fatty liver. DME, drug-metabolizing enzymes; FFA, free fatty acids; HNF4α, hepatocyte nuclear factor 4α.

2.2 METHOD

2.2.1 Animals, Drug Treatment, Body Composition Analysis, and Histological Evaluation

Mice were housed under a 12-h light-dark cycle and given regular RMH3000 diet from PMI Nutrition International (St. Louis, MO) or high fat diet (catalog number S3282) from Bio-serv (Frenchtown, NJ). Wild type C57BL/6J mice and ob/ob mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The creation of CAR−/− mice has been described previously (4). CAR−/− mice were maintained in the C57BL/6J-SvJ129 mixed background. When necessary, mice received once per week intraperitoneal injections of TCPOBOP (0.5 mg/kg) or vehicle (DMSO). Mice were sacrificed 24 h after the last dose of drug. Body composition was analyzed in live animals using EchoMRI-100TM from Echo Medical Systems (Houston, TX). For histological analysis, tissues were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with hemotoxylin and eosin. Frozen sections (10 μm) were used for Oil-red O staining. The use of mice in this study has complied with relevant federal guidelines and institutional policies.

2.2.2 Measurement of Serum and Fecal Chemistry

Blood samples were collected from fed or fasted mice. Serum levels of bile acids (Bio-Quant, San Diego, CA), alanine aminotransferase (Calchem, Huntington, NY), total triglycerides and cholesterol (Stanbio Laboratory, Boerne, TX), free fatty acids (Biovision, Mountain View, CA), and insulin and leptin (Crystal Chem, Downers Grove, IL) were measured by using commercial

assay kits according to the manufacturer's instructions. Feces were collected by using mouse metabolic cages and subjected to the measurement of triglycerides.

2.2.3 Gene Expression Analysis

Total RNA was isolated using the TRIZOL reagent from Invitrogen. For real time PCR analysis, reverse transcription was performed with random hexamer primers and Superscript RT III enzyme from Invitrogen. SYBR Green-based real time PCR was performed with the ABI 7300 real time PCR system, as I have described previously (Lee et al. 2008). Data were normalized against control cyclophillin.

2.2.4 Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

For GTT, mice were fasted for 16 or 4 h before receiving an intraperitoneal injection of D-glucose at 2 g/kg body weight. Blood samples were taken at different time points, and the concentrations of glucose were measured with a glucometer. For ITT, mice were fasted for 4 h before receiving an intraperitoneal injection of human insulin (Novolin) from Novo Nordisk (Princeton, NJ) at 0.5 or 1.5 units/kg body weight. Blood samples were taken, and blood glucose levels were measured.

2.2.5 VLDL Secretion Assay

VLDL secretion rate in vivo was measured as described previously (Dresner et al. 1999). In brief, mice were fasted for 4 h before receiving a tail vein injection of tyloxapol from Sigma at 500 mg/kg body weight. Plasma samples were collected at 0, 1, 2, and 4 h after tyloxapol injection, and triglyceride levels were then measured as described above.

2.2.6 Immunofluorescence Microscopy

Tissues were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 μm, tissue sections were deparaffinized and rehydrated, followed by pre-incubated in blocking buffer (1X PBS, 5% normal donkey serum, and 0.3% Triton X-100) for 60 min. Tissue sections were then incubated with diluted primary antibody overnight at 4℃, and fluorochrome-conjugated secondary antibody for 1-2 hours at room temperature in dark the next day. Antibodies used include rabbit anti-mouse PGC1a (H300) polyclonal antibody (Santa Cruz), rabbit anti-mouse CAR (M-150); Mouse Anti-PML (clone 36.1-104, Millipore); Mouse anti-flag M2 (sigma); Rabbit anti-HA (C29F4, cell signaling).

2.2.7 Mouse Primary Hepatocyte Isolation

Mouse was anesthetized and placed on a warm surface to keep the body temperature at 37℃. The abdominal and chest cavities were opened, and the inferior vena cava, portal vein, and heart were exposed. The inferior vena cava was cannulated with a 21-gauge catheter attached to a perfusion line. The livers were perfused consecutively with 0.5 mM EGTA at a rate of 5

ml/min for 5 min, PBS at 5 ml/min for 2 min, and liver digest medium containing an additional 0.15 mg/ml collagenase at 2 ml/min for 10 min. The digested liver was excised and disaggregated by gentle rubbing with a cell scraper in a Petri dish with hepatocyte wash medium. The cells were washed twice with hepatocyte wash medium with centrifugation at 50 g. The hepatocyte pellet was resuspended in Williams' medium E supplemented with 5% FBS, counted in a hemocytometer after staining with Trypan blue, and seeded at a density of 1 *106 hepatocytes per well in 6-well plate coated with collagen.

2.2.8 Analysis of Oxygen Consumption and Measurement of Skeletal Muscle Mitochondrial Fatty Acid Oxidation

Oxygen consumption in live animals was measured as described previously (Hoover-Plow et al. 1985). Skeletal muscle mitochondrial fatty acid oxidation was measured as described previously (Koves et al. 2008). In brief, fresh gastrocnemius muscles were excised and placed in ice-cold modified Chappell-Perry buffer. Muscle homogenates were prepared, and mitochondria were isolated using differential centrifugation. Oxidation assays were performed using 0.2 mM [^{14}C]-oleate in the absence or presence of 2 mM sodium pyruvate. Reactions were terminated by adding 100 µl of 70% perchloric acid, and [^{14}C]-CO2 was trapped in 200 µl of 1 N NaOH. [^{14}C]-CO2 and ^{14}C-labeled acid-soluble metabolites were assessed by liquid scintillation counting.

2.2.9 Statistical Analysis

Results are presented as means ± SD. Statistical analysis was determined using the unpaired student's *t* test for comparison between two groups, with *P* values of less

than 0.05 considered significant; One-way ANOVA was used to compare the means of two or more groups; Repeated-measures ANOVA was used to compare the means of two or more groups across multiple time points.

2.3 RESULTS

2.3.1 Activation of CAR Prevented and Reversed Obesity

To determine the effect of CAR on obesity, 8-week-old male C57BL/6J mice were fed with a high fat diet (HFD) for 8 weeks and simultaneously treated with the CAR agonist TCPOBOP (0.5 mg/kg, once a week) or vehicle (DMSO). As shown in Fig. 3a, TCPOBOP significantly inhibited the gain of body weight after 2 weeks of drug treatment. MRI analysis showed that TCPOBOP inhibited the gain of fat mass (Fig. 3b), whereas the lean masses between the TCPOBOP and vehicle groups were not significantly different (Fig. 3d). As such, the lean mass/body weight ratio decreased in vehicle-treated mice, whereas the ratio remained steady in the TCPOBOP group (Fig3c). Fig. 1f shows a representative pair of mice HFD-fed and drug-treated for 5 weeks, at which the body weight difference between TCPOBOP group and vehicle group was completely accountable by the gain of fat mass in the vehicle group (Fig. 3d). The TCPOBOP effect was unlikely to be due to toxicity of the drug, because the differences in the serum levels of bile acids (Fig. 3e, left) and alanine aminotransferase (ALT) activity (Fig. 3e, right) between the vehicle and TCPOBOP groups were not statistically significant. A similar pattern of TCPOBOP effect was observed in female mice (Fig. 4a), and similar to male mice, TCPOBOP treatment inhibited the gain of fat mass (Fig. 4b), whereas the lean masses between the TCPOBOP and vehicle groups were not significantly different. These results suggesting that the CAR agonist administration can prevent the gain of adiposity in high-fat diet condition without significantly affecting the lean mass and the phenotype was not gender-specific. As expected, the hepatic mRNA expression of CYP2B10, a prototypical CAR target gene (Honkakoski et al. 1998; Wei et al. 2000), was induced by TCPOBOP (Data not shown).

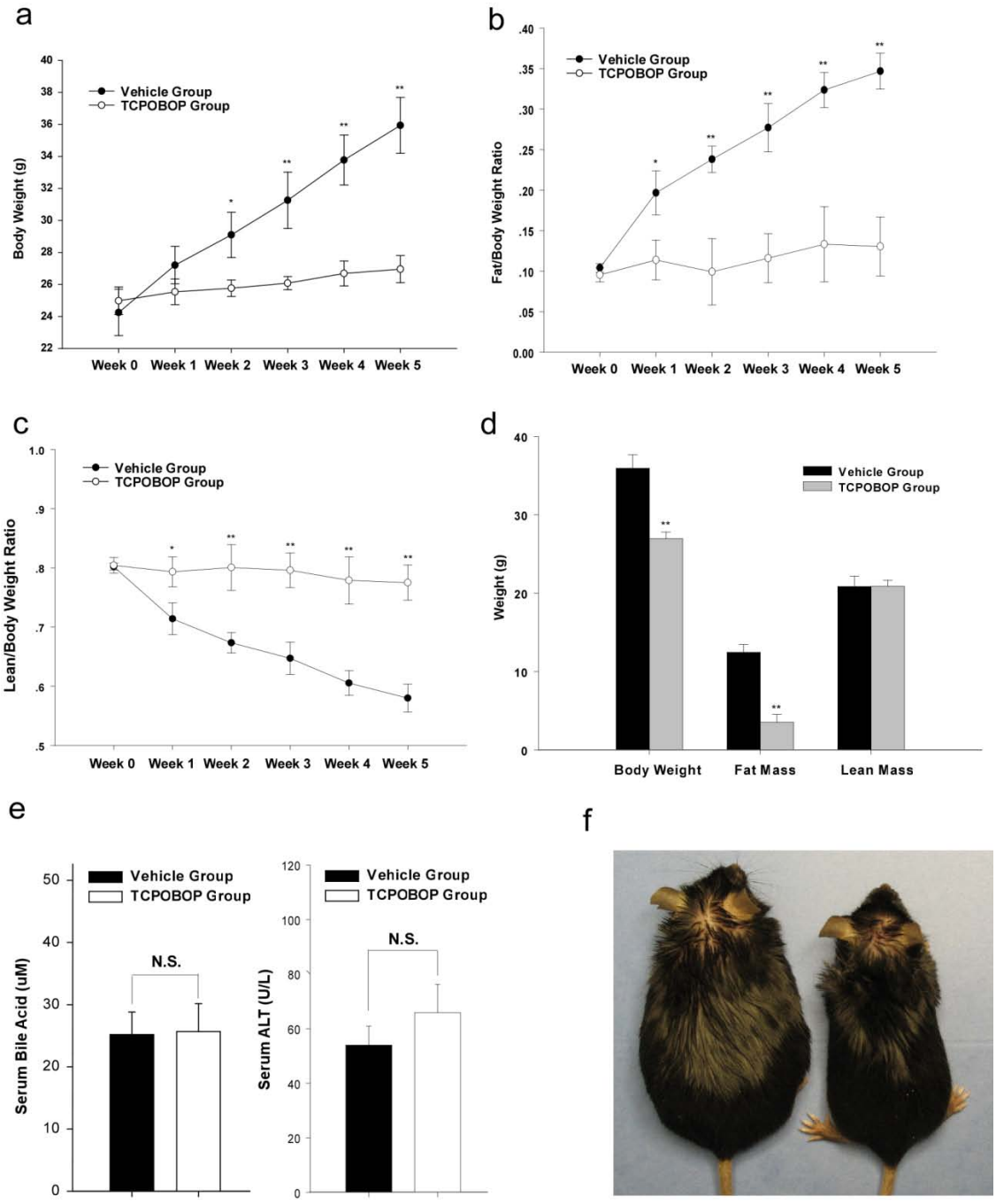

Figure 3. Treatment of mice with CAR agonist prevents obesity in high-fat diet fed mice.

Growth curve (a), fat mass/body weight (b), lean mass/body weight (c) in male C57bl/6j mice fed with HDF for 6 weeks, in the present or absence of TCPOBOP treatment (0.5mg/kg, intraperitoneal, once per week). Fat and lean masses were determined by MRI, n=5 for each group. (d) Body weight and body composition of HFD fed mice at the end of the 8-week drug treatment. (e) Serum levels of bile acids (left) and ALT activity (right) at the end of an 8-week diet and drug treatment. (f) a representative pair of vehicle- and TCPOBOP-treated mice. Results are expressed as means±S.D. *, $p \leq 0.05$; **, $p \leq 0.01$, compared with the vehicle group.

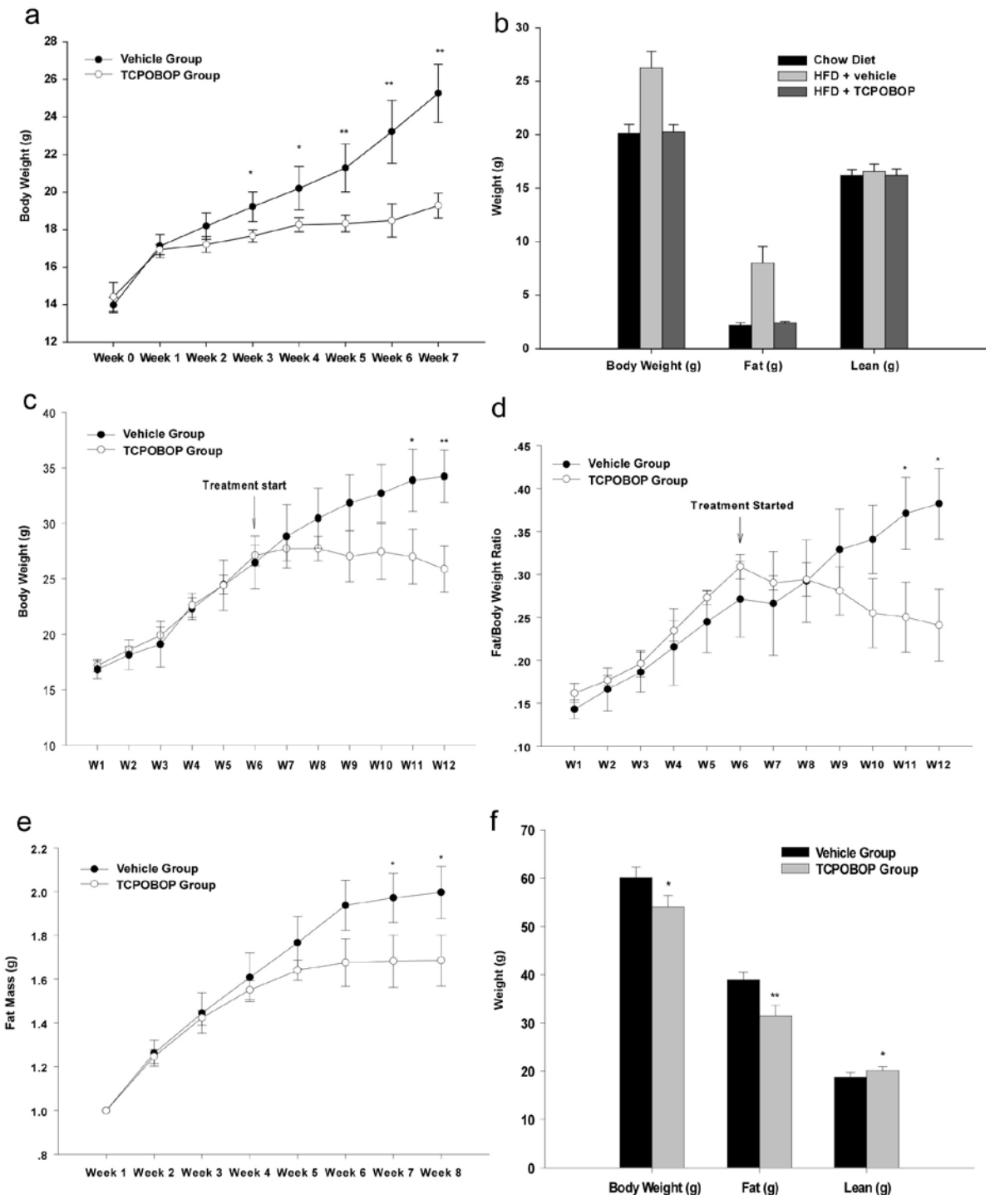

Figure 4. Treatment of mice with CAR agonist reverse obesity in high-fat diet fed mice.

Growth curve (a) Body composition (b) of C57bl/6j female mice fed with HDF with/without TCPOBOP. Body weight (c), fat mass/body weight (d), fat mass (e), body composition of C57bl/6j female mice fed with HDF for 6 weeks, followed by the treatment of TCPOBOP treatment (0.5mg/kg, intraperitoneal, once per week) or vehicle. Fat and lean masses were determined by MRI, n=5 for each group. Results are expressed as means±S.D. *, $p \leq 0.05$; **, $p \leq 0.01$, compared with the vehicle group.

To determine whether CAR activation was effective in mice with preexisting obesity, female C57BL/6J mice were fed with HFD for 6 weeks, followed by 6 weeks of TCPOBOP treatment while remaining on HFD. As shown in Fig. 4c, treatment with TOPOBOP stabilized the body weight, whereas the vehicle group continued to gain body weight (Fig. 4c). Again, the TCPOBOP effect was largely attributed to the inhibition of gain of fat mass (Fig. 4d,e). TCPOBOP was also effective in inhibiting obesity in the leptin deficient *ob/ob* mice. In this experiment, 7-week-old female ob/ob mice were maintained on a chow diet and treated with TCPOBOP or vehicle for 8 weeks. The TCPOBOP group had less gain of fat mass (Fig. 5c). By the end of the 8-week treatment, the TCPOBOP group had significantly lower body weight compared with the vehicle group, accompanied by a decrease in fat mass (Fig. 5d). Interestingly, the TCPOBOP-treated ob/ob group had a modest but statistically significant gain of lean mass (Fig. 5d). Taken together, these results suggest that CAR has an anti-obesity effect.

Table 1. Serum chemistry in HFD-fed C57BL/6J mice and ob/ob mice treated with vehicle or TCPOBOP

	HFD-fed C57BL/6J		**Chow-fed *ob/ob***	
	Vehicle	**TCPOBOP**	**Vehicle**	**TCPOBOP**
Fasting Glucose (mg/dL)	**153±18.3**	**102.8±17.7****	**183.9±21.3**	**128.7±23.4****
Insulin (ng/mL)	**3.26±0.68**	**1.57± 0.37***	**17.89±0.35**	**15.38.9±1.85**
Total Triglyceride (mg/dL)	**230.2±21.3**	**132.8±17.7****	**162.5±14.1**	**126.3±13.7***
Total Cholesterol (mg/dL)	**100.8±9.6**	**89.7±5.3**	**118.4±5.9**	**90.2±9.9***
Free Fatty Acid (mM)	**0.84±0.11**	**0.68±0.06**	**2.59±0.21**	**2.14±0.15**

*The durations of TCPOBOP treatment are 5 and 8 weeks for the C57BL/6J mice and *ob/ob* mice, respectively. Mice were fasted overnight before blood sampling. *, $P<0.05$; **, $P<0.01$

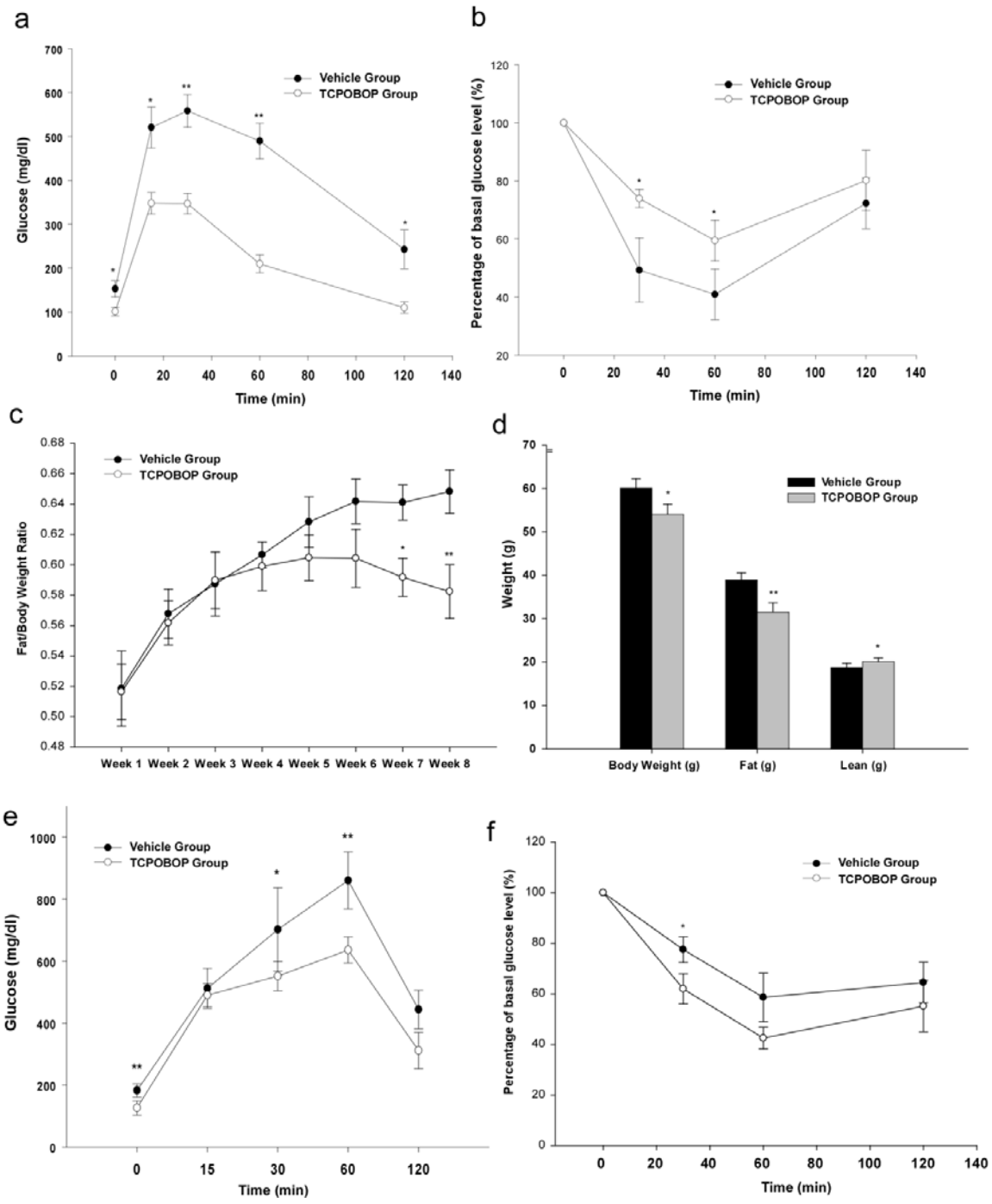

Figure 5. Treatment of mice with CAR agonist ameliorated diabetic phenotype on mice model of Type 2 diabetes.

Treatment with TCPOBOP ameliorated insulin resistance induced by HFD in male C57BL/6J mice, as determined by GTT (a) and ITT (b). Mice were fed with HFD for 8 weeks in the presence or absence of TCPOBOP (0.5 mg/kg, once per week). Body weight (c), body composition (d), GTT (e) and ITT (f) of *ob/ob* mice maintained on chow diet treated with TCPOBOP or vehicle for 8 weeks *, $p \leq 0.05$; **, $p \leq 0.01$,

2.3.2 Activation of CAR Improve Insulin Sensitivity

Because obesity is strongly associated with insulin resistance, a typical characteristic of type 2 diabetes, I further examined the effect of CAR on insulin sensitivity. Treatment of HFD-fed male C57BL/6J mice with TCPOBOP for 8 weeks significantly improved insulin sensitivity, as confirmed by both GTT (Fig. 5a) and ITT (Fig. 5b). The insulin-sensitizing effect of TCPOBOP was also evident in ob/ob mice (Fig. 5e and f), although the changes were consistently smaller than those observed in the C57BL/6J mice. When the serum chemistry was analyzed, I found that treatment with TCPOBOP in HFD-fed C57BL/6J mice decreased the levels of fasting glucose, insulin, and triglycerides (Table 1). In *ob/ob* mice, TCPOBOP treatment decreased the levels of fasting glucose, serum triglyceride, and cholesterol, but the changes in insulin and free fatty acid were not statistically significant (Table 1). Of note, compared with the HFD-fed C57BL/6J mice, ob/ob mice had a much higher basal level of insulin. The TCPOBOP-induced reductions of insulin and triglycerides were smaller in *ob/ob* mice (14 versus 52% and 23 versus 42%, respectively).

In the loss-of-function CAR−/− mice, I found that the chow-fed CAR−/− mice in C57BL/6J-SvJ129 mixed background had spontaneously impaired insulin sensitivity when compared with their wild type counterparts, and the insulin insensitivity in CAR−/− mice was not responsive to TOPOBOP (Fig. 6).

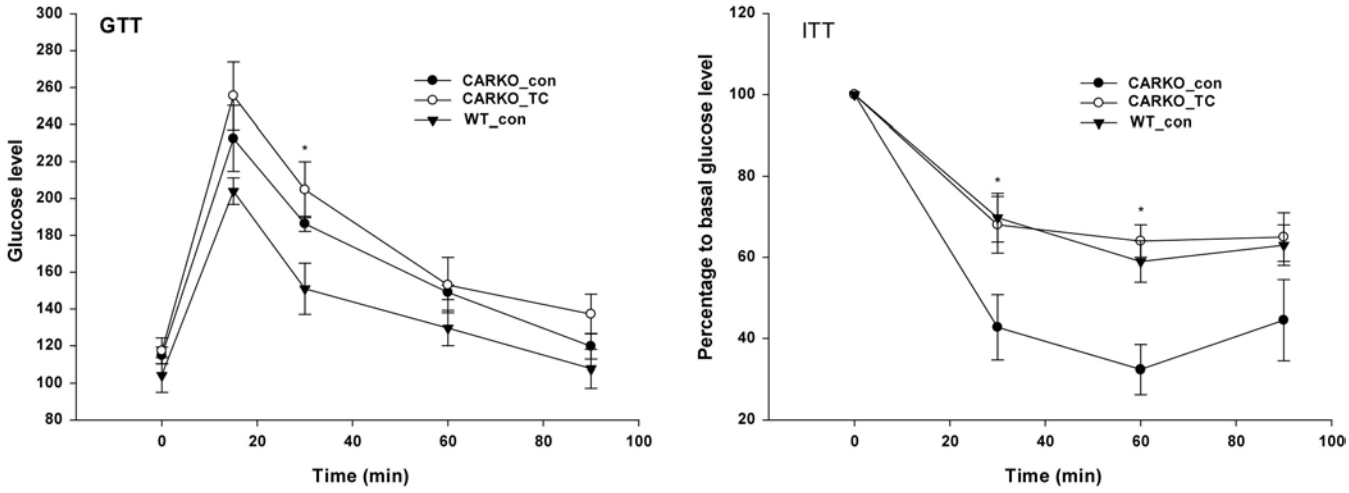

Figure 6. CAR null mice showed spontaneous glucose intolerance and insulin resistance.

GTT (E) and ITT (F) tests on CAR null mice maintained on chow diet in the presence or absence of 5-week TCPOBOP treatment. *, $p \leq 0.05$; **, $p \leq 0.01$,

2.3.3 Activation of CAR Suppressed the Expression of Gluconeogenic and Lipogenic Enzyme Genes, Inhibited Hepatic Steatosis, and Inhibited Adiposity

To understand the mechanism by which CAR inhibits obesity and improves insulin sensitivity, I profiled the expression of metabolic genes in HFD-fed C57BL/6J mice treated with TOPOBOP for 1 week. In the liver, TCPOBOP lowered the expression of lipogenic genes, including Srebp-1c, Acc-1, Fas, and Scd-1 (Fig. 7a). Significant inhibition of Fas and Scd-1 gene expression was also observed in the skeletal muscle (Fig. 7a), brown adipose tissue (BAT) (Fig. 7a), and white adipose tissue (WAT) (Fig. 7a) of TCPOBOP-treated mice. The expression of Acc-1 was also significantly inhibited by TCPOBOP in BAT. A similar pattern of gene expression was observed in mice fed with HFD and treated with TCPOBOP for 8 weeks (data not shown). The inhibition of lipogenesis was supported by the ameliorated hepatic steatosis

(Fig. 7c), decreased sizes of abdominal WAT and BAT (Fig. 7b), and adipocyte hypotrophy (Fig. 8a and b) in HFD-fed mice treated with TCPOBOP for 8 weeks. Treatment with TCPOBOP also inhibited VLDL-triglyceride secretion (Fig. 8c), consistent with the reduced serum triglyceride level in these mice (Table 1). In TCPOBOP-treated mice, the hepatic expression of two important gluconeogenic enzymes, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, was also significantly inhibited (Fig. 8d), consistent with the decreased fasting glucose in these animals (Table 1). The effect of TCPOBOP on lipogenic and gluconeogenic gene expression was CAR-dependent, because this regulation was abolished in ~~C/AR~~ mice (Fig. 9), consistent with a previous report (Ueda et al. 2002).

2.3.4 Activation of CAR Had Little Effect on Lipid Absorption in the Small Intestine

Since CAR is highly expressed in the small intestine, I went on to determine whether activation of CAR affected lipid digestion and absorption in this tissue. Feces were collected by using metabolic cages over a 24-h period in parallel with food intake measurement. As shown in Fig. 5, neither fecal weight (Fig. 10a) nor fecal triglyceride content (Fig. 10a) was affected by TCPOBOP treatment. Moreover, the intestinal expression of genes involved in lipid absorption, synthesis, assembly, and secretion was not significantly affected by TCPOBOP (Fig. 10b). Genes evaluated included the fatty acid translocase Cd36, fatty acid-binding proteins Fabp1 and Fabp6, and the microsomal triglyceride transport protein. The intestinal expression of Cyp2b10 was induced by TCPOBOP, as expected. These results suggest that activation of CAR may not have a major effect on fat absorption and metabolism in the intestine.

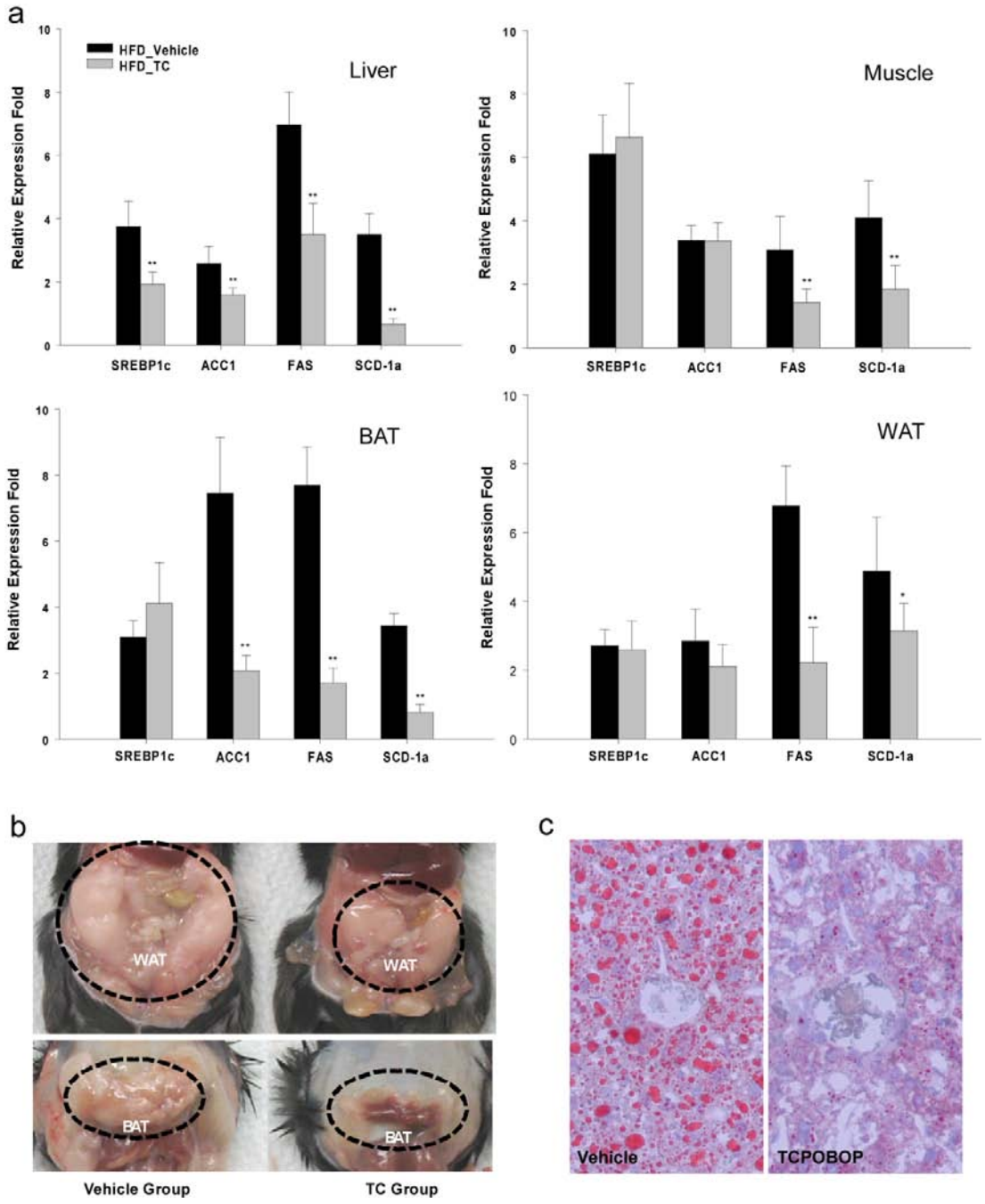

Figure 7. Treatment of a CAR agonist suppresses lipogenesis and hepatic steatosis.

(a) Liver, skeletal muscle, BAT, and WAT were measured for the mRNA expression of genes involved in lipogenesis by real time PCR analysis. Male C57BL/6J mice were HFD-fed and drug-treated for 1 week. n=4 for each group. (b) Gross appearance and (c) Oil-red O staining of liver sections from male C57BL/6J mice fed with HFD and treated with TCPOBOP for 8 weeks.. *, $p \leq 0.05$; **, $p \leq 0.01$,

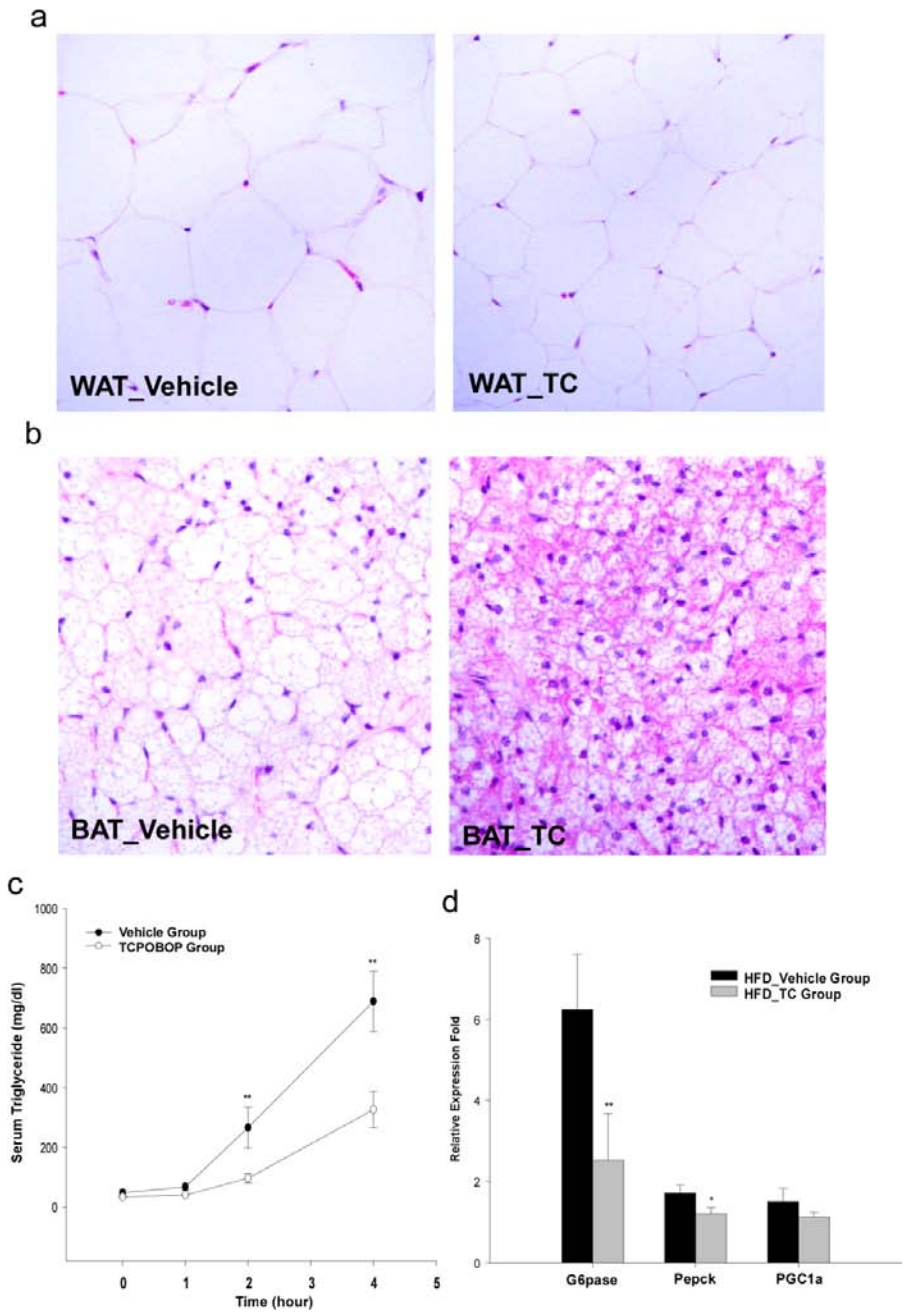

Figure 8. Treatment of a CAR agonist suppresses adiposity gain, hepatic VLDL secretion and gluconeogenesis.

hematoxylin/eosin staining of abdominal WAT (a) and BAT (b) of mice in high-fat diet for 8 weeks, (c) VLDL-triglyceride secretion rate in male C57BL/6J mice treated with vehicle or TCPOBOP for 8 weeks. (d) Liver mRNA expression of genes involved in gluconeogenesis was measured by real time PCR analysis.*, $p \leq 0.05$; **, $p \leq 0.01$.

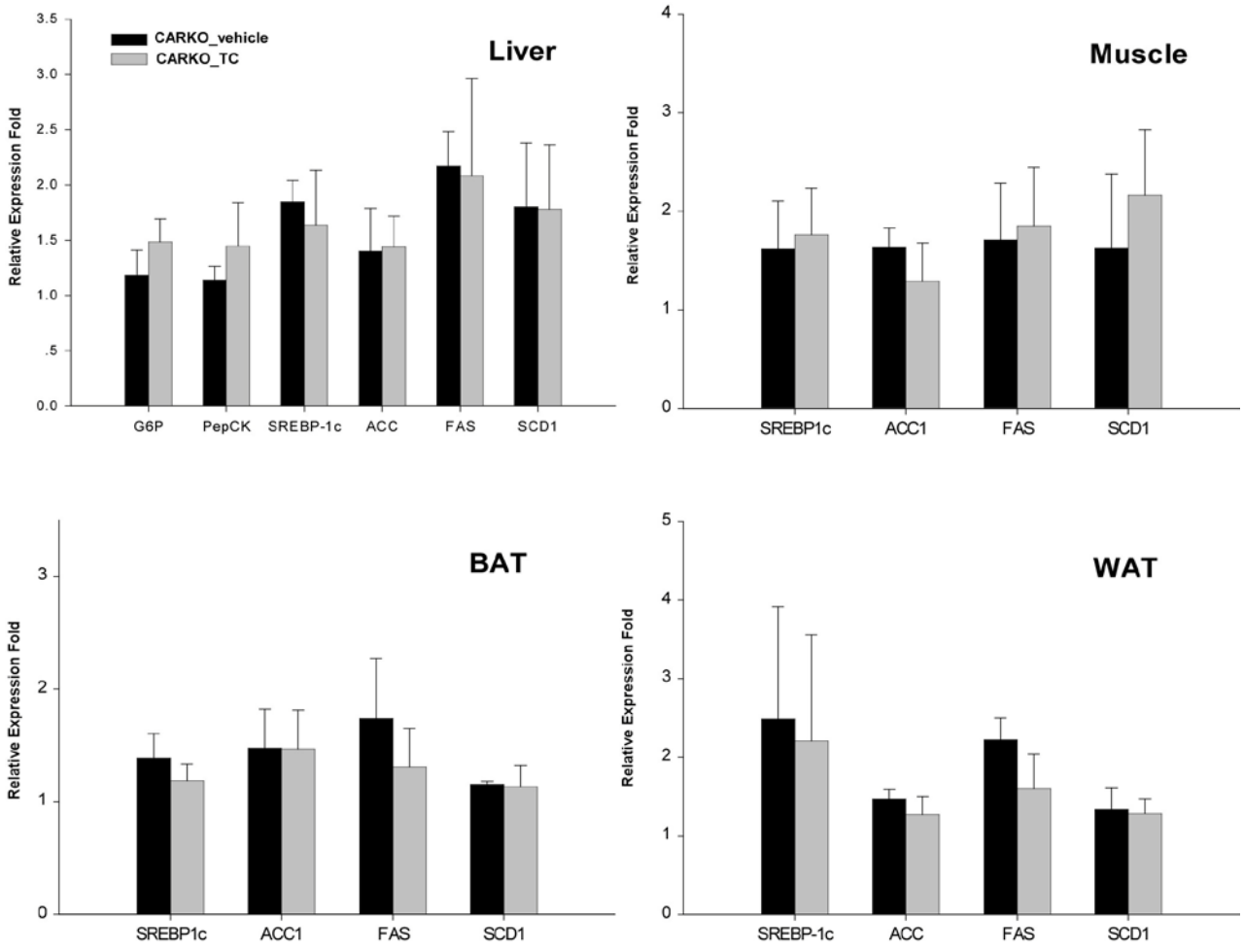

Figure 9. The suppression of lipogenic and gluconeogenic gene expression by TCPOBOP was abolished in CAR null mice.

Male CAR null mice were HFD-fed and TOPOBOP-treated for 1 week before tissue harvesting. liver , skeletal muscle , BAT , and WAT were measured for the mRNA expression of lipogenic and gluconeogenic genes by real time PCR analysis. n=5 for each group.

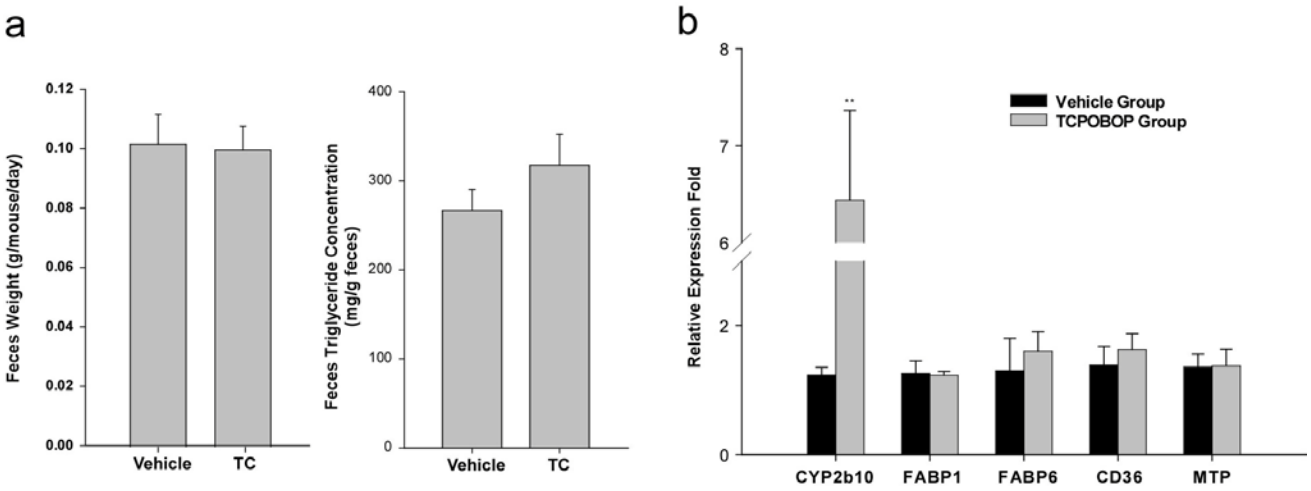

Figure 10. Activation of CAR had little effect on lipid absorption in the small intestine.

Male C57BL/6J mice were HFD-fed and TOPOBOP-treated for 8 weeks. Fecal output and fecal concentration of triglyceride (a). hepatic gene expression (b) as measured by real time PCR. *, $p \leq 0.05$; **, $p \leq 0.01$ compared with the vehicle group.

2.3.5 Activation of CAR Prevented Fatty Acid Overloading and Incomplete β-Oxidation in Skeletal Muscle

Obesity-related insulin resistance in skeletal muscle is characterized by excessive fatty acid β-oxidation and impaired switch to carbohydrate substrate during the fasted-to-fed transition (Koves et al. 2008). When the expression of genes involved in fatty acid oxidation was analyzed, I found that an 8-week treatment of TCPOBOP significantly suppressed the expression of PPARα and its target genes involved in β-oxidation and ketogenesis in both the liver (Fig. 11a) and skeletal muscle (Fig. 11b). These include the long-chain acyl-CoA dehydrogenase, medium-chain acyl CoA dehydrogenase, and HMG-CoA synthase (HMGCS2). Interestingly, the expression of β-oxidation genes was largely unaffected in mice treated with TCPOBOP for only 1 week, although the expression of PPARα in the liver was significantly suppressed. When the

mitochondrial β-oxidation rate was measured in the gastrocnemius muscle of HFD-fed mice, I found that mice treated with TCPOBOP had a modest but significant decrease in the rate of [^{14}C]oleate oxidation to CO_2, an indicator of complete β-oxidation (Fig. 11c). In contrast, TCPOBOP treatment resulted in a more dramatic reduction in the accumulation of radiolabeled intermediates in the acid-soluble metabolite (ASM), an indicator of incomplete β-oxidation (Fig. 11d), suggesting that TCPOBOP-treated mice had reduced incomplete β-oxidation. When pyruvate was added to the incubation buffer as a competing carbon source to induce a substrate switch from fatty acid to carbohydrate (Randle et al. 1963), vehicle-treated mice failed to exhibit a significant substrate switch, which was suggestive of insulin resistance. In contrast, TCPOBOP-treated mice showed a significant switch by having decreased CO2 formation (Fig. 11c). The TCPOBOP group also showed a modest but statistically significant decrease in incomplete β-oxidation (Fig. 11d), which is suggestive of improved insulin sensitivity.

2.3.6 Activation of CAR Increased BAT Energy Expenditure and Promoted Peripheral Fat Mobilization

BAT plays an important role in thermogenesis. As shown in Fig. 12a, TCPOBOP treatment significantly increased BAT expression of Pgc-1α, muscle-type carnitine palmitoyltransferase I, long-chain acyl coenzyme A dehydrogenase, thyroid hormone-activating type 2 iodothyronine deiodinase, and uncoupling protein-1, -2, and -3. Mice treated with TCPOBOP also had increased oxygen consumption (Fig. 12b), supporting the notion of increased energy expenditure adapted to a high fat diet. Interestingly, the rectal temperatures between the vehicle and TCPOBOP groups were not significantly different (data not shown).

When the WAT was analyzed, I found that the expression of adipose triglyceride lipase but not the hormone-sensitive lipase, was induced in WAT of TCPOBOP-treated mice (Fig. 12c), suggesting that an increased peripheral fat mobilization may have also contributed to the decreased fat mass and adipocyte hypotrophy.

Among other metabolic changes, 8-week treatment of TCPOBOP under HFD conditions modestly but significantly suppressed the food intake (Fig. 12d, left). However, the food intake was unchanged when normalized against the body weight (Fig. 12d, right). TCPOBOP treatment also had little effect on the food intake in chow-fed ob/ob mice (Fig. 12e). Analysis of the expression of food intake-related neuropeptides in the hypothalamus showed decreased expression of agouti-related peptide (Fig. 12f), a neuropeptide that increases appetite and decreases metabolism and energy expenditure (Ollmann et al. 1997). The expression of neuropeptide Y, pro-opiomelanocortin, and SOCS3 (suppressor of cytokine signaling 3), three hypothalamic genes also known to influence appetite and/or metabolism (Hanson et al. 1995; Fan et al. 1997; Bjorbaek et al. 1998), was unchanged. It is worthy of notice that the control group has a higher hypothalamic agouti-related peptide expression, even in the condition of high serum leptin level, which indicates that the mice subjected to HFD gradually developed leptin resistance and hyperphagia, whereas TCPOBOP treatment significantly preserved the leptin sensitivity (Korner et al. 2001).

2.3.7 Activation of CAR Had Little Effect on Endoplasmic Reticulum (ER) Stress in the Liver

ER stress has been shown to contribute to the pathogenesis of metabolic dysfunction and hepatic steatosis. In an effort to determine whether activation of CAR impacts ER stress, I

showed that treatment of HFD-fed wild type mice (Fig. 13a) or chow-fed ob/ob mice (Fig. 13b) with TCPOBOP had no significant effect on the expression of several ER stress marker genes, such as the G protein-coupled receptors Grp78 and Grp94, and Xbp1 (X-box-binding protein 1) (Ozcan et al. 2004). Among other detoxifying genes, the expression of Ugt1a1, a CAR target gene (Sugatani et al. 2001), was induced as expected, whereas the expression of HMG-CoA reductase was not significantly affected. These results suggest that the suppression of ER stress may not be a major mechanism by which CAR inhibits obesity and improves insulin sensitivity.

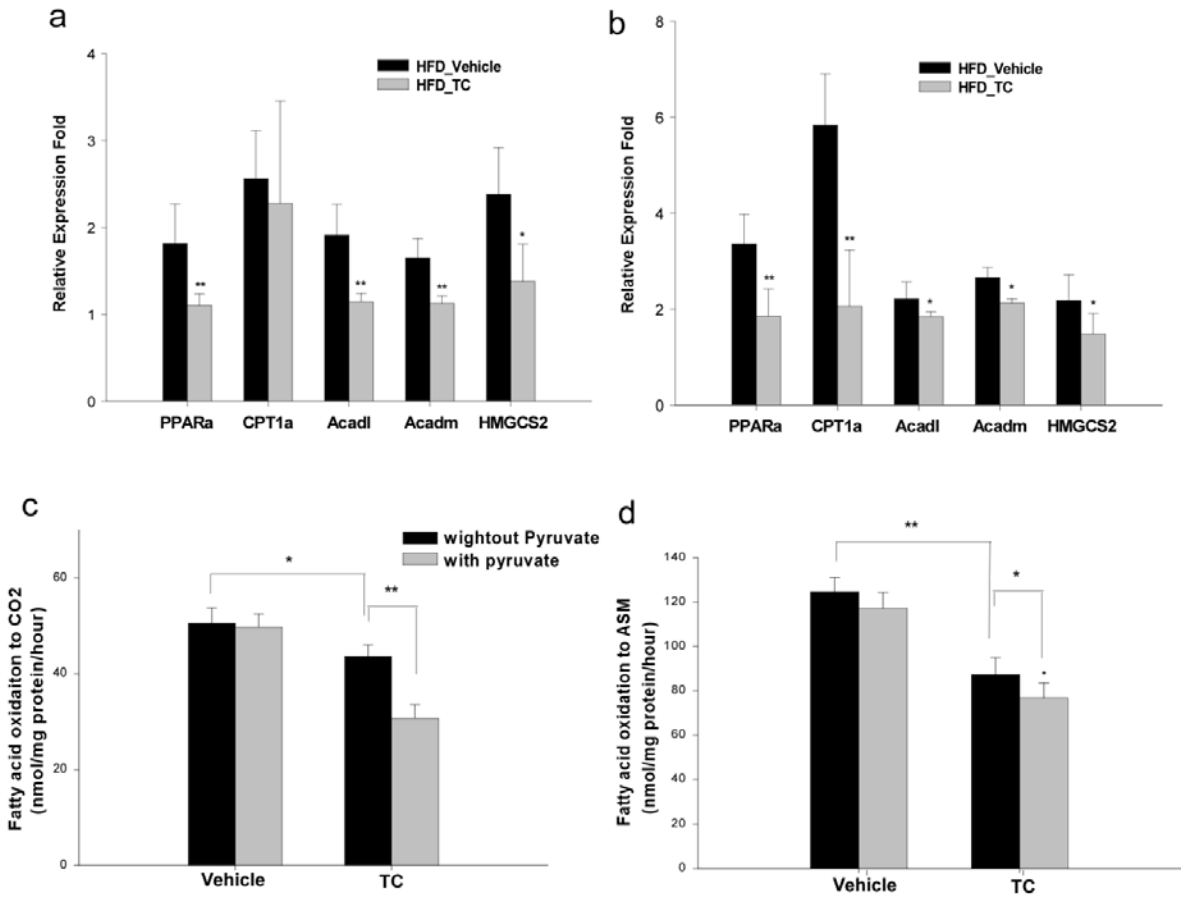

Figure 11. Activation of CAR prevented fatty acid overloading in the skeletal muscle.

Liver (a) and skeletal muscle (b) mRNA expression of genes involved in β-oxidation. Male C57BL/6J mice were HFD-fed and TOPOBOP-treated for 8 weeks. n = 4 for each group. Complete oxidation of [^{14}C]oleate to CO_2 (c) or incomplete oxidation of [^{14}C]oleate to acid-soluble metabolite (ASM) (d) in mitochondria isolated from the gastrocnemius muscle, in the absence or presence of pyruvate. *, $p \leq 0.05$; **, $p \leq 0.01$ compared with the vehicle group.

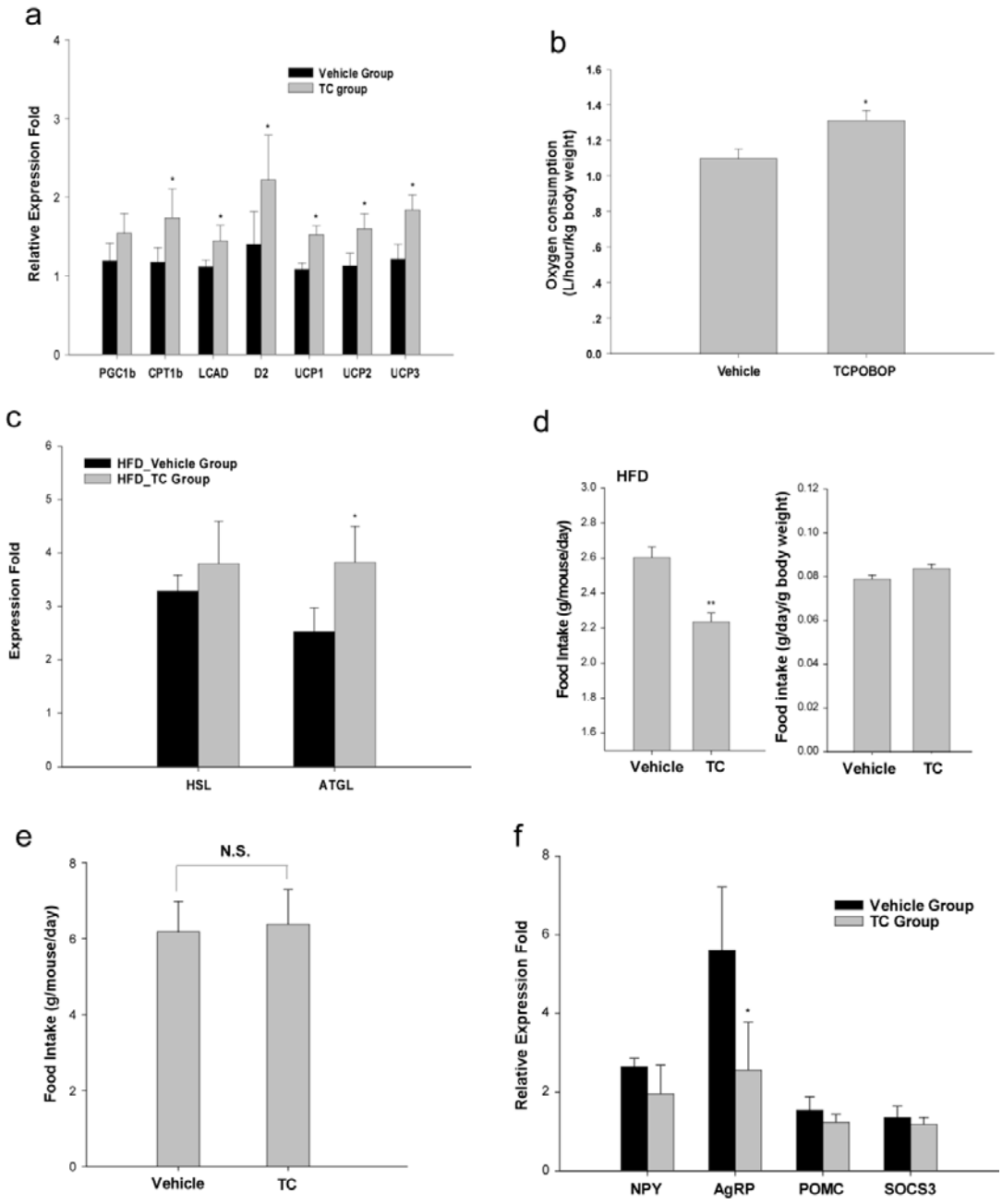

Figure 12. Activation of CAR increased BAT energy expenditure and promoted peripheral fat mobilization.

(a) BAT mRNA expression of genes involved in energy expenditure and thermogenesis. Male C57BL/6J mice were HFD-fed and TOPOBOP-treated for 1 week. n = 4 for each group. (b) oxygen consumption in male C57BL/6J mice HFD-fed and TOPOBOP-treated for 5 weeks. n = 4 for each group. (c) WAT mRNA expression of lipase genes. (d) Food intake without (left) or with (right) normalization against the body weight. Mice are the same as those described in B. (e) Food intake of ob/ob mice maintained on a chow diet and treated with vehicle or TCPOBOP for 8 weeks. n=5 for each group. (f) Hypothalamic mRNA expression of neuropeptides. *, $p \leq 0.05$; **, $p \leq 0.01$ compared with the vehicle group.

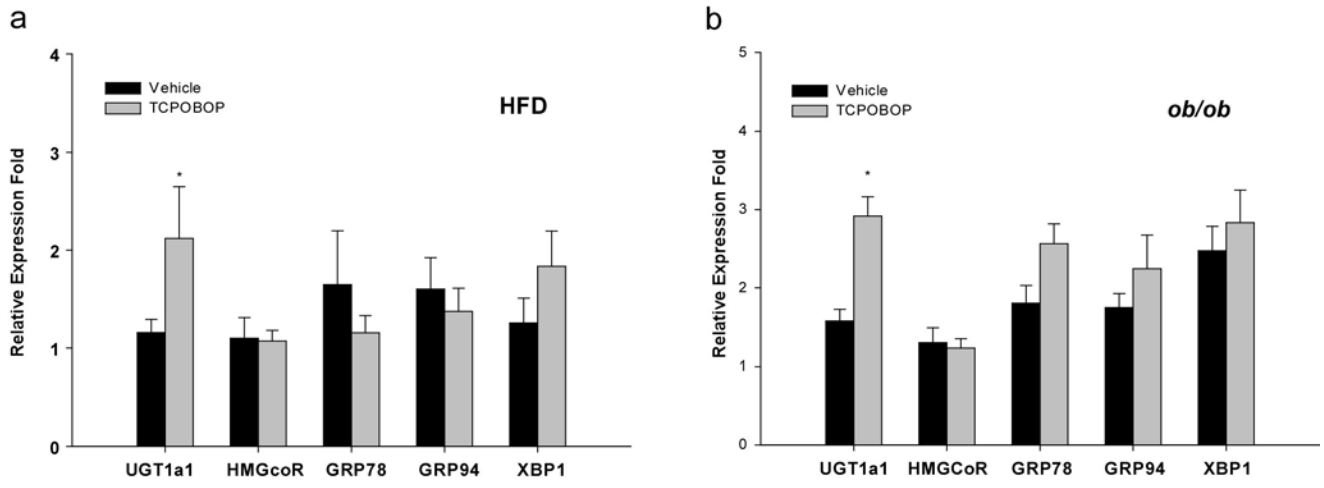

Figure 13. Activation of CAR had little effect on ER stress in the liver.

Liver mRNA expression of genes involved in ER stress as determined by real time PCR analysis. (a) male C57BL/6J mice were HFD-fed and TOPOBOP-treated for 8 weeks. n = 4 for each group. (b) *ob/ob* mice maintained on a chow diet and treated with vehicle or TCPOBOP for 8 weeks. n =5 for each group. *, $p \leq 0.05$; **, $p \leq 0.01$ compared with the vehicle group.

2.3.8 CAR Interacts with PGC-1α and Inhibits Its Transcriptional Activity

In the process of further investigating the molecular mechanism through which CAR activation suppresses hepatic gluconeogensis, I hypothesized that CAR activation can inhibit the transcriptional activity of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α), the key transcriptional coactivator controlling hepatic gluconeogenic genes induction during fasting and diabetic conditions (Finck et al. 2006). Consistent with my hypothesis, I found CAR and PGC-1α can interact with each other and immunoprecipitated together when co-expressed in HEK293 cell line (Fig. 14a and b). In G6pase reporter assay, CAR dose dependently inhibited the transcriptional activity of PGC-1α and the suppression effect can be further enhanced in the presence of CAR agonist TCPOBOP (Fig. 14c). The suppression effect

of CAR on the transcriptional activity of PGC-1α is unique among several nuclear receptors which are known to interact with PGC-1α (Fig. 14d) (Puigserver et al. 2003). The suppression effect of CAR and its specificity on transcriptional activity of PGC-1α had also been confirmed in Gal4 reporter system and peroxisome proliferator-activated receptor response element (PPRE) reporter system (Fig. 14f). Adenovirus vectors expressing HA-tagged PGC-1α and Flag-tagged CAR were constructed for further evaluating the suppression effect of CAR on PGC-1α in mouse primary hepatocyte, an more physiologically relevant setting. The transduction efficiency and the expression of target proteins were confirmed by immunofluorescence and Western blot (Fig. 15). Primary hepatocytes were treated with forsklin, a chemical known to raise cellular cAMP level, for 2.5 hours to induce the expression of gluconeogenic genes Pepck and G6pase. In the presence of CAR agonist TCPOBOP, the induction of gluconeogenic genes by forsklin was significantly compromised (Fig. 16a), which is consistence with previous reports that PGC-1α serves as a coactivator of HNF-4α, and FoxO1 to induce hepatic gluconeogenesis in response to increased cellular cAMP level (Herzig et al. 2001; Puigserver et al. 2003). The suppression effect of TCPOBOP on gluconeogenic genes is CAR dependent, and TCPOBOP displayed no such suppression effect on the primary hepatocytes isolated from CAR null mice (Fig. 16b). When CAR was reconstituted in the CAR null primary hepatocytes, TCPOBOP regained the suppression effect on gluconeogenic genes upon forsklin challenge (Fig. 16c). Adenovirus mediated PGC-1α overexpression alone was capable of mimicking full effect of forsklin treatment on the induction of gluconeogenic genes as previous reported (Herzig et al. 2001), and CAR co-expression can significantly suppress the induction of gluconeogenic genes by PGC-1α (Fig. 17a and b). Interestingly, CAR had little effects on the expression of other PGC-1α target genes such as CycS and Cox4i, two mitochondria genes involved into ATP production (Fig. 17c

and d) (Puigserver et al. 2003). Consistent with the suppression effect on gluconeogenic genes, CAR over-expression significantly inhibited glucose output from the primary hepatocyte at basal condition or when PGC-1α was over-expressed (Fig. 17e).

2.3.9 CAR Inhibits Transcriptional Activity of PGC-1α *via* Sequestrating It into PML Nuclear Body.

To explain the underlying mechanism through which CAR suppressed transcriptional activity of PGC-1α, I found CAR, when co-expressed, induced sub-nuclear redistribution of PGC-1α into nuclear loci, where CAR and PGC-1α are co-localized (Fig. 18a). The effect of inducing protein redistribution is mutual between PGC-1α and CAR, and both are evenly distributed over the nucleus when expressed individually (Fig. 18b). The nature of the nuclear loci where both PGC-1α and CAR localized were identified as promyelocytic leukemia (PML) nuclear body, which was demonstrated by the co-immunofluorescence staining of PML with PGC-1α in the present of CAR (Fig. 19 a and b).

Finally, to demonstrate that the suppression effect of CAR on PGC-1α is conserved between human and mice. I tested the suppression effect of human CAR agonist CITCO on gluconeogenic genes in primary hepatocytes. Similar to what I observed in mouse primary hepatocytes, CITCO significantly suppressed forsklin-induced gluconeogenic genes expression in three cases of human primary hepatocyte (Fig. 20).

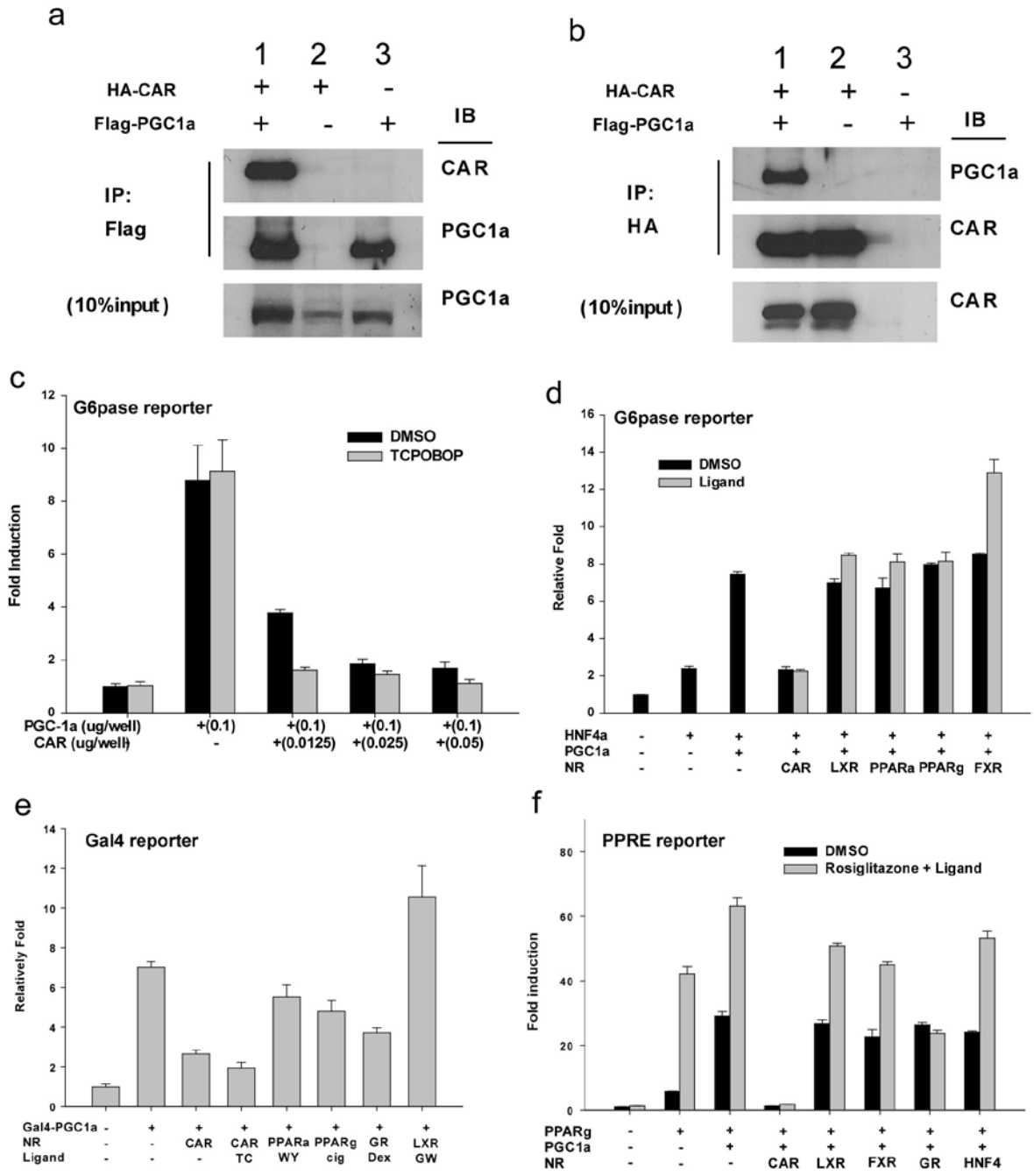

Figure 14. CAR interacts with PGC-1α and inhibits its transcriptional activity.

HA-tagged CAR or Flag-tagged PGC-1α are expressed in HEK293 cell, and immunoprecipitated with anti-Flag (a) or anti-HA (b) and immunoblotted with anti-PGC-1α or anti-CAR antibody. CAR dose dependent inhibits PGC-1α transcriptional activity in G6pase reporter (c), and the suppression effect is specific among several nuclear receptors (d). Assay are performed in triplicates *, $p \leq 0.05$; **, $p \leq 0.01$ compared with the vehicle group.

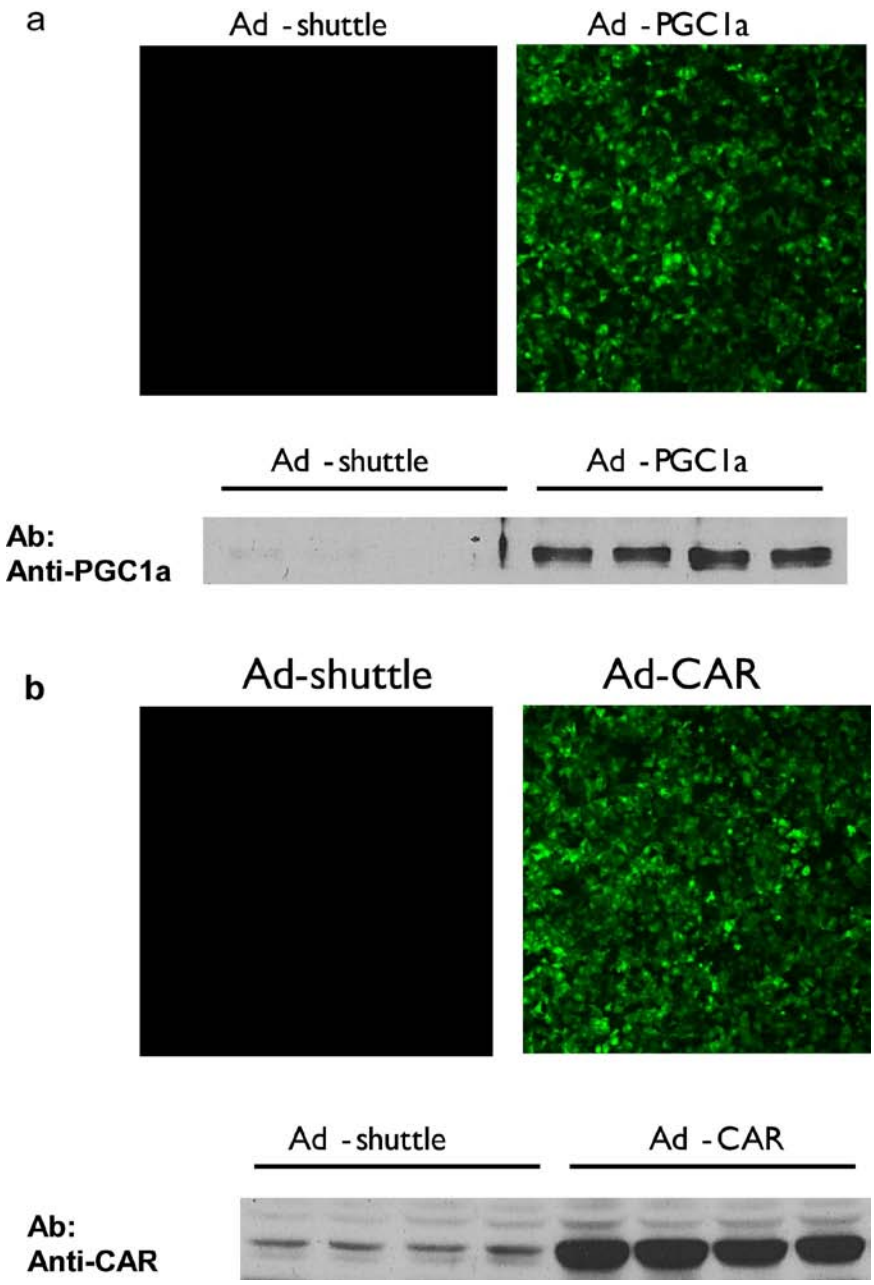

Figure 15. Adenovirus mediated expression of PGC-1α and CAR .

Adenovirus vector expressing PGC-1α (a) and CAR (b) are produced to infect primary mouse hepatocytes. Image showed the transduction efficiency 24 hours after virus infection.

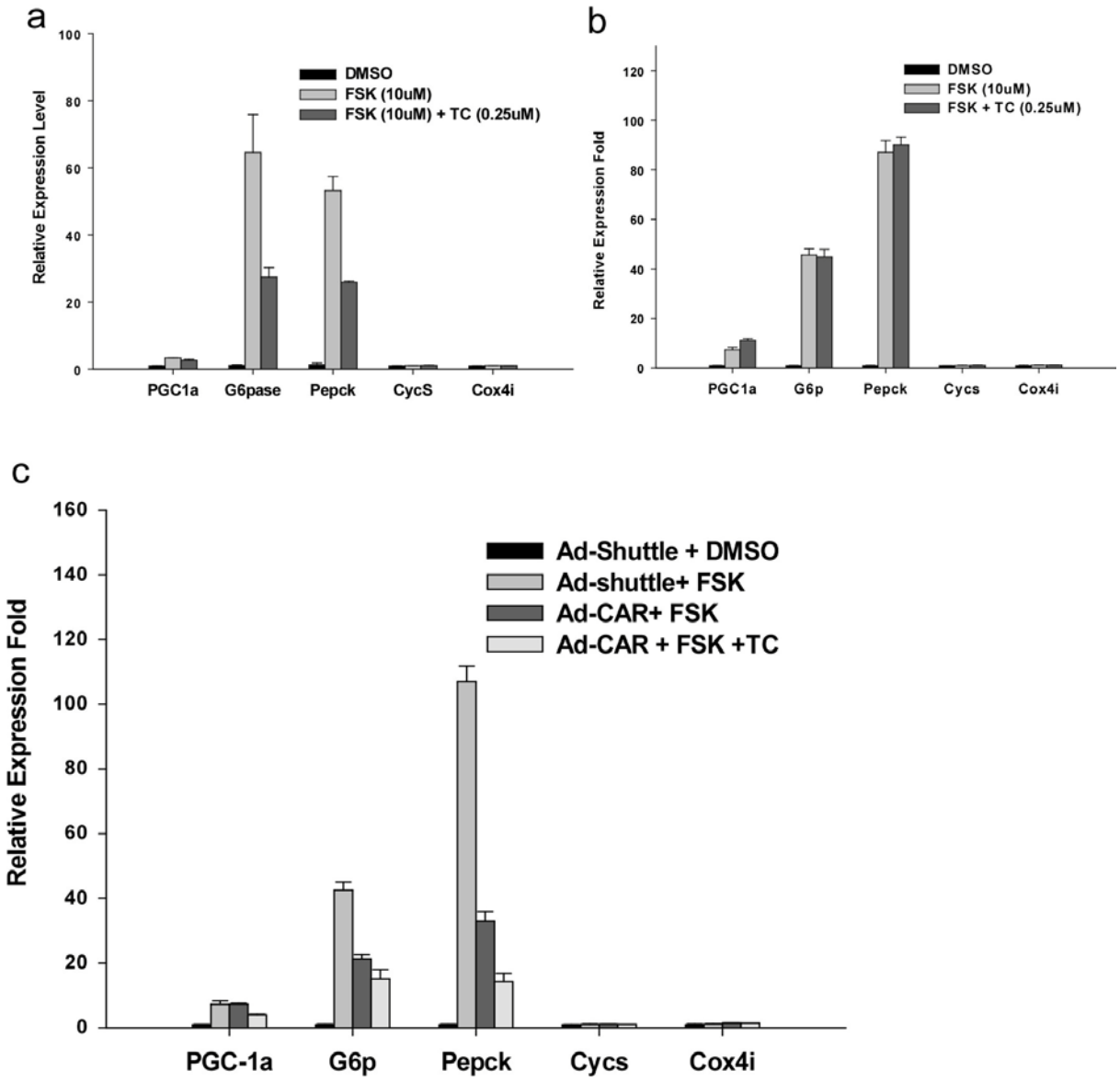

Figure 16. CAR activation inhibited PGC-1α transcriptional activity in mouse primary hepatocytes.

TCPOBOP (250 nM) treatment inhibits gluconeogenic genes Pepck and G6pase in primary hepatocytes (a), while the effect was abolished in the primary hepatocytes from CAR null mice, and reconstituted CAR expression in the primary hepatocytes from CAR null mice restore the suppression effect of TCPOBOP on PGC-1α (c). Gene expression was measured 48 hours after virus infection.

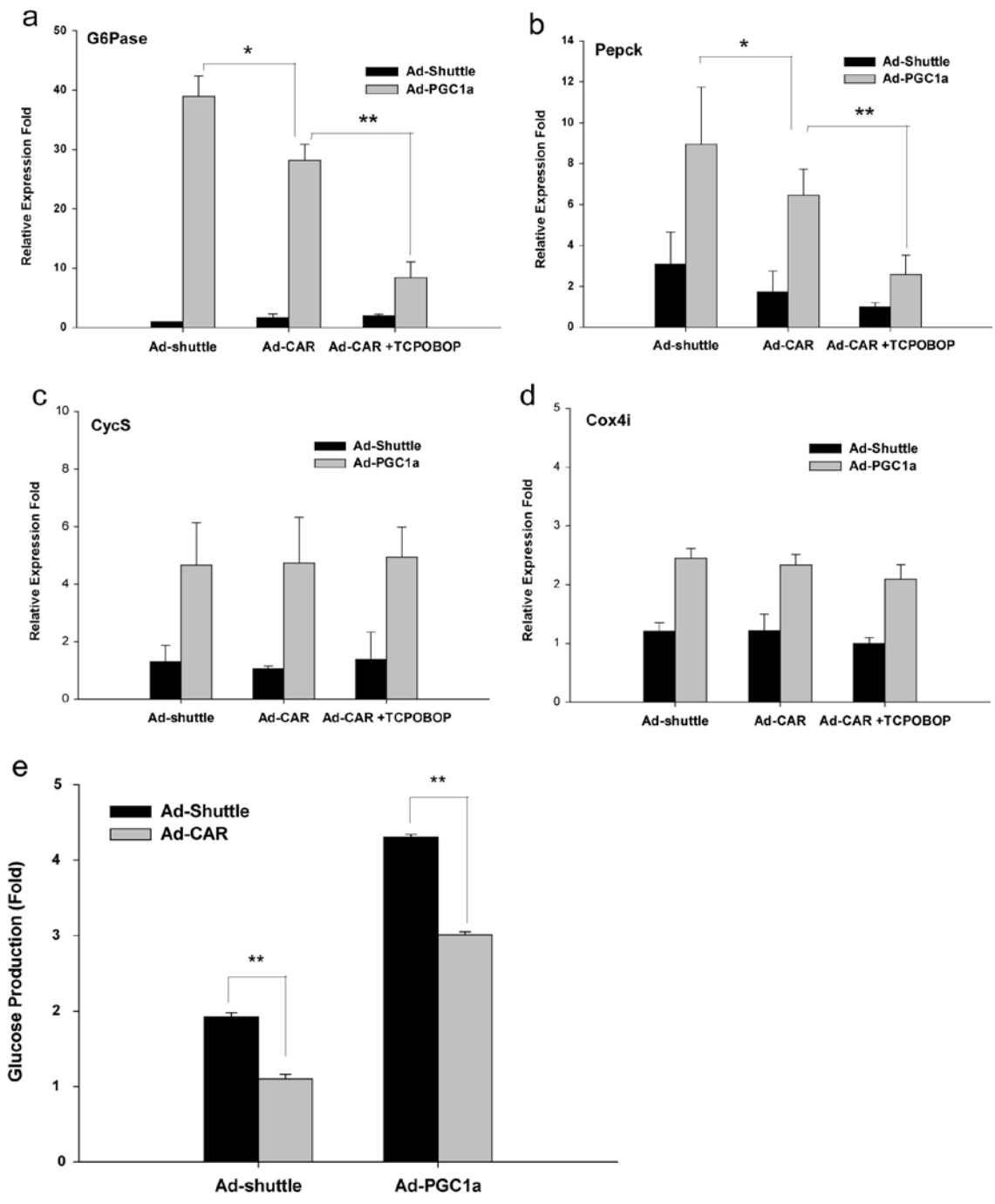

Figure 17. CAR overexpression suppressed PGC-1α induced gluconeogenic gene expression and glucose output.

Adenovirus mediated CAR overexpression suppressed gluconeogenic genes G6pase (a) and Pepck (b) expression in primary hepatocytes induced by PGC-1α, while the expression of mitochondria oxidation genes CycS (c) and Cox4i (d) was not affected by CAR overexpression. (e) CAR overexpression suppressed PGC-1α induced hepatocyte glucose output.

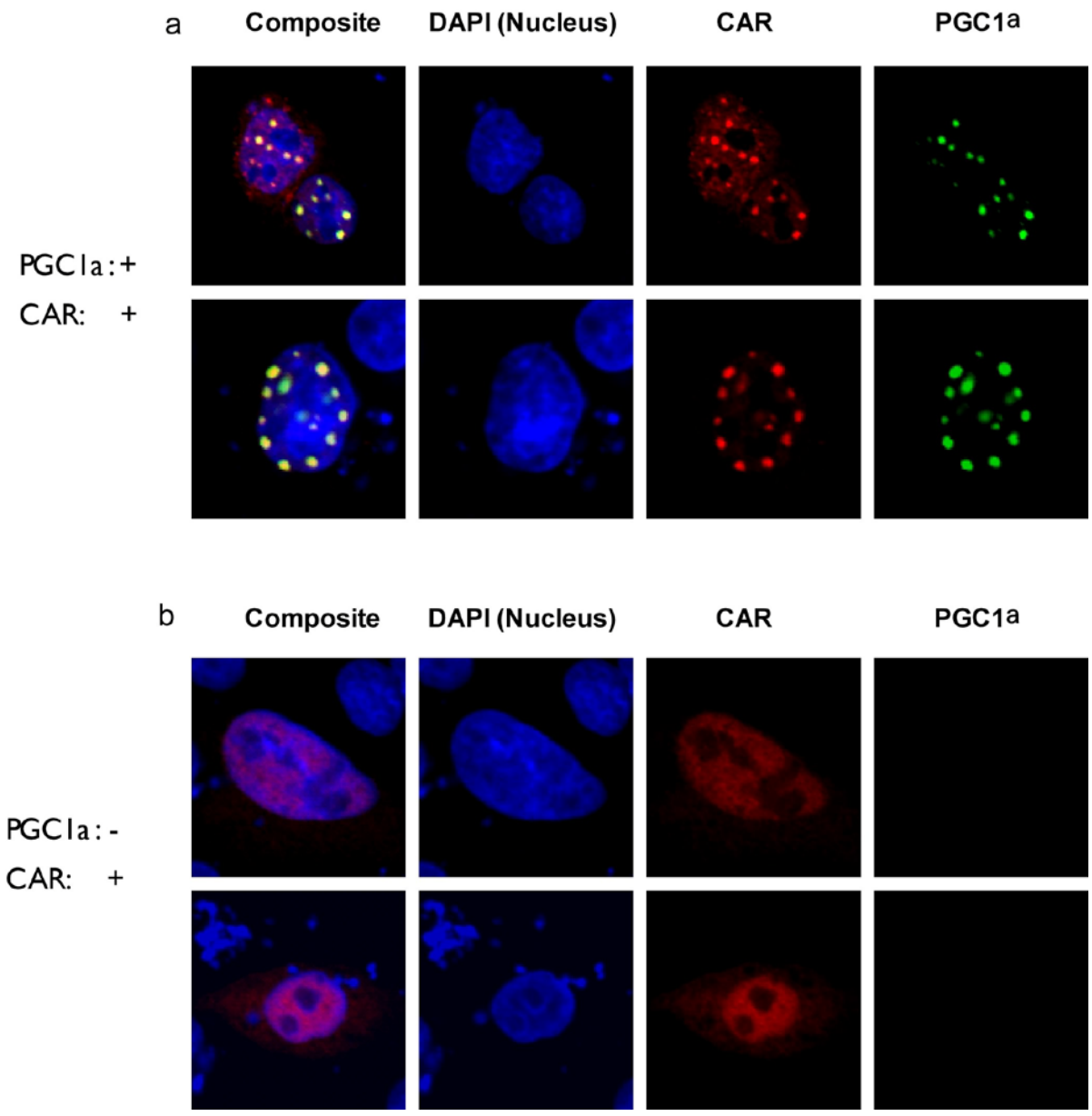

Figure 18. CAR led to sub-nuclear redistribution of PGC-1α in mouse primary hepatocytes.

Adenovirus mediated CAR and/or PGC-1α overexpression in mouse primary hepatocytes. PGC-1α and CAR are immunostained with (a) or without (b) PGC-1α transduction *via* adenovirus.

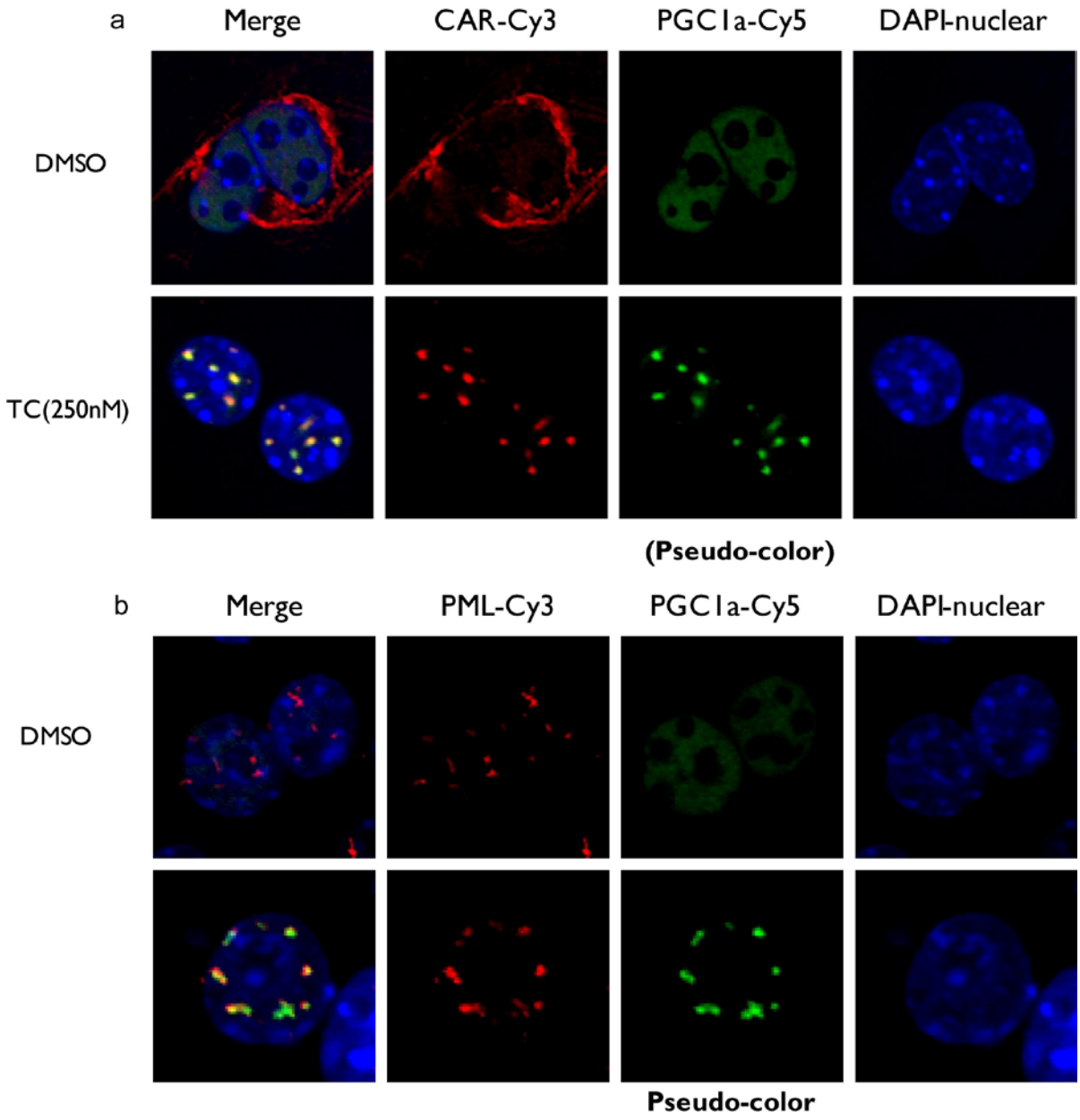

Figure 19. CAR led to sub-nuclear redistribution of PGC-1α into PML nuclear body in mouse primary hepatocytes.

Adenovirus mediated CAR and/or PGC-1α overexpression in mouse primary hepatocytes. PGC-1α and CAR are immunostained (a) or PGC-1α and PML are immunostained 48 hours after virus transduction.

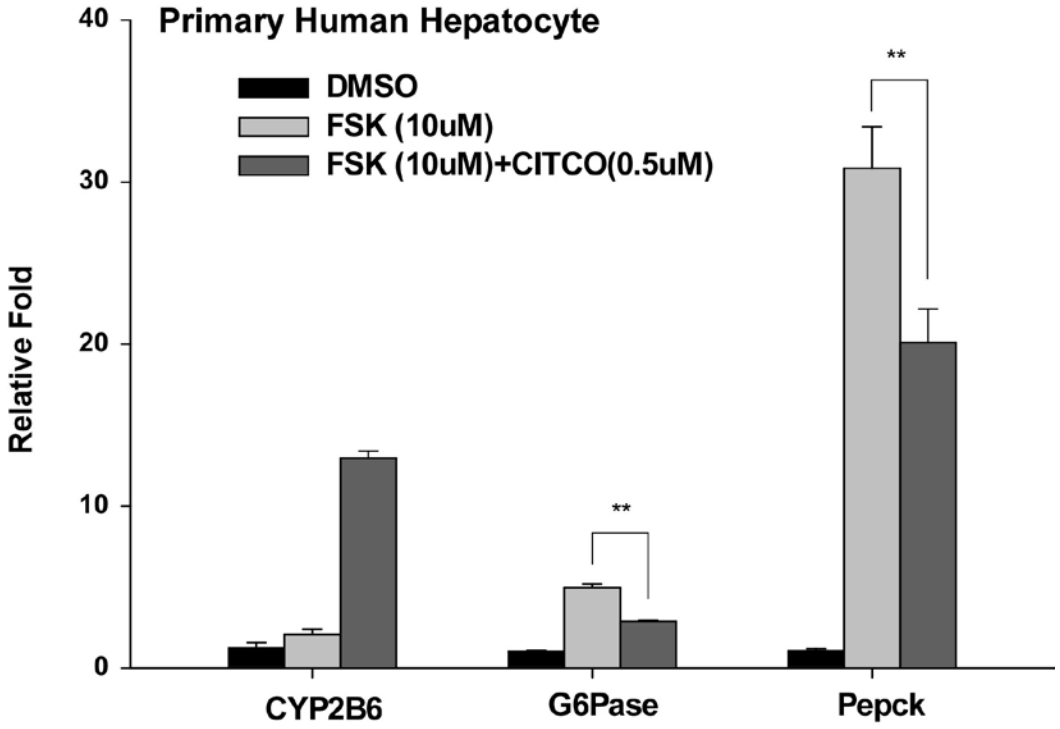

Figure 20. CAR agonist treatment suppressed gluconeogenic gene expression in human primary hepatocytes.

mRNA expression of genes involved in gluconeogenesis was measured by real-time PCR analysis 2.5 hours after FSK treatment in the presence or absence of TCPOBOP treatment. *, $p \leq 0.05$; **, $p \leq 0.01$.

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2.4 DISCUSSION

Patients of obesity and diabetes often suffer from both hyperglycemia and hyperlipidemia. Although the inhibition of gluconeogenesis and lipogenesis has been suggested largely through the gene expression analysis (Ueda et al. 2002; Roth et al. 2008), whether the activation of CAR is beneficial in relieving obesity and diabetes has not been reported. In the

current study, I showed that CAR was effective in preventing obesity and alleviating type 2 diabetes. The metabolic benefits of CAR may have resulted from the combined effect of inhibition of lipogenesis, VLDL secretion and export of triglycerides, and gluconeogenesis as well as increases in BAT energy expenditure and peripheral fat mobilization.

It is interesting to note that treatment with the CAR agonist inhibited lipogenic gene expression not only in the liver, but also in the WAT, BAT, and skeletal muscle. Among lipogenic enzymes, it has been reported that mice deficient in Acc-2 or Scd-1 were protected from obesity and hepatic steatosis induced by HFD (Miyazaki et al. 2007). Inhibition of Fas was also shown to inhibit food intake and prevent obesity (Loftus et al. 2000). Thus, I reason that the inhibition of lipogenesis may have played an important role in the anti-obesity effect of CAR.

The pleiotropic effect of CAR on energy metabolism revealed in my study is rather intriguing. My preliminary studies showed that the skeletal muscle, hypothalamus, and pituitary gland have a low but appreciable expression of CAR, whereas the adipose tissues do not express CAR (data not shown). It remains to be determined whether the phenotypes in gene regulation and fatty acid oxidation in the skeletal muscle were mediated by the local CAR effect. Whether and how the CAR activation in the hypothalamus and pituitary gland affects food intake also remain to be determined. The future creation of tissue-specific CAR knock-out or CAR transgenic mice will help to shed light on the extrahepatic function of CAR in energy metabolism. I also cannot exclude the possibility that the hypothalamic and pituitary function of CAR may have contributed to the hepatic phenotype. Such brain-liver communications have been reported. The lack of CAR expression in the adipose tissues suggests that the inhibition of lipogenesis, increased BAT energy expenditure, and activation of adipose triglyceride lipase

gene expression in WAT were probably secondary to CAR activation in tissues outside of the adipose tissues.

Recent studies have linked muscle insulin resistance with fatty acid overload and the consequent incomplete fatty acid β-oxidation. In the presence of HFD, skeletal muscles undergo transcriptional remodeling adapting to an increased supply of lipid substrates. However, in the absence of exercise, the tricarboxylic acid cycle remains inactivated at the transcriptional level. As a result, although the complete fat oxidation is decreased or remains unchanged, the rate of incomplete oxidation rises significantly, leading to "mitochondrial stress" and insulin resistance (Kraegen et al. 2008). Accordingly, inhibition of β-oxidation and fatty acid influx by using CPT1 inhibitors has been shown to improve insulin sensitivity (Koves et al. 2008). In the current study, I showed that activation of CAR inhibited the expression of PPARα and its target genes involved in β-oxidation and fatty acid influx, thus reducing incomplete fatty acid β-oxidation of HFD-fed mice. The mechanism by which CAR suppresses the expression of PPARα and its target β-oxidation genes remains to be defined. I showed that a short term (1-week) treatment of TCPOBOP did not affect the expression of many of the β-oxidation genes. It is possible that the inhibition of PPARα and its target gene expression is secondary to the reduced plasma triglyceride and free fatty acid levels in TCPOBOP-treated mice. It has been reported that PPARα knock-out mice were resistant to HFD-induced diabetes, and dexamethasone induction of hypertension and diabetes was PPARα-dependent (Bernal-Mizrachi et al. 2003). Therefore, the suppression of PPARα gene expression by CAR may have contributed to the metabolic benefit of CAR.

There was an ~15% decrease in food intake in mice HFD-fed and TCPOBOP-treated for 8 weeks, which correlated with a similar body weight and fat mass decrease. However, the food

intake was unchanged when normalized against the body weight. TCPOBOP also had little effect on the food intake in chow-fed ob/ob mice. Moreover, the inhibition of gluconeogenic and lipogenic gene expression by TCPOBOP was also observed in chow-fed mice (data not shown), whose body weights were not affected by this drug. Taken together, although I cannot completely rule out the possibility that the improved insulin sensitivity and steatosis may be secondary to body weight and fat mass loss in TCPOBOP-treated mice, my results suggest that the inhibition of food intake may not be the primary mechanism by which CAR improves insulin sensitivity. It is more likely that the CAR-mediated suppression of gluconeogenesis and lipogenesis preceded and was responsible for the anti-diabetic effect of TCPOBOP.

Dong et al. (Dong et al. 2009) recently reported that CAR mediates the induction of drug metabolism in a streptozotocin-induced mouse model of type 1 diabetes, which was associated with the induction of Pgc-1α in the liver. In the current study, I showed that hepatic Pgc-1α was induced by HFD feeding (data not shown), consistent with the notion that Pgc-1α promotes gluconeogenesis and insulin resistance in the liver (Koo et al. 2004). Together, these results suggest that both type 1 and type 2 diabetes are associated with the induction of Pgc-1α. I showed that compared with the vehicle group, treatment with TCPOBOP in HFD-fed mice had the tendency to suppress the expression of PGC-1α, although the suppression did not reach a statistical significance. The lack of PGC-1α induction and effective suppression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase gene expression in TCPOBOP-treated mice were consistent with the metabolic benefit of CAR.

The underlying mechanism though which CAR activation suppressed gluconeogenic gene expression seems mediated through PGC-1α, the key transcriptional co-activator for the induction of hepatic gluconeogenesis in the condition of fasting or diabetic status. My data also

suggested the CAR induced PGC-1α redistribution to PML nuclear bodies, and therefore suppressing its transcriptional activity. The detail molecular switch triggering the redistribution events in both CAR and PGC-1α still needs further investigation. One possibility is that the conformation change upon the interaction between CAR and PGC-1α will confer the ability of CAR/PGC-1α complex to interact with certain component of PML nuclear body. All normal tissues expressed PML protein, and its expression is frequently lost in human cancers of various histological origins, and its loss associates with tumor grade and progression in some tumor histotypes (Gurrieri et al. 2004). My results suggested the novel function of PML nuclear bodies in metabolic homeostasis, and it's interesting to further investigate the significance of PML nuclear bodies on glucose/lipid metabolism in various physiological conditions.

In summary, my results have uncovered an important metabolic function for CAR in preventing and relieving obesity and type 2 diabetes. It is interesting to note that the anti-diabetic and anti-obesity effect appeared to be unique for CAR, because the same effect was not observed for pregnane X receptor (data not shown), a sister xenobiotic receptor of CAR that has also been shown to inhibit lipogenesis and gluconeogenesis (Zhou et al. 2006; Kodama et al. 2007). It is tempting for me to propose that CAR may represent a novel therapeutic target to manage obesity and type 2 diabetes. Indeed, the CAR agonist phenobarbital has been shown to enhance insulin sensitivity and improve glucose and lipid metabolism in diabetic rats (Venkatesan et al. 1994) and in diabetic patients (Lahtela et al. 1984; Lahtela et al. 1985). Obesity is a medical problem of high prevalence. It is encouraging to note that CAR-activating activities have been found not only in clinical drugs but also in neutraceuticals, such as herbal medicines (Huang et al. 2004), raising the hope that CAR could be a target for neutraceutical prevention and relief of metabolic syndrome.

3.0 CHAPTER III: GENDER-SPECIFIC EFFECT OF ESTROGEN SULFOTRANSFERASE ON MOUSE MODELS OF TYPE 2 DIABETES

3.1 NUCLEAR RECEPTORS REGULATE ESTROGEN HOMEOSTASIS

Estrogens are implicated in various physiological functions besides reproduction. In recent years, the importance of estrogens in regulating energy and glucose homeostasis has gained increasing attention. Mice lacking aromatase, the enzyme that converts androgens to estrogens, developed obesity due to reduced physical activity and decreased lean body mass (Jones et al. 2000). The estrogen receptor α (ERα) deficient mice also developed obesity with decreased energy expenditure (Heine et al. 2000). The mechanism by which estrogens stimulated energy expenditure is not fully understood. It was suggested that ERα signaling in the ventromedial nucleus of the hypothalamus played an important role in regulating food intake, systemic insulin sensitivity, and energy expenditure in female mice (Musatov et al. 2007). Estrogen deficiencies also led to impaired insulin sensitivity in both aromatase knockout (Jones et al. 2000) and ERα knockout (Ribas et al. 2010) mice but without a defined mechanism. Administration of estrogens, on the other hand, improved insulin sensitivity in HFD fed female mice (Riant et al. 2009) and *ob/ob* mice (Gao et al. 2006).

The molecular mechanisms through which estrogen offers beneficial effects on metabolic functions, especially on improving insulin sensitivity in peripheral tissue, had only been partially

revealed. In muscle, estrogens may modulate glucose disposal through several proteins of the insulin signaling pathway and the expression and translocation of GLUT4. E2 stimulates the phosphorylation of Akt, AMPK in soleus muscle in rats (Rogers et al. 2009). Administration of E2 to insulin-resistant rats or mice increases IRS-1 content and the concentration of the phosphorylated form of Akt in muscle and increase insulin sensitivity (Riant et al. 2009). E2 treatment may also improve glucose homeostasis through its ability to increase muscle GLUT4 content on the cell membrane In aging female rats (Barros et al. 2006). ERα-selective agonist PPT treatment also increases GLUT4 translocation to the cell membrane in L6 myoblasts, while ERα knocking down led to decreased GLUT4 translocation (Gorres et al. 2011).

Human subcutaneous and visceral adipose tissues express both ERα and ERβ, whereas only ERa mRNA has been identified in brown adipose tissue (Rodriguez-Cuenca et al., 2007). In adipose tissue, E_2 seems to prevent accumulation of visceral fat, increase central sensitivity to leptin, and increase the expression of insulin receptors in adipocytes, thus preventing the development of obesity and insulin resistance (Clegg et al. 2006). In the condition of estrogen deficiency such as post-menopause, female patients usually have increased visceral fat mass, lipid accumulation and decreased lipid utilization, all of which led to increased risk of cardiovascular diseases and insulin resistance (Wohlers et al. 2010) In mice, ovariectomy also increased adipose triglyceride lipase signaling, resulting in increased serum free fatty acids level, while E_2 replacement will largely reverse these effects (Wohlers et al. 2010). In both male and female mice, deletion of ERα caused large adipocyte size and increased adipocyte number, and both are insulin resistant with impaired glucose tolerance (Heine et al. 2000). However, most of the experiments are carried out in ER whole knockout mice, while the phenotype observed in adipose tissue are unavoidably affected by other tissues such as brain. The direct effects of

estrogens on adipose tissue need may require further investigation through characterizing adipose tissue specific transgenic mice.

Current experimental evidences suggested the critical role of estrogen signaling in hepatic glucose/lipid metabolism. In the aromatase null mice, insulin resistance develops secondary to the hepatic steatosis (Takeda et al. 2003). In ERα null mice, there is increased gluconeogenesis genes expression and hepatic glucose production, as well as increased expression of genes involved in lipid synthesis genes and decreased expression of genes involved in lipid transport (Bryzgalova et al. 2006). The metabolic benefit of estrogens may due to its protection effects against liver inflammation (Evans et al. 2002). When ovariectomized mice were treated with IL-1B to induce hepatic inflammation, E_2 administration antagonized the induction of cytokines, adhesion molecules only in WT mice, but no in ERα null mice (Lemieux et al. 2005).

Estrogen homeostasis is tightly regulated through balanced biosynthesis and metabolism, especially in individual tissues. Sulfation is a dominant estrogen transformation and inactivation pathway, because the sulfonated estrogens can no longer bind to ER (Hobkirk 1993). Estrogen sulfotransferase (EST, or SULT1E1) is a cytosolic enzyme catalyzing the transfer of sulfate from 3'-phosphoadenosine-5'-phosphosulfate to available hydroxyl groups of the estrogens. EST has been proposed to be the primary enzyme responsible for sulfonation and inactivation of estrogens at physiological concentrations. Consistent with the proposed function of EST in estrogen homeostasis, *Est-/-* males exhibited structural and functional lesions in their reproductive system, a phenotype resulting from chronic estrogen stimulation (Qian et al. 2001). In female *Est-/-* mice, the defect of estrogen sulfation caused estrogen excess, leading to placental thrombosis and spontaneous fetal loss (Tong et al. 2005). Consistent with its known

role in embryogenesis, EST are highly expressed in the reproductive systems such as ovary and testis in both male and female mice. EST also highly expressed in the male abdominal fat tissue, where the expression of EST is hardly detected in female mice.

Under normal conditions, the hepatic expression of *Est* in mice was rather low (Gong et al. 2007; Gong et al. 2008). An aberrant and marked induction of *Est* has been reported in the liver of the obese and diabetic C57BL/KsJ-*db/db* mice (Leiter et al. 1994), whereas the expression of *Est* in the testis was not affected in the same mice (Song et al. 1995). Positive associations between diabetic phenotype and hepatic *Est* induction were also reported in several other strains of female obese mice (Leiter et al. 1991). However, whether and how Est plays a role in energy metabolism and pathogenesis of type 2 diabetes have not been reported.

Mechanistically, the *Est* induction in obese and diabetic mice might have been mediated by the glucocorticoids and glucocorticoid receptor (GR). It has been shown that activation of GR by dexamethasone (DEX) induced the hepatic expression and activity of EST. EST induction by DEX was completely abolished in GR null mice. Interestingly, the DEX effect on EST expression appeared to be tissue specific, since DEX had little effect on EST expression in the testis and adipose tissue, tissues known to express GR. Treatment of female mice with DEX lowered circulating estrogens, compromised uterine estrogen responses, and inhibited estrogen-dependent breast cancer growth in *vitro* and in a MCF-7 xenograft model. It has also been shown that both mouse and human EST genes are transcriptional targets of GR, and deletion of EST/ SULT1E1 in mice abolished the DEX effect on estrogen responses (Gong et al. 2008). The *ob/ob* and *db/db* phenotype is known to be associated with an increased glucocorticoid level (Wittmers et al. 1983). Chronic treatment of wild type mice with dexamethasone (DEX), a synthetic glucocorticoid, was sufficient to induce hyperglycemia and hyperinsulinemia (Gill et al. 1994).

Although the positive association between diabetic phenotype and hepatic *Est* induction among several strains of female obese mice had been reported, and It had been speculated that the induction of hepatic Est leads to decreased liver estrogen availability which in turn cause decreased hepatic insulin sensitivity and hyperglycemia. However the definitive proof for this hypothesis has not been provided in previous publication to my best knowledge. In order evaluate the role of Est in the pathogenesis of type 2 diabetes, I will investigate the impact of EST deficiency in several type 2 diabetes models, including *ob/ob* mice, DEX and high-fat diet induced insulin resistant mice models. I hypothesize that *Est* ablation will increase hepatic estrogen signaling, improve liver insulin sensitivity, and impact overall energy expenditure. I also hypothesize that the effect of *Est* deficiency will exert sexual dimorphism with more significant effects on female mice, and beneficial effect of *Est* ablation will be abolished in ovariectomized female mice.

3.2 METHOD

3.2.1 Animals

ob/ob mice lacking *Est* (*obe*) were generated by crossing heterozygous B6.V-Lepob/J mice from the Jackson Laboratory with *Est-/-* mice in C57BL/6J background (Qian et al. 2001) until both alleles reach homozygosity. Food intake and weights were determined weekly. All studies were performed on age-matched mice. High-fat diet (Cat # S3282) was purchased from Bio-serv (Frenchtown, NJ). Body composition was analyzed in live animals using EchoMRI-100 from Echo Medical Systems (Houston, TX). The use of mice in this study has complied with all relevant federal guidelines and institutional policies.

3.2.2 Indirect Calorimetry

This was performed using an Oxymax Indirect Calorimetry System from Columbus Instruments (Columbus, OH). Mice were individually housed in the chamber with a 12-h light/12-h dark cycle in an ambient temperature of 22–24℃. Metabolic rate (VO_2), respiratory quotient (ratio of VCO_2/VO_2), and physical activity were evaluated over a 48-h period. Mice were acclimatized 4-6 hours before data collection.

3.2.3 Euglycemic-hyperinsulinemic Clamp

The clamp experiments were performed as described by others (Kim 2009). Briefly, right jugular veins of 7-8 weeks old *ob/ob* or *obe* mice were catheterized 4 days before the

experiment, and mice were fasted 16-h before euglycemic clamps. On the day of the clamp experiment, the mouse was placed in a rat-size restrainer with its tail tape-tethered at one end at least 2-h in order for the mouse to acclimatize to the restrained state. D-[3-^{3}H] glucose (0.05 μCi/min) was infused for 2-h and blood samples were collected from the tail vein to assess the basal rate of whole body glucose turnover. Following the basal period, the mouse was infused with a primed (300 mU/kg body weight) and continuous infusion (12.5 mU/kg/min) of human insulin (Novolin) from Novo Nordisk (Princeton, NJ). Blood glucose levels were measured in 10 min intervals, and a variable 20% glucose was infused to maintain glucose at 120-150 mg/dl. Blood samples (20 μl) at 100, 110, and 120 min were collected for the measurement of plasma [3-^{3}H] glucose, and insulin levels were measured in the final blood sample.

3.2.4 GTT, ITT and GSIS

For Glucose tolerance (GTT), mice were fasted for 16-h before receiving an i.p. injection of D-glucose at 1 g/kg body weight (for *ob/ob* mice), or 2 g/kg body weight (for other genotypes). Blood glucose concentrations were measured with a glucometer. For insulin tolerance test (ITT), mice were fasted for 4-h before receiving an i.p. injection of insulin at 0.5 or 1.5 U/kg body weight. For glucose stimulated insulin secretion test (GSIS), additional 20 μl blood was collected from 30, 60 and 120 time points during GTT for the measurement of plasma insulin levels. Mouse islets were isolated and *in vitro* GSIS was performed as previously described (Garcia-Ocana et al. 2001).

3.2.5 Histochemistry and Immunofluorescence Microscopy

Tissues were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (Gao et al. 2009). For immunofluorescence, tissue sections were deparaffinized and rehydrated, followed by pre-incubated in blocking buffer (1X PBS, 5% normal donkey serum, and 0.3% Triton X-100) for 60 min. Tissue sections were then incubated with diluted primary antibody overnight at 4°C, and fluorochrome-conjugated secondary antibody for 1-2 hours at room temperature in dark the next day. Antibodies used include rabbit anti-human insulin (C27C9) monoclonal antibody (Cat #3014 from Cell signaling), goat anti-human glucagon (N-17) polyclonal antibody (Cat #sc-7780 from Santa Cruz), and goat anti-mouse CD68 (M-20) polyclonal antibody (Cat #sc-7084 from Santa Cruz). Pancreatic β cell proliferation and apoptosis were determined by Ki67 immunostaining (Ki67 antibody Cat #SP6 from Neomarkers) and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay from Promega (Madison, WI), respectively (Velazquez-Garcia et al. 2011).

3.2.6 Estrogen Sulfotransferase Enzymatic Assay

This was performed as described previously (Gong et al. 2007). Briefly, 20 μg/ml total liver cytosolic protein extract was incubated with 1 μM of estrone substrate and [^{35}S]-phosphoadenosine phosphosulfate from PerkinElmer (Boston, MA) at 37°C for 30 min. The reaction was terminated by adding ethyl acetate, and the aqueous phase was then counted in a scintillation counter.

3.2.7 Serum Chemistry

Serum levels of E_2 (Cat #DSL-4800), E_1 (Cat #DSL-8700), E_1S (Cat #DSL-5400), T_4 (Cat #DSL-3200), T_3 (Cat #DSL-3100) were measured using RIA assay kits form Diagnostic Systems Laboratories (Webster, TX). Serum level of triglyceride (Cat #2100-430, Stanbio), cholesterol (Cat #1010-430, Stanbio), free fatty acids (Cat #11383175001, Roche), insulin (Cat #90080, Crystal Chem), and IGF-1 (Cat #MG100, R&D Systems) were measured by using commercial assay kits according to the manufacturer's instructions.

3.2.8 Quantitative RT-PCR

Total RNA was isolated using the TRIZOL reagent from Invitrogen (Carlsbad, CA). Reverse transcription was performed with random hexamer primers and Superscript RT III enzyme from Invitrogen. SYBR Green-based real-time PCR was performed with the ABI 7300 Real-Time PCR System. Data was normalized against internal control cyclophillin.

3.2.9 Statistical Analysis

Results are presented as means ± SD. Statistical analysis was determined using the unpaired student's *t* test for comparison between two groups, with *P* values of less than 0.05 considered significant; One-way ANOVA was used to compare the means of two or more groups; Repeated-measures ANOVA was used to compare the means of two or more groups across multiple time points.

3.3 RESULTS

3.3.1 *Ob/ob* Mice Lacking *Est* had Reduced Adiposity and Increased Energy Expenditure

Est is known to have a low basal expression in the mouse liver (Gong et al. 2007; Gong et al. 2008). I showed that *ob/ob* mice had a dramatic and liver-specific induction of *Est* (Fig. 21a), consistent with the hepatic induction of the same gene in *db/db* mice (Leiter et al. 1994; Song et al. 1995). To determine the role of *Est* in the pathogenesis of type 2 diabetes in *ob/ob* mice, I cross-bred *Est-/-* mice with heterozygous *ob/ob* mice to generate *ob/ob* mice lacking *Est* that were termed *obe* mice. Obe mice exhibited hyperphagia and early onset of obesity similar to *ob/ob* mice before 6 weeks of age. During adulthood, however, female, but not male, *obe* mice showed a modest but significant decrease in body weight compared to age-matched *ob/ob* females (Fig. 21b). Magnetic resonance imaging analysis revealed favorable body composition changes in *obe* mice that included decreased fat mass and increased lean mass (Fig. 21b), which was achieved without significant changes in food intake and physical activity (Table 3). *Ob/ob* mice were reported to be acyclic (Ng et al. 2010), suggesting that estrous cycle may not be an important factor in affecting food intake. Consistent with their increased lean mass, *obe* females showed increased skeletal muscle fiber bundle size (Fig. 21c) and increased gastrocnemius and soleus muscle weight (Table 3), which was associated with an increased serum level of IGF-1 and increased *IGF-1* mRNA expression (Fig. 21d) in the liver, a tissue known to contribute up to 90% of circulating IGF-1 (Ohlsson et al. 2009). A decreased IGF-1 level has been proposed to contribute to the decreased muscle mass in *ob/ob* mice (Bartell et al. 2011). The improved body composition and unchanged food intake led to my hypothesis that *obe* females may have increased energy expenditure. Indeed, compared to their *ob/ob* counterparts, *obe* females had

higher oxygen consumption (Fig. 21e, Fig. 22), higher energy expenditure when normalized against the lean body mass (Butler et al. 2010) (Fig. 21f), and approximately 1.5 ^{o}C increase in resting rectal temperature (Fig. 21g). The metabolic benefit of loss of *Est* was not associated with altered serum levels of triiodothyronine (T_3) and thyroxine (T_4) (Table 2), although thyroid hormones have been reported as low-affinity EST substrates (Kester et al. 1999).

Table 2. Metabolic profiles of *ob/ob* and *ob/ob_Est null* female mice

	ob/ob	***Ob/ob_Est null***
Triiodothyronine (nM)	1.43±0.09	1.39±0.19
Thyroxine (nM)	61.8±7.2	50.8±8.7
Triglyceride (mg/dl)	184±32	110±19*
Total cholesterol (mg/dl)	129±6	121±11
Free fatty acid (mM)	0.96±0.11	0.51±0.15**
Estrone (E1, pg/ml)	36.13±2.40	44.37±1.84*
Estrone sulfate (E1S, pg/ml)	644.2±53.1	459.3±78.5*
17β-estradiol (E_2, pg/ml)	9.09±2.02	11.13±2.14
Fasting glucose (mg/dl)	132.7±10.5	87.0±6.4**
Fasting insulin (ng/ml)	5.73±1.54	2.01±0.43**
Liver triglyceride (mg/g tissue)	95.7±5.9	32.0±7.3**
Liver total cholesterol (mg/g tissue)	9.43±1.64	1.64±0.35**

***, P<0.05; **, P<0.01**

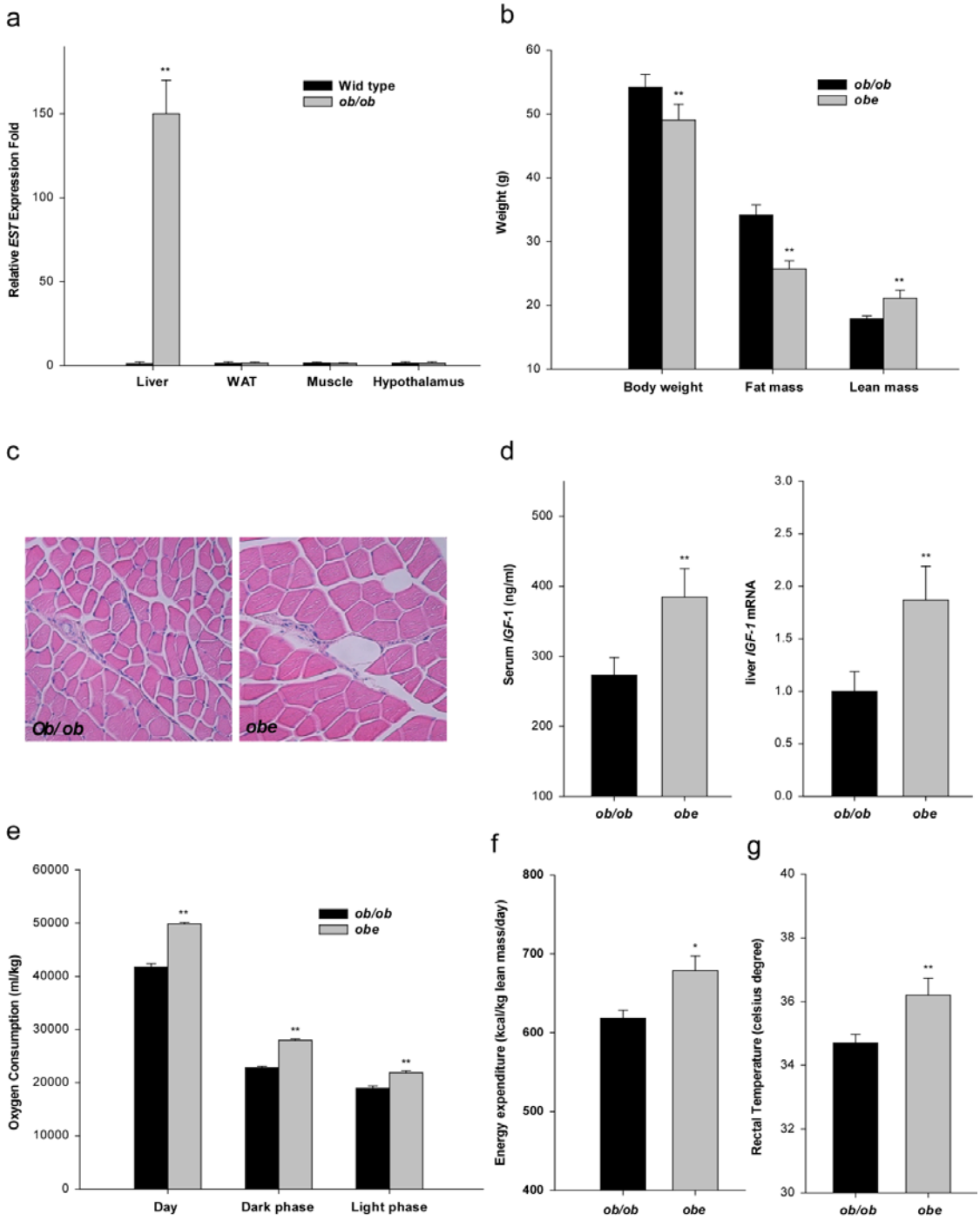

Figure 21. Loss of *Est* inhibited adiposity and improved metabolic functions in *ob/ob* female mice.

(a) Expression of *Est* in female wild type C57BL/6J and *ob/ob* mice as determined by real-time PCR analysis. (b) Body composition analysis of 12-week old female mice. (c) H&E staining of gastrocnemius muscle. (d) Serum IGF-1 level and hepatic *IGF-1* mRNA expression. (e and f) Oxygen consumption (e) and energy expenditure (f) were measured by CLAMS. (g) Measurement of rectal temperature. N≥ 4 for each group. *, $P < 0.05$; **, $P < 0.01$; *ob/ob* versus *obe*.

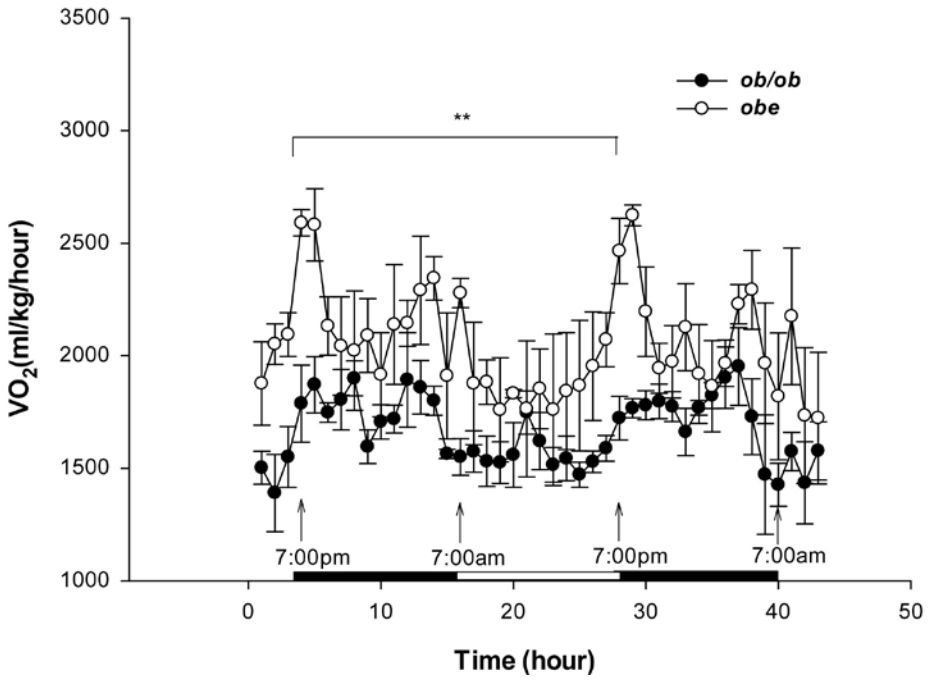

Figure 22. Oxygen consumption of *obe* and *ob/ob* female mice.

Oxygen consumption were measured by CLAMS for 48 hour time period . n ≥ 4 for each group. *, $P < 0.05$; **, $P < 0.01$; *ob/ob* versus *obe*.

Table 3. Metabolic profiles of *ob/ob* and *ob/ob_Est null* female mice

	ob/ob	***Ob/ob_Est null***
Food intake (g/ day/mice)	5.69±0.48	6.02±0.33
Food intake (g/day/kg lean mass)	317.6±22.6	310.3±18.1
Physical activity (X-total counts/day)	25079.3±3872.4	30037.5±5186.8
Gastrocnemius and soleus muscles weight (g)	0.20±0.02	0.27±0.04*
Gastrocnemius muscles index (% Body weight)	0.35±0.04	0.53±0.08**

***, P<0.05; **, P<0.01**

3.3.2 *Obe* Female Mice Displayed Improved Insulin Sensitivity and Reduced Hepatic Steatosis.

In understanding the metabolic benefit of *Est* ablation, I found that *obe* females, but not males, showed reduced fasting hyperglycemia and improved performance in glucose tolerance (GTT) and insulin tolerance (ITT) tests (Fig. 23a and b). *Obe* females showed inhibition of hepatic gluconeogenesis as supported by their improved performance in pyruvate tolerance test (Fig. 23c). Using hyperinsulinemic-euglycemic clamp test, I found that 8-9 weeks old *obe* females showed significantly lower fasting glucose and insulin levels (Table 2) and a nearly 3-fold increase of glucose infusion rates during clamp period (Fig. 23d), suggesting a markedly improved insulin sensitivity. *Obe* females showed lower basal and clamp hepatic glucose production (HGP) (Fig. 23f), and significant suppression of HGP during clamp stage compared with *ob/ob* mice (Fig. 23g), which indicated improved hepatic insulin sensitivity. Consistent with reduced HGP, *obe* females showed a decreased hepatic expression of gluconeogenic genes, including *Pgc-1α*, *Pepck*, and *G6pase* (Fig. 24a). Female *obe* mice also exhibited relief of hepatic steatosis as shown by histology (Fig. 24c) and measurement of liver levels of triglycerides and cholesterol (Fig. 24b). The expression of hepatic lipogenic and adipogenic genes *Srebp-1c*, *Acc1*, *Fas*, *Scd-1* and *aP2* was decreased (Fig. 24d), whereas the expression of fatty acid oxidative genes *Ppar-α*, *Cpt-1α*, *Lcad* and *Mcad* was increased in *obe* females (Fig. 24e). The improved metabolic function of *obe* females was also manifested at the serum chemistry level, which included decreased circulating levels of triglycerides and free fatty acids (Table 2).

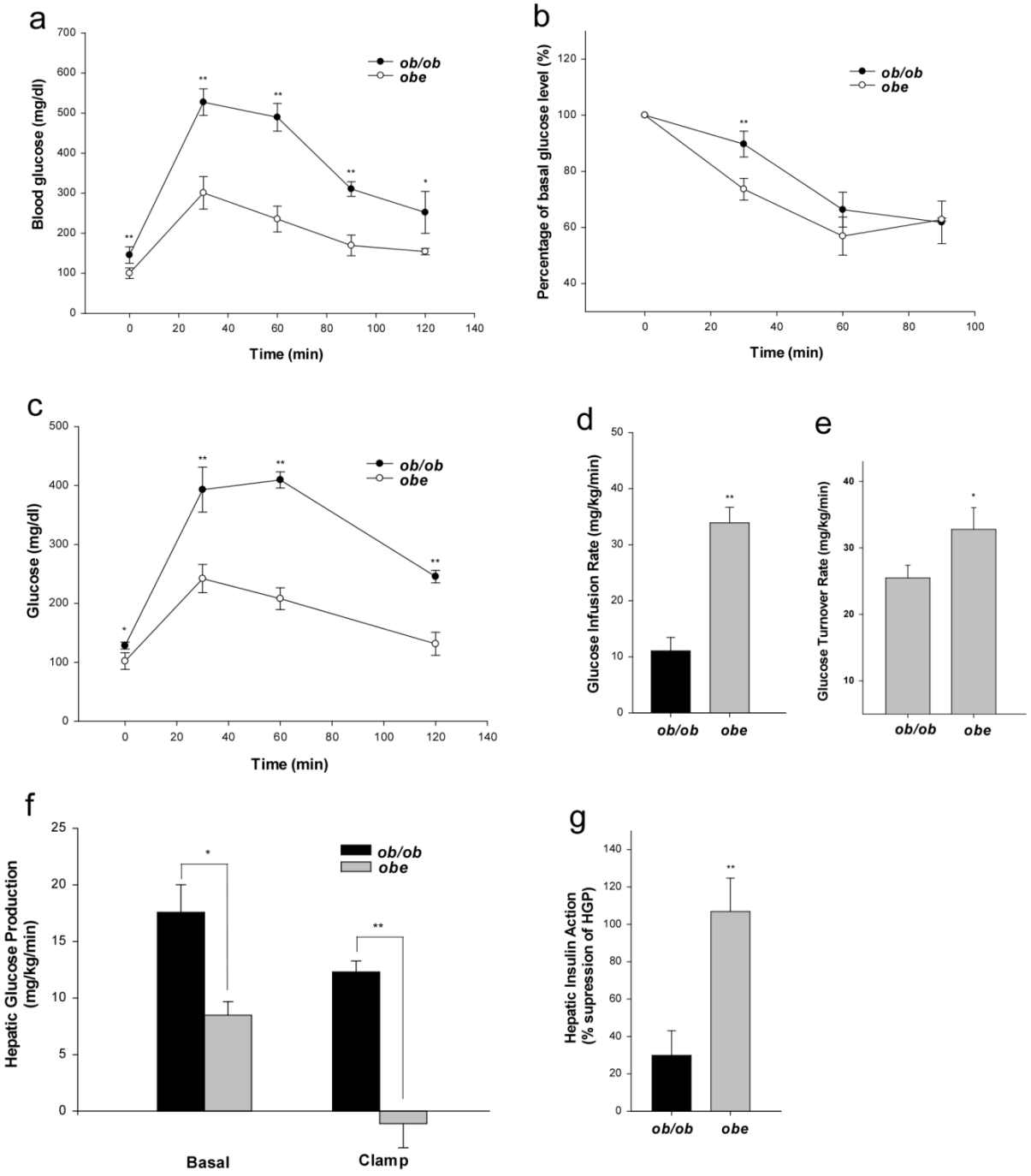

Figure 23. *Obe* female mice showed improved insulin sensitivity and reduced hepatic glucose output.

(a) Glucose tolerance test (GTT) , (b) insulin tolerance test (ITT) and (c) pyruvate tolerance test (PTT) on 9- week old *ob/ob* and *obe* female mice. (d) Glucose infusion rate, (e) glucose turnover rate, (f) hepatic glucose production and suppression of hepatic glucose production. (g) Hepatic insulin action calculated based on suppression percentage in (f). $n \geq 4$ for each group. *, $P < 0.05$; **, $P < 0.01$; *ob/ob* versus *obe*.

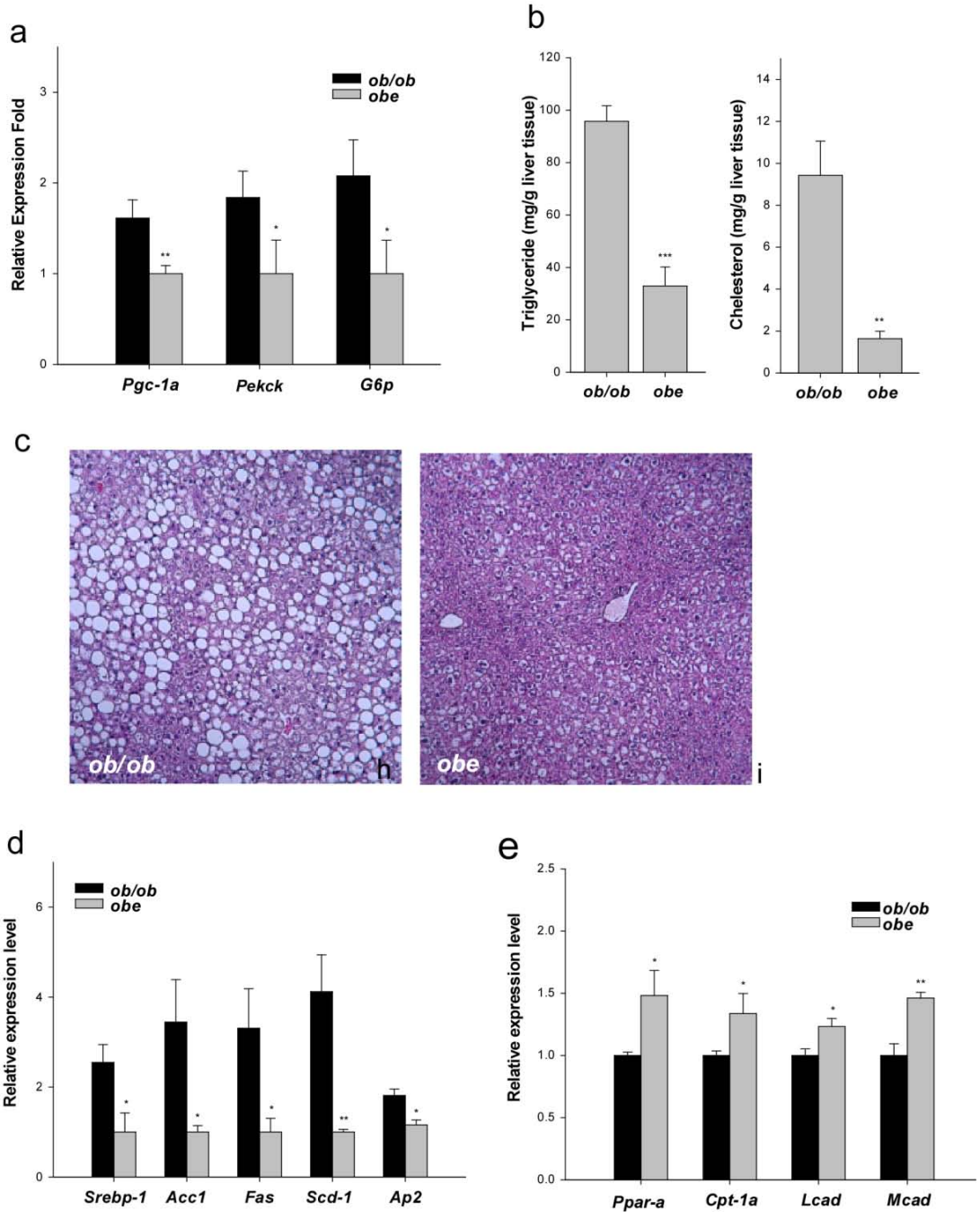

Figure 24. *Obe* female mice showed reduced hepatic steatosis and inhibition of gluconeogenic genes expression.

(a) hepatic gluconeogenesis genes expression, (b) total triglyceride and cholesterol concentration in liver tissue extracts, (c) H&E staining of liver tissue section of 9- week old *ob/ob* and *obe* female mice. (d) lipogenesis genes and (e) lipid oxidation genes expression in liver tissue measured by RT-PCR. n ≥ 4 for each group. *, $P < 0.05$; **, $P < 0.01$; *ob/ob* versus *obe*.

3.3.3 The Metabolic Benefit of *Est* Deficiency was Mediated Through the Estrogen Pathway.

Because the primary function of Est is to sulfonate and deactivate estrogens, and estrogens are known to improve the metabolic functions of *ob/ob* mice (Gao et al. 2006), I went on to determine whether the improved metabolic function in *obe* females was due to increased estrogenic activity in the liver. As shown in Fig. 25a, compared to *ob/ob* females, the hepatic expression of a panel of estrogen responsive genes was induced in *obe* females, which was consistent with the observation that the liver extracts of *obe* mice showed a substantially lower estrogen sulfotransferase activity (Fig. 25b). The estrogenic effect was liver specific, because the expression of estrogen responsive genes was not affected in the skeletal muscle and white adipose tissue (Fig. 26b), despite modest increases in the serum levels of estrone (E_1) and its sulfated metabolites estrone sulfate (E_1S). The circulating level of estradiol (E_2), the most potent estrogen, was not significantly affected in *obe* females (Table 2).

To further evaluate the estrogen pathway, I performed ovariectomy on 5-week old *ob/ob* and *obe* female mice and examined their metabolic functions 3 weeks after the surgery. Ovariectomy completely abolished the metabolic benefits of *obe* mice in body composition (Fig. 25c), IGF-1 protein and mRNA expression (Fig. 26c), GTT and ITT performance (Fig. 25d and e), hepatic steatosis (Fig. 26a), and the expression of lipogenic, gluconeogenic and fatty acid oxidative genes (Fig. 25f). The activation of hepatic estrogen responsive genes in *obe* mice was also abolished upon ovariectomy (Fig. 26d).

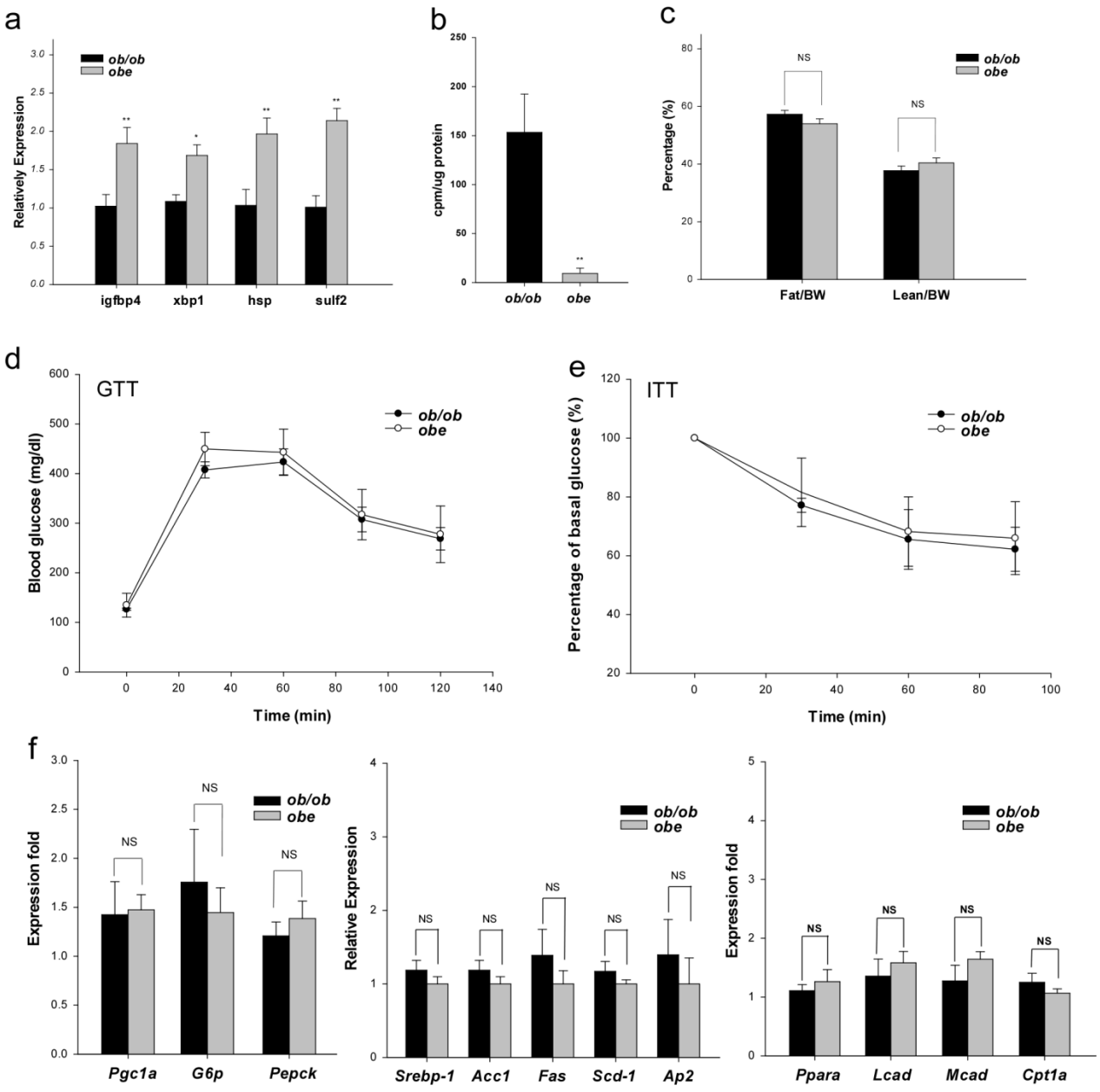

Figure 25. The improved insulin sensitivity was abolished in ovariectomized *obe* female mice.

Hepatic mRNA expression of estrogen responsive genes (a) and measurement of estrogen sulfotransferase activity in the liver extracts (b) of intact *ob/ob* and *obe* mice. Body composition analysis (c), GTT (d) and ITT (e), and the expression of gluconeogenic, lipogenic and fatty acid oxidation genes (f) in ovariectomized mice as measured by real-time PCR analysis. n ≥ 4 for each group. *, $P < 0.05$; **, $P < 0.01$; NS, statistically not significant; *ob/ob* versus *obe*.

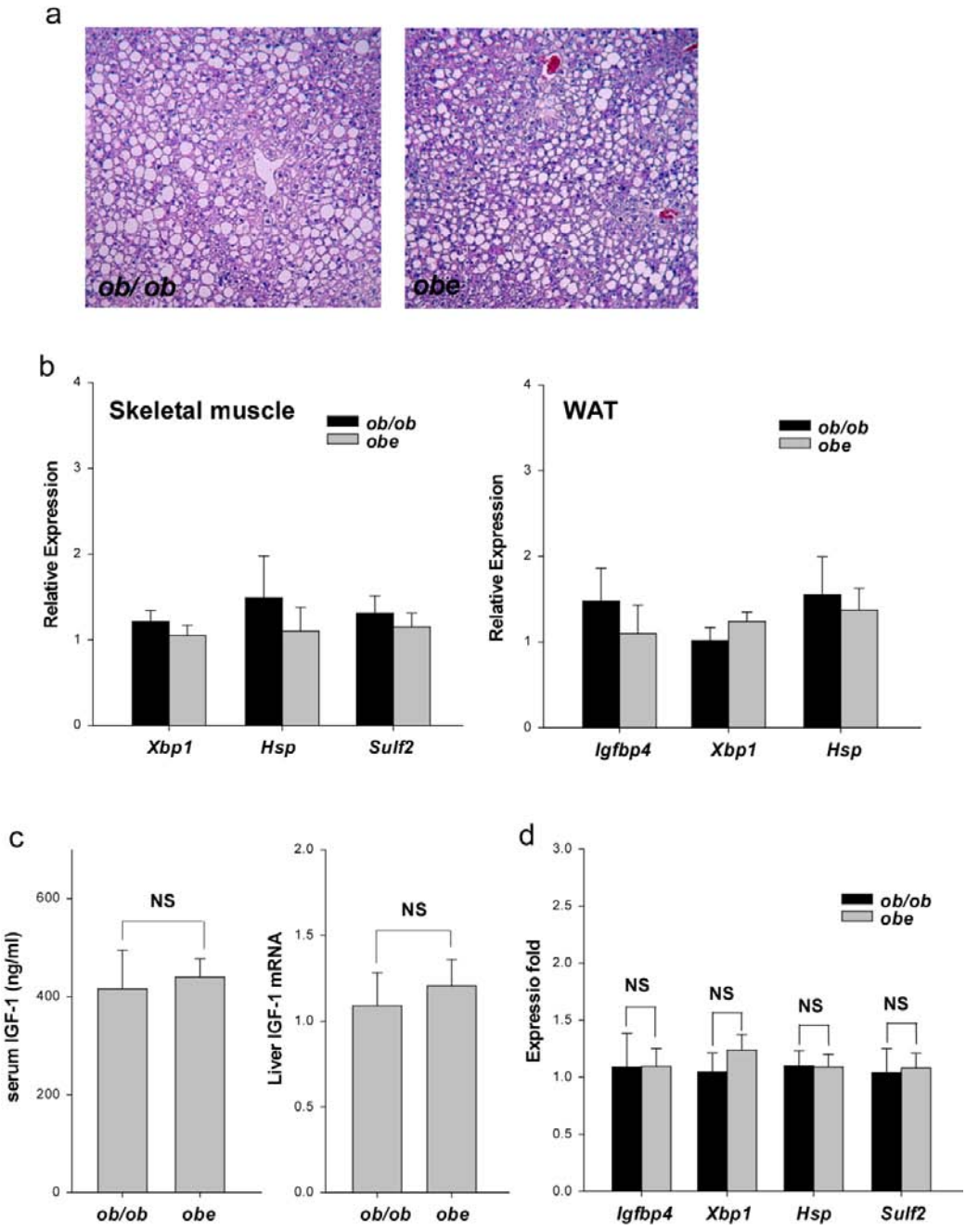

Figure 26. Induction of liver estrogenic genes was abolished in ovariectomized *obe* female mice.

(a) H&E staining of liver section from ovariectomized *ob/ob* and *obe* female mice. **(b)** Expression of estrogen responsive genes in skeletal muscle and white adipose tissue (WAT) were measured by real-time PCR. **(c)** Serum IGF-1 and hepatic IGF-1 mRNA level in ovariectomized mice. **(d).** The expression of estrogen responsive genes in ovariectomized mice as measured by real-time PCR analysis. N≥ 4 for each group. *, $P < 0.05$; **, $P < 0.01$; NS, statistically not significant; *ob/ob* versus *obe*. NS, statistically not significant.

3.3.4 Loss of *Est* Inhibited Dexamethasone (DEX)- and HFD-induced Insulin Intolerance in Female Mice.

A chronic treatment of WT mice with DEX was sufficient to induce hyperglycemia with female mice showing a higher sensitivity (Gill et al. 1994). To determine whether DEX-induced hyperglycemia and insulin resistance was *Est* dependent, I administrated DEX for two weeks to both WT and *Est-/-* mice. As expected, DEX treatment resulted in fasting hyperglycemia and glucose intolerance in WT females (Fig. 27a), which was associated with a marked induction of *Est* in the liver (Fig. 27b). In contrast, *Est-/-* females were resistant to DEX-induced hyperglycemia and showed improved GTT performance (Fig. 27a). Ovariectomy abolished the protective effect of *Est* ablation on DEX-induced insulin resistance (Fig. 27d). The loss of *Est* effect in the DEX model was female specific, because *Est-/-* males remained sensitive to DEX-induced hyperglycemia (Fig. 27c). The anti-diabetic effect of *Est* ablation was also observed in the HFD model. *Est-/-* females showed improved GTT and ITT performance upon 20 weeks of HFD feeding (Fig. 27e and f), which was associated with a hepatic induction of *Est* (Fig. 27b). My results suggested the EST may play key role in mediating hyperglycemia and insulin resistant phenotype after DEX treatment, especially in female mice, and highlighted the importance of liver estrogens signaling in protecting against metabolic disease in woman.

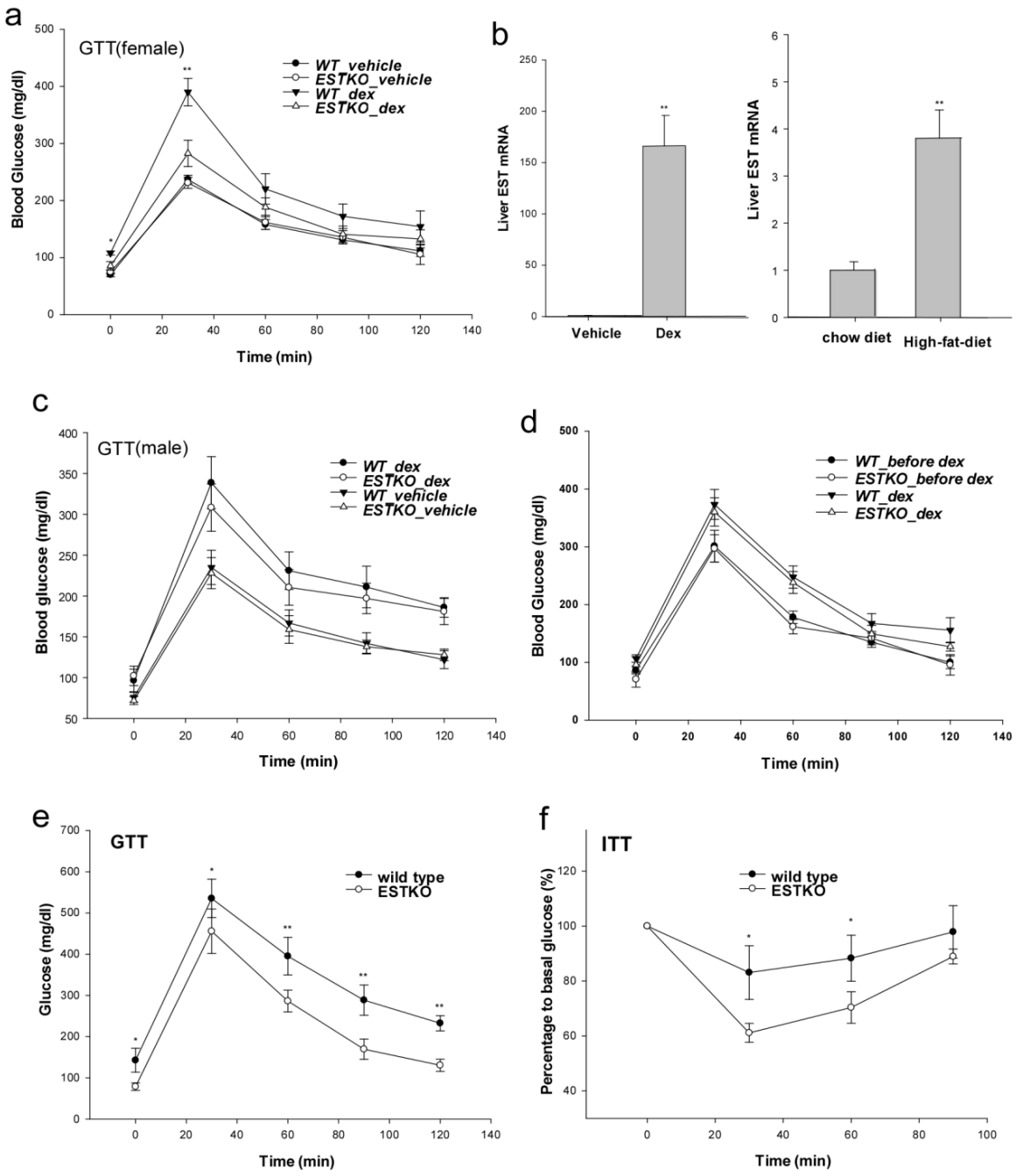

Figure 27. Loss of *Est* inhibited dexamethasone (DEX)- and high fat diet (HFD)-induced insulin intolerance.

(a) GTT in WT and *Est*$^{-/-}$ female mice treated with vehicle or DEX (daily i.p. injection at 1 mg/kg) for two weeks. **(b)** Hepatic *Est* expression in DEX or vehicle treated (left), 20 weeks high-fat diet fed (right) wild type female C57BL/6J. **(c)** GTT in WT and *Est*$^{-/-}$ male mice treated with vehicle or DEX. **(d)** GTT in ovariectomized WT and *Est*$^{-/-}$ female mice treated with vehicle or DEX. **(e)** GTT and ITT in WT and *Est*$^{-/-}$ female mice fed with HFD for 20 weeks. n =5 for each group. *, $P < 0.05$; **, $P < 0.01$.

3.3.5 Loss of *Est* in *ob/ob* Male Mice Aggravated Diabetic Phenotype, Caused a Loss of Pancreatic β Cell Mass, and Increased White Adipose Tissue (WAT) Inflammation.

Interestingly, the loss of *Est* effect on the *ob/ob* phenotype was gender specific, because *obe* males had worsened diabetic phenotype compared to *ob/ob* males. As shown in Fig. 28a, *obe* males showed a higher fasting glucose level and worse GTT performance. The expression of estrogen responsive genes was not affected in the liver of *obe* males (Fig. 28c), and castration failed to improve the metabolic function (Fig. 28d). In addition, *obe* males showed impaired glucose stimulated insulin secretion (GSIS), although the basal insulin level was indistinguishable between *obe* and *ob/ob* males (Fig. 28b). Histological analysis showed dramatically reduced islet size, total islet area, and β cell mass (Fig. 28e, and Fig 29) in *obe* males. Insulin and glucagon double staining revealed a normal distribution of endocrine cells in both *ob/ob* and *obe* males (Fig. 30a). The insulin-producing β cells were evenly distributed and the glucagon-producing α cells were mainly located peripherally in the islets. There was a relative increase of α cell in *obe* mice possibly due to the decreased β cell number. Islets of *obe* male mice were deformed with an increased infiltration of CD68 positive macrophages (Fig. 30b), which was suggestive of increased inflammation. Interestingly, islets isolated from *obe* males exhibited *in vitro* GSIS similar to those isolated from *ob/ob* males (Fig. 30c). These results suggested that the reduced β cell mass might be responsible for the impaired insulin secretion upon glucose challenge. No expression of *Est* was detected in islets (data not shown), suggesting that the impaired insulin secretion was not due to the intrinsic loss of *Est* effect on β cells. The loss of β cell mass was not observed in *obe* females (Fig. 29).

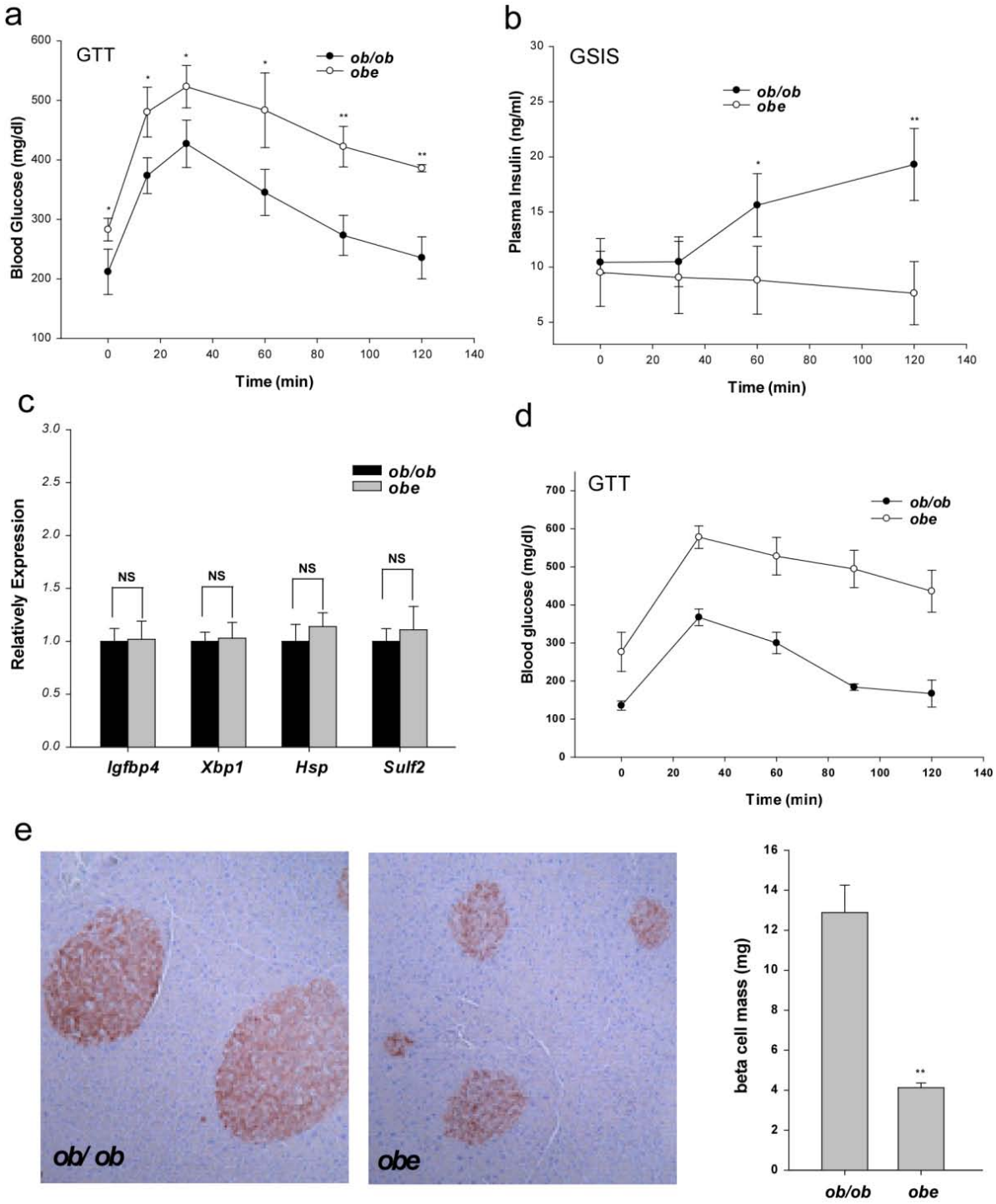

Figure 28. Loss of Est in *ob/ob* male mice aggravated diabetic phenotype.

(a) GTT in *ob/ob* and *obe* male mice. (b) *In vivo* glucose stimulated insulin secretion (GSIS) test, (c) Hepatic expression of estrogen responsive genes in intact male mice as measured by real-time PCR analysis. (d) GTT in castrated *ob/ob* and *obe* male mice.. (e) Immunostaining of insulin and quantification of total islet area. n =5 for each group. *, $P < 0.05$; **, $P < 0.01$.

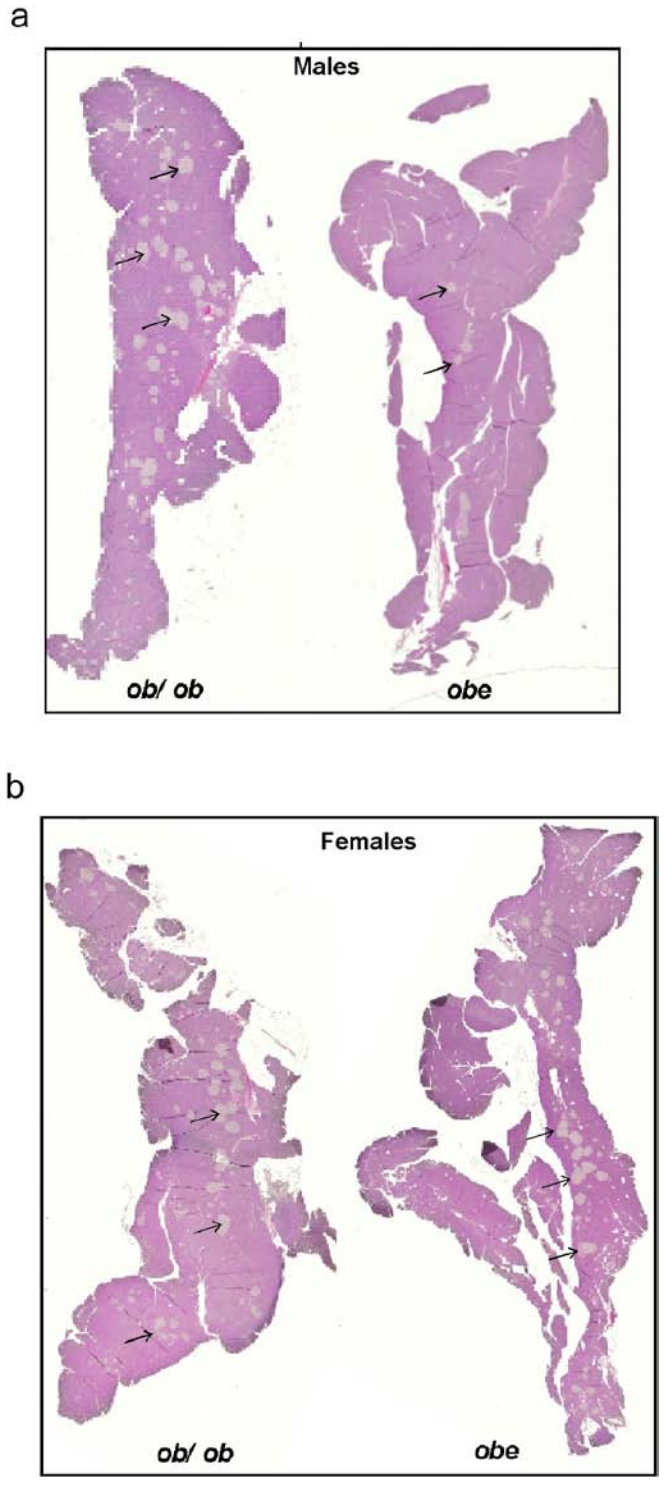

Figure 29. H&E staining of the whole pancreas section of *ob/ob* and *obe* mice

H&E staining of pancreas section from **(a)** Male and **(b)** female *ob/ob* and *obe* mice. Arrow indicates islets.

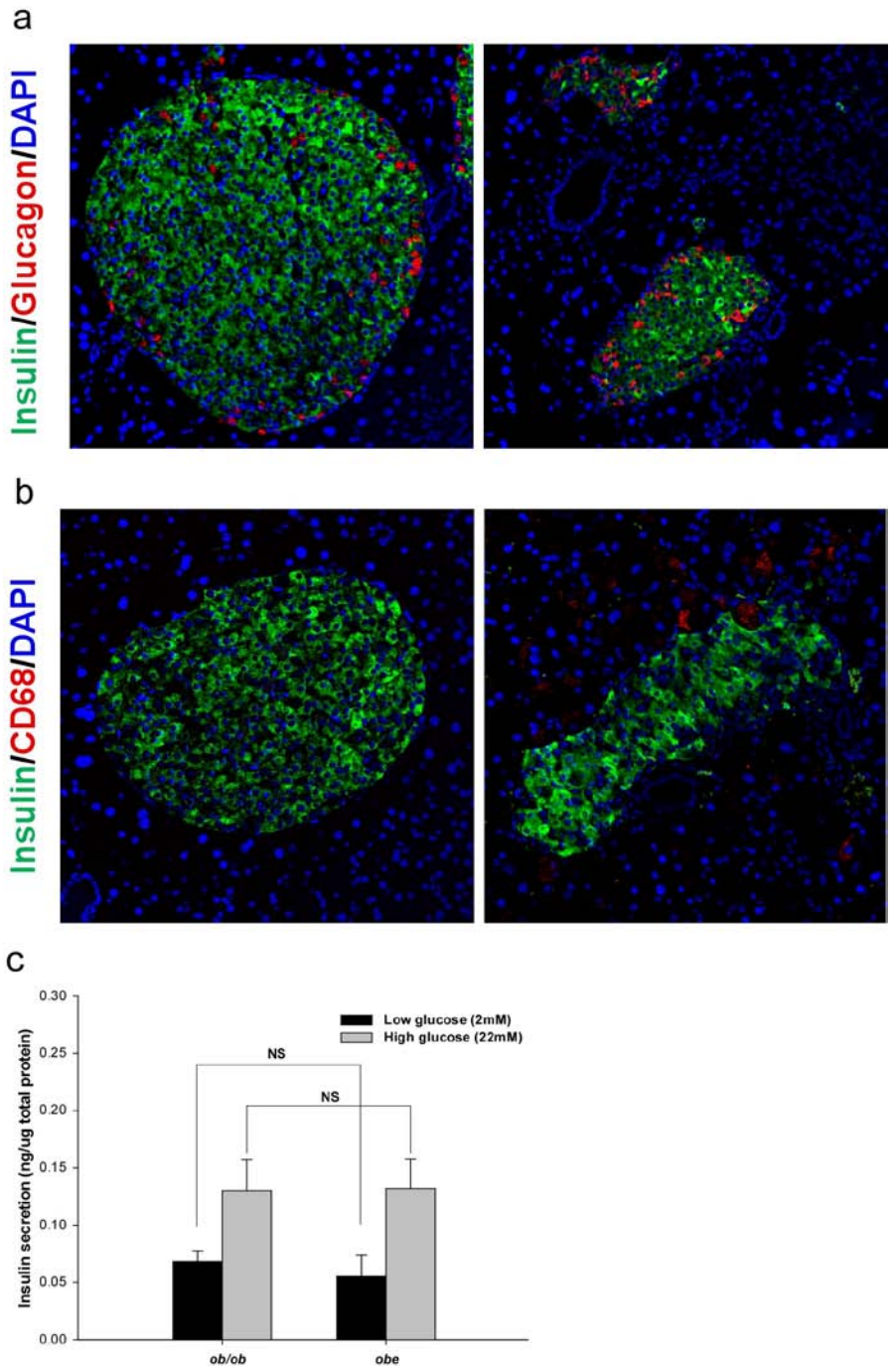

Figure 30. Defect on glucose-stimulated insulin secretion in obe mice are due to reduced pancreatic β cell mass.

(a) Immunofluorescence analysis of insulin, glucagon and (b) CD68 expression. (c) *In vitro* GSIS test on isolated pancreatic islets from *ob/ob* and *obe* male mice. n =4 for each group. *, $P < 0.05$; **, $P < 0.01$.

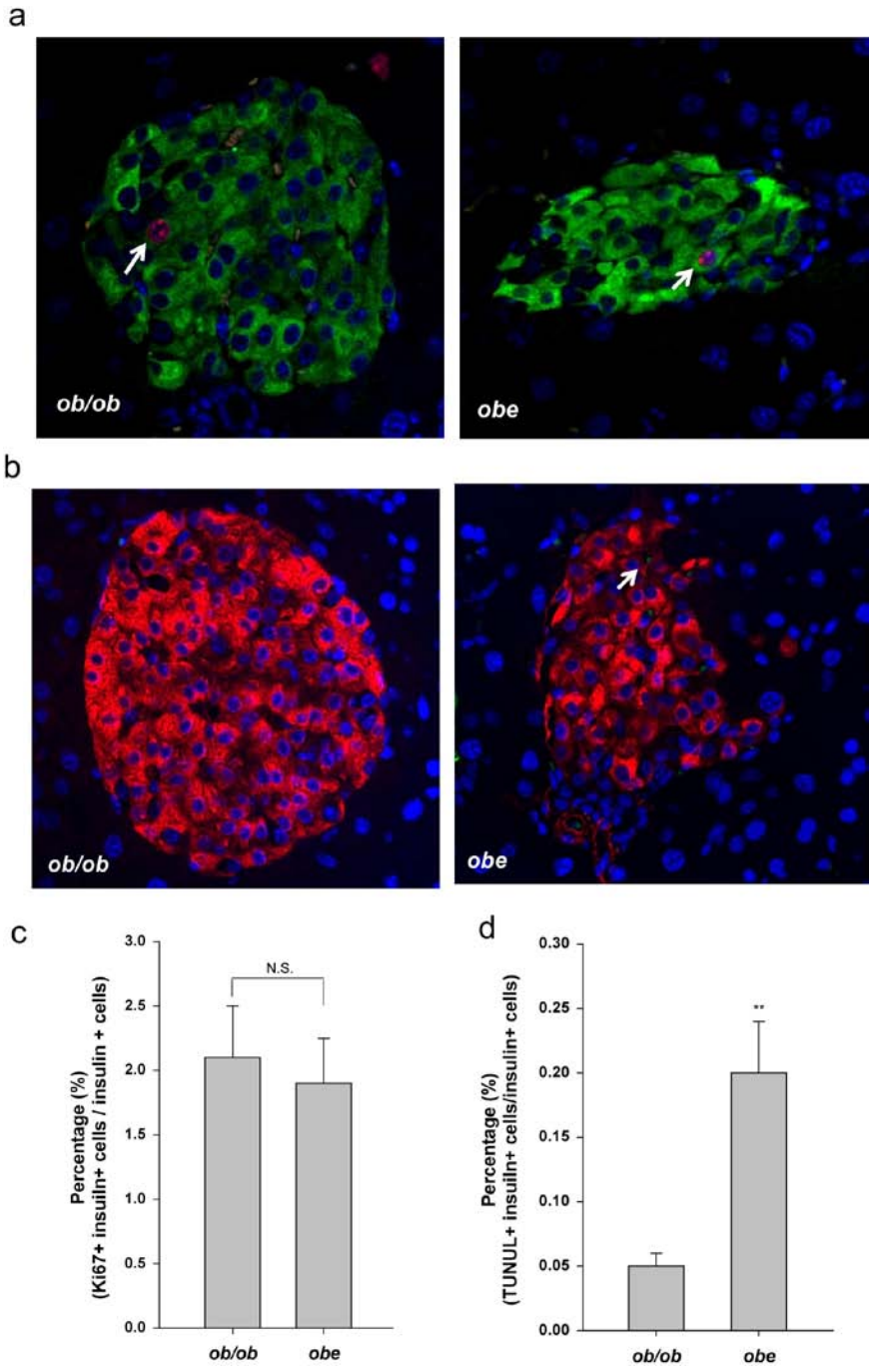

Figure 31. Defect on glucose-stimulated insulin secretion in obe mice are due to reduced pancreatic β cell mass.

Proliferation and apoptosis was measured by Ki67 immnostaining (a) and TUNEL assay (b) respectively. In both assays, the sections were co-stained with insulin. (c) Quantification of proliferation and apoptosis rate in pancreatic section from *ob/ob* and *obe* male mice. n>=4 for each group; *, P < 0.05; **, P < 0.01. n =4 for each group. *, *P* < 0.05; **, *P* < 0.01.

Reduced islet size in *obe* male mice suggested alterations in β cell proliferation and/or death in obe mice. Indeed, when β cell turnover was evaluated, I found that obe male mice displayed increased β cell apoptosis and no alteration in β cell proliferation compared with *ob/ob* male mice (Fig. 31). In understanding the mechanism by which *Est* ablation exacerbated metabolic phenotype in *ob/ob* males, I found that *obe* males showed increased macrophage infiltration and inflammation in WAT as supported by increased density and size of the crown-like structures (Fig. 32a), as well as increased expression of several macrophage markers (*F4/80* and *CD68*) and inflammatory markers (*Mcp1*, *Mac1*, *Adam8*, *Mip1*, and *Tnfα*), and decreased expression of the beneficial adipokine *adiponectin* (Fig. 32b) in the abdomen fat. The effect of *Est* ablation on WAT inflammation was absent in *obe* females. Female ob/ob and obe mice showed no significantly difference in adipose size and signs of macrophage infiltration (Fig. 32c, and d). These results consistent with the sexual dimorphism expression pattern of EST in adipose tissue, and suggested a potential link between β cell loss and increased adipose inflammation caused by *Est* ablation in *obe* male mice.

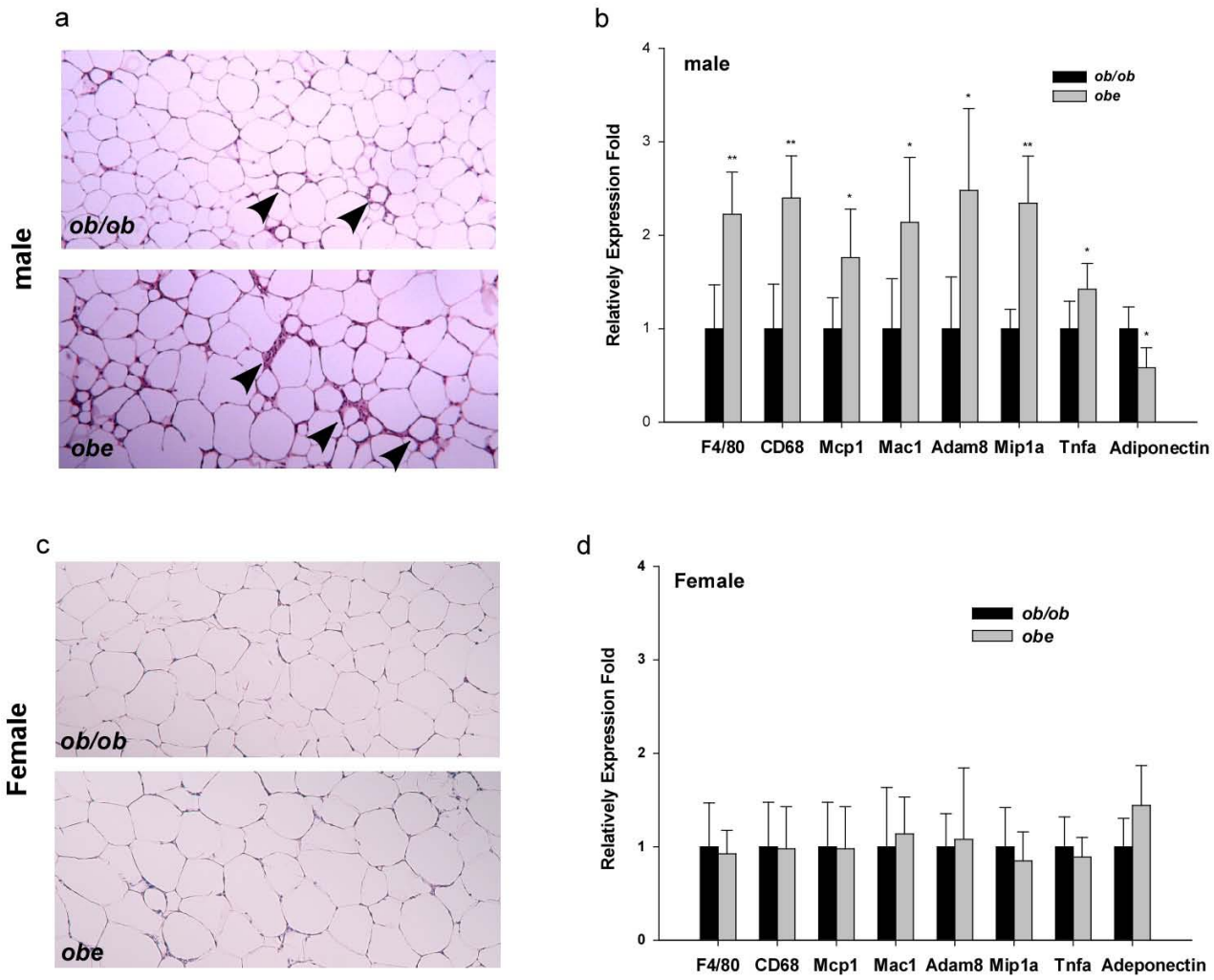

Figure 32. Loss of Est in ob/ob male mice displayed increased white adipose tissue (WAT) inflammation.

(a) H&E staining of abdomen adipose tissue from male *ob/ob* and *obe* mice. Arrowheads indicate "crown-like" structures. **(b)** Expression of macrophage markers and inflammatory genes in the adipose tissue from male *ob/ob* and *obe* mice as determined by real-time PCR. **(c)** H&E staining of abdomen adipose tissue from female *ob/ob* and *obe* mice. **(d)** Expression of macrophage markers and inflammatory genes in the adipose tissue from female *ob/ob* and *obe* mice. The expression of each gene was arbitrarily set as 1 in *ob/ob* mice. N ≥ 4 for each group. *, $P < 0.05$; **, $P < 0.01$; NS, statistically not significant; *ob/ob* versus *obe*.

3.4 DISCUSSION

Here, I showed that induction of hepatic *Est* was a phenotype shared by several type 2 diabetes mouse models. The induction of *Est* most likely played a pathogenic role in type 2 diabetes because loss of *Est* in female mice improved metabolic function in *ob/ob*, DEX and HFD models of type II diabetes. The metabolic benefit of *Est* ablation in female *obe* mice seemed attributed to decreased estrogen deprivation and increased estrogenic activity in the liver, underscoring the importance of liver estrogen signaling in protecting females from developing metabolic disease. I had shown previously that EST is a GR target gene (Gong et al. 2008). The pathogenic role of EST in type 2 diabetes is consistent with the observations that *ob/ob* mice had an increased glucocorticoid level, and DEX was sufficient to induce hyperglycemia. It is clear that estrogens and glucocorticoids have beneficial and detrimental effect on insulin sensitivity, respectively. I have shown that glucocorticoids can antagonize estrogenic activity through the regulation of *EST* (Gong et al. 2008). It is possible that in women, the detrimental effect of glucocorticoids on energy metabolism may become dominant with the onset of menopause because of the major reduction in estrogen production.

Several other lines of anecdotal evidence also suggested that EST may be an important mediator in the pathogenesis of type 2 diabetes in rodents and humans. The expression of *Est* was elevated in the liver of cystic fibrosis transmembrane conductance regulator knockout mice (Li et al. 2007). Cystic fibrosis patients are known to have a higher risk of diabetes (Austin et al. 1994; Moran et al. 1994). In contrast, several anti-diabetic phytoestrogens, such as equol and genistein, have been reported as potent enzymatic inhibitors of EST (Harris et al. 2004). It has been reported that *Est* is expressed in abdominal subcutaneous adipose tissue of both obese males and females in association with expression of TNF-α and suppressor of cytokine signaling

3 (SOCS3), suggesting potential roles in inflammation (Ahima et al. 2011). The causal effect of the expression and/or regulation of EST on the pathogenesis of type 2 diabetes in human patients remain to be confirmed, and further studies are needed to determine the specific metabolic roles of EST expression in human adipose tissue

It is interesting to note that *Est* deficiency led to increased lean mass and skeletal muscle fiber bundle size, which was associated with an increased expression of liver IGF-1, an important growth-promoting endocrine factor (Musaro et al. 1999; Ohlsson et al. 2009). IGF-1 has been implicated in skeletal muscle growth and regeneration, and a viral delivery of the *IGF-1* gene attenuated skeletal muscle atrophy and restore muscle mass and strength in mice (Ohlsson et al. 2009). The production of IGF-1 is regulated by estrogens. Specifically, activation of ERα was necessary for a systemic production of IGF-1, whereas ablation of ERα in the liver decreased the circulating level of IGF-1 (Della Torre et al. 2011). My data suggested that the relieved muscle hypotrophy in female *obe* mice was likely due to the increased IGF-1 secretion as a result of increased estrogenic activity in the liver.

I were surprised to find that *Est* ablation exacerbated the diabetic phenotype in male *ob/ob* mice. Although sexual dimorphism has been documented in diabetic models, and female mice are in general less susceptible to type 2 diabetes (Liu et al. 2009; Riant et al. 2009), previous reports on aromatase and ERα knockout mice suggested that estrogens also exert metabolic benefits in male mice. The discrepancy may be due to the tissue specific effect of estrogens. My *obe* mouse model mainly reflected the hepatic effect of estrogens, because both the induction of *Est* and activation of estrogen responsive genes in *obe* females were liver-specific. The exacerbated diabetic phenotype in male *obe* mice seemed to be due to compromised insulin secretion. Since the expression of *Est* cannot be detected in the islets, and

isolated islets from *ob/ob* and *obe* male mice showed a similar insulin secretion upon glucose stimulation, the lack of insulin secretion was most likely driven by factors other than the intrinsic loss of *Est* in β cells. Castration of male *obe* mice failed to improve metabolic function, suggesting that the phenotype was not simply due to changes in sex hormones. My results, however, were consistent with the observation that estrogens usually protect β cell mass and function in male models of diabetes (Tiano et al. 2011). I showed that the loss of β cell mass in *obe* males was associated with increased macrophage infiltration and inflammation in WAT, providing a plausible mechanism by which *Est* ablation exacerbated the metabolic phenotype in *ob/ob* males. The basal expression of *Est* in adipose tissue was high in male mice but barely detectable in female mice, and overexpression of *Est* inhibited adipogenesis (Khor et al. 2008; Wada et al. 2011), which may help to explain the male-specific effect of *Est* ablation on adipose tissue inflammation.

In summary, I have uncovered a critical role of *Est* in energy and glucose homeostasis during the pathogenesis of type 2 diabetes. My results suggested that hepatic estrogen signaling modulated by *Est* induction may represent an important mediator of gender-specific phenotypes usually observed in type 2 diabetes mouse models, even without apparently affecting systemic circulating estrogens. Hepatic *Est*, at least in females, may represent a therapeutic target for the management of type 2 diabetes.

4.0 PTER IV: SUMMARY AND PERSPECTIVES

In this dissertation study, I have uncovered an unexpected role of CAR in preventing obesity and alleviating type 2 diabetes. Activation of CAR prevented obesity and improved insulin sensitivity in both the HFD-induced type 2 diabetic model and *ob/ob* mice. The metabolic benefits of CAR activation may have resulted from inhibition of hepatic lipogenesis and gluconeogenesis. The underlying molecular mechanism through which CAR suppressed hepatic gluconeogenesis might be mediated *via* peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) by sequestrate it into promyelocytic leukemia (PML) nuclear bodies, thus preventing the PGC-1α from binding to the promoter region of gluconeogenic genes in the diabetic conditions (Fig. 33). In summary, my results have uncovered an important metabolic function for CAR in preventing and relieving obesity and type 2 diabetes. It is interesting to note that the anti-diabetic and anti-obesity effect appeared to be unique for CAR, because the same effect was not observed for pregnane X receptor, a sister xenobiotic receptor of CAR. My results established this "xenobiotic receptor" as a novel therapeutic target for the prevention and treatment of obesity and type 2 diabetes. It is encouraging to note that CAR-activating activities have been found not only in clinical drugs but also in neutraceuticals, such as herbal medicines, raising the hope that CAR could be a target for neutraceutical prevention and relief of metabolic syndrome.

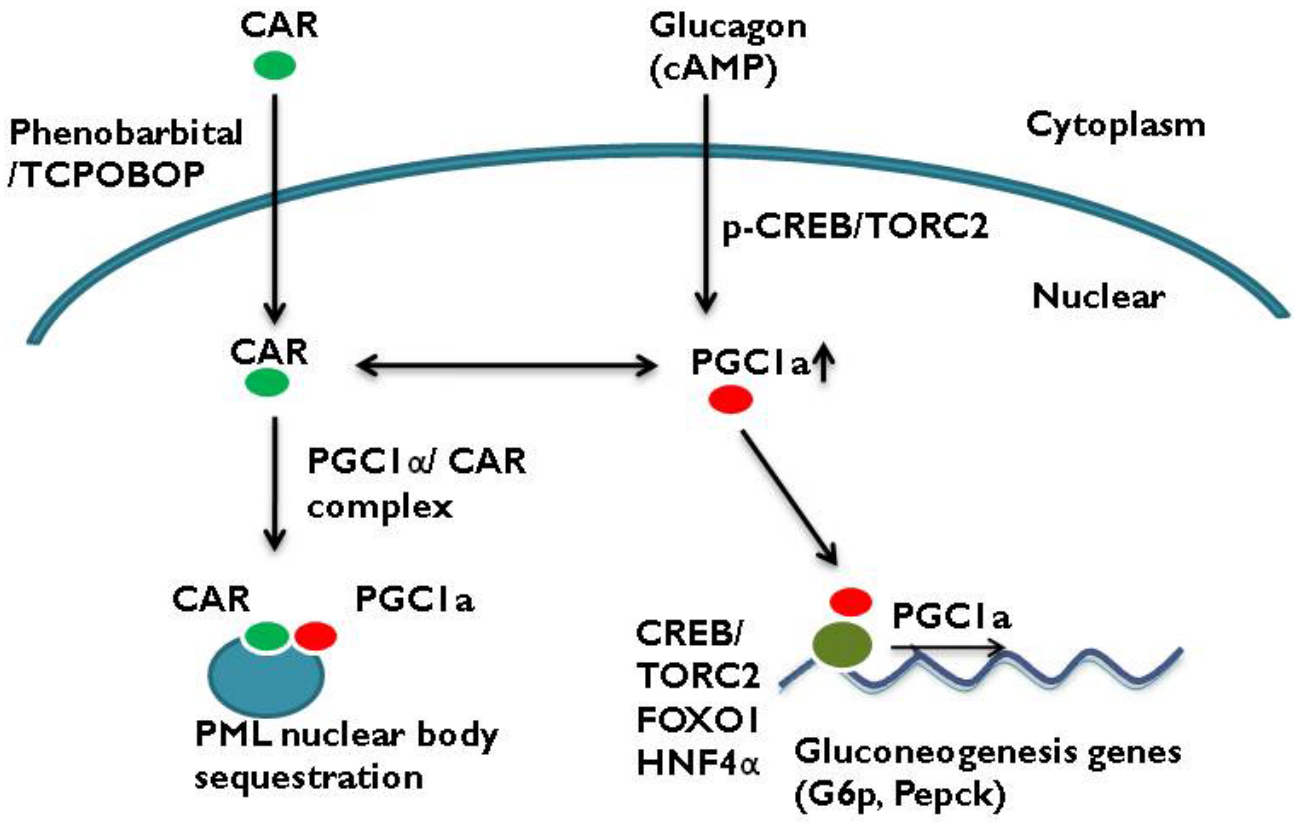

Figure 33. Model for CAR to sequestrate PGC-1α and inhibit its transcriptional activity.

CAR, upon activation by its agonist, translocates from cytoplasm to nuclear, where it binds to and forms complex with PGC-1α, and subsequently induces subnuclear redistribution of PGC-1α/CAR complex into PML nuclear bodies. The sequestration of PGC-1α prevents it from co-activating transcriptional factors which are recruited and bind to the promoter regions of gluconeogenic genes in the fasting condition or pathological condition of type 2 diabetes.

For future studies, to what extent the anti-obesity and anti-diabetic effect of CAR activation are mediated through sequestrating and suppressing PGC-1α activity requires further investigation. Our data has suggested CAR activation may suppress hepatic gluconeogenesis through PGC-1α, which provide one potential explanation for the anti-diabetic effect of CAR. More experiment need to be conducted to test whether the suppression of lipogenic genes and VLDL secretion by CAR activation are also mediated through PGC-1α. Our data suggested the CAR activation lead to PGC-1α redistribution and sequestration to PML nuclear bodies, thus inhibiting transcriptional activity of PGC-1α. This model suggests the transcriptional activity of CAR are not required for its suppression effect on PGC-1α, which has been partially supported by our data showed that ligand binding domain (LBD) of CAR alone also exert suppression effect on PGC-1α activity. For further experiments, adenovirus vector expression LBD fragment of CAR need to be constructed and tested in CAR null mice to confirm our model *in vivo*.

The interaction between CAR and PGC-1α indicates the cross-talk between energy homeostasis and drug metabolism. Since CAR can be activated by many clinical drugs, such as phenobarbital, it's interesting to investigate how these drugs may potentially influence energy metabolism through CAR activation. On the other hand, it's equally interesting to evaluate whether change of PGC-1α activity during physio-pathological conditions may also impact drug metabolizing capacity.

Since CAR is highly expressed in the liver, my current studies is mainly focused on the hepatic effect of CAR activation, and assumes the phenotype observed in other tissues, such as adipose tissue and muscle, are secondary. Although my data suggests CAR activation directly impact on hepatic gluconeogenesis and lipogenesis, I cannot rule out the possibility that CAR effect in tissues other than liver might also contribute to the anti-obesity/diabetic phenotype. For

further experiments, tissue-specific CAR null mice need to be generated to delineate the contribution of each organ to the overall phenotype observed. Although CAR was first identified as ‘xenobiotic receptors’, emerging evidence suggests equally important roles for this receptor in endobiotic homeostasis. Further studies on the endobiotic roles of CAR are expected to drive this ‘old’ receptor into a new era

Estrogens are implicated in various physiological functions besides reproduction. In recent years, the importance of estrogens in regulating energy and glucose homeostasis has gained increasing attention. Estrogen homeostasis is tightly regulated through balanced biosynthesis and metabolism, and sulfation is a dominant estrogen transformation and inactivation pathway. Estrogen sulfotransferase (EST, or SULT1E1) is the primary enzyme responsible for the sulfonation and inactivation of estrogens, and plays an important role in estrogen homeostasis. Here, I showed that induction of hepatic Est is a common feature of type 2 diabetes. Loss of Est in female mice improved metabolic function in ob/ob, dexamethasone- and high-fat diet-induced mouse models of type 2 diabetes. The metabolic benefit of Est ablation included improved body composition, increased energy expenditure and insulin sensitivity, and decreased hepatic gluconeogenesis and lipogenesis. This metabolic benefit appeared to have resulted from decreased estrogen deprivation and increased estrogenic activity in the liver, whereas such benefit was abolished in ovariectomized mice (Fig. 34). Interestingly, the effect of Est was gender specific, as Est ablation in *ob/ob* males exacerbated the diabetic phenotype, which was accounted for by the decreased islet β cell mass and failure of glucose-stimulated insulin secretion *in vivo*. The loss of β cell mass in obe males was associated with increased macrophage infiltration and inflammation in white adipose tissue.

In summary, I have uncovered a critical role of Est in energy and glucose homeostasis during the pathogenesis of type 2 diabetes. My results suggested that hepatic estrogen signaling modulated by Est induction may represent an important mediator of gender-specific phenotypes usually observed in type 2 diabetes mouse models, even without apparently affecting systemic circulating estrogens. Hepatic Est, at least in females, may represent a therapeutic target for the management of type 2 diabetes.

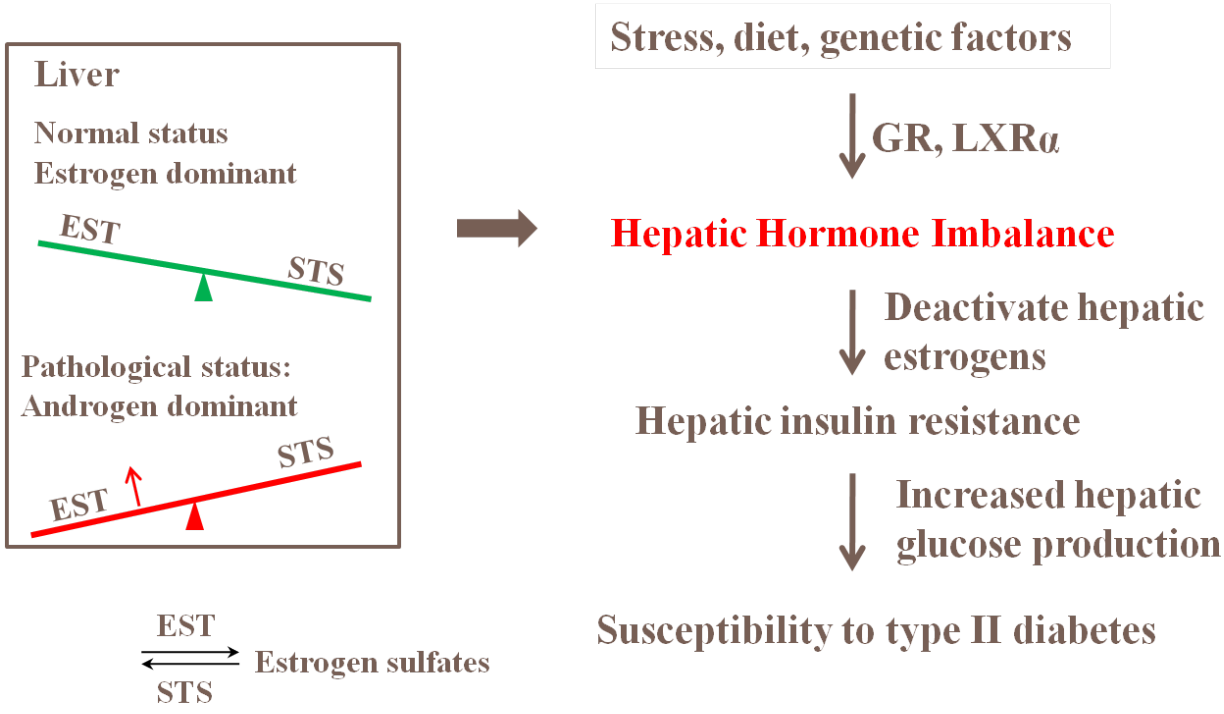

Figure 34. Model for hepatic estrogen sulfotransferase (EST) to regulate hepatic estrogen homeostasis and metabolic functions.

Hepatic estrogen homeostasis is well balanced through the reversible reaction of sulfation and desulfation by estrogen sulfotransferase (EST) and sulfatase (STS). In normal physiological condition, the STS activity dominates in the liver and estrogen signaling protects liver from developing dyslipidemia and insulin resistance. While in the pathological condition, especially with the rise of stress hormone glucocorticoids, EST expression is dramatically induced, and surpasses the STS activity. Under this condition, liver, especially in females, are "androgenized", and lost the protection from estrogen signaling, and become vulnerable to diet and environmental induced metabolic dysfunction such as type 2 diabetes.

For future study, the mechanism underlying increased ß cell death and inflammatory cell infiltration in adipose tissue requires further investigation. Based on our results, reduced ß cell mass in *obe* mice are mainly caused by increased ß cell death which is associated with enhanced macrophage infiltration. Since islet phenotypes in *obe* male mice are likely driven by extrapancreatic factors, it is with great interests that I observed increased macrophage infiltration and inflammation specifically in male white adipose tissue, given the facts that basal expression of *Est* in WAT follows a sexually dimorphic manner: abundantly expressed in male but barely detectable in female mice. It's tempting to speculate that increased inflammation along with degreased adiponectin synthesis, a beneficial adipokine, in WAT lead to low-grade systemic inflammatory response, exaggerated insulin resistance in *obe* male mice, and followed by failure of β cell compensation in the late stage. More experiment need to be conducted to confirm the causality relationship between them.

The association between *EST* ablation and increased inflammation in male WAT under diabetic condition is another intriguing topic for our further investigation. Although estrogen had been reported to suppress lipogenesis and exert anti-inflammatory effects in WAT, most of the studies are conducted in ovariectomized or estrogen receptor whole knockout mice, and the direct action of estrogens in adipose tissue especially regarding its sexually dimorphic manner, to our best knowledge, has not been carefully examined. The facts that *Est* highly expressed in male adipose tissue and EST null male displayed large adipocyte size as reported previously suggests that estrogens signaling in male WAT may be strictly regulated under normal physiological conditions and excess estrogens may cause adverse effects, instead of beneficial ones as we asserted currently, to males especially under extremely obese condition such as *ob/ob* mice. In fact, several lines of evidence suggested that testosterone has anti-obesity effects and its

deprivation in men contributes to the development of metabolic syndrome. For future study, the adipose-specific EST null mice currently under developing in our lab may serve as a valuable tool to further illustrate the direct effect of estrogens signaling in male adipose tissue.

APPENDIX A

SEQUENCES OF REAL-TIME PCR PRIMERS

ACC1_F: GCCTCTTCCTGACAAACGAG; ACC1_R: TGACTGCCGAAACATCTCTG

ADAM8_F: GCAGCCGACTCCTGCTATAC; ADAM8-R: CTCCATGGTTGGAGGAGAAA

AgRP_F: GGCCTCAAGAAGACAACTGC; AgRP_R: GACTCGTGCAGCCTTACACA

ATGL_F: TCCGAGAGATGTGCAAACAG; ATGL_R: CTCCAGCGGCAGAGTATAGG

CD68_F: CCAATTCAGGGTGGAAGAAA; CD68_R: CTCGGGCTCTGATGTAGGTC

CPT1b_F: GGTCGCTTCTTCAAGGTCTG; CPT1b_R: CGAGGATTCTCTGGAACTGC

Cyc1_F: TTCCCTGCTCACTGGCTACT; Cyc_R: GGGGTGCCATCATCATACTC

Cycs_F: CCAAATCTCCACGGTCTGTT; Cycs_R: CCAGGTGATGCCTTTGTTCT

CYP2B10_F: CATTTGTCTTGGTFAAAGCATT; CYP2B10_R: GGATGGACGTGAAGAAAAG

CYP4A10_F: CCACAATGTGCATCAAGGAG; CYP4A10_R: TTGGGTAAAGAGCGTGCATC

CYP4A14_F: TTGCCAGAATGGAGGATAGG; CYP4A14_R: CAGGAAATTCCACTGGCTGT

DIO2_F: GATGCTCCCAATTCCAGTGT; DIO2_R: TGAACCAAAGTTGACCACCA

F4-80_F: GCTGTGAGATTGTGGAAGCA; F4-80_R: ATGGCCAAGGCAAGACATAC

FABP1_F: AAATCGTGCATGAAGGGAAG; FABP1_R: GTCTCCAGTTCGCACTCCTC

FASN_F: CCCTTGATGAAGAGGGATCA; FASN_R: ACTCCACAGGTGGGAACAAG

G6p_F: TCTGCCCCAGGAATCAAAAAT; G6P_R: TGGGCAAAATGGCAAGGA

GcK_F: GGATGCAGAAGGAGATGGAC ; GcK_R: GGTTCCTCCCAGGTCTAAGG

HMGCS2-F: ATCAACTCCCTGTGCCTGAC; HMGCS2-R: GCAATGTCACCACAGACCAC

HSL_F: GCTTGGTTCAACTGGAGAGC; HSL_R: GCCTAGTGCCTTCTGGTCTG

LCAD_F: TCAATGGAAGCAAGGTGTTCA; LCAD_R: GCCACGACGATCACGAGA

MAC1_F: GACTCAGTGAGCCCCATCAT; MAC1_R: AGATCGTCTTGGCAGATGCT

MCAD-F: TTGAGTTGACGGAACAGCAG; MCAD-R: CCCCAAAGAATTTGCTTCAA

MIP-1a_F: TGCCCTTGCTGTTCTTCTCT; MIP-1a_R: GATGAATTGGCGTGGAATCT

NPY_F: TGGACTGACCCTCGCTCTAT; NPY_R: TAGTGTCGCAGAGCGGAGTA

PEPCK_F: AGGAGGAGTACGGGCAGTTG; PEPCK_R: CTTCAGCTTGCGGATGACA

PGC1A_F: GACTCAGTGTCACCACCGAAA; PGC1A_R: TGAACGAGAGCGCATCCT

PGC1b_F: TTGTAGAGTGCCAGGTGCTG; PGC1b_R: GATGAGGGAAGGGACTCCTC

POMC_F: CATTAGGCTTGGAGCAGGTC; POMC_R: CTTCTCGGAGGTCATGAAGC

PPARA_F: TGTCGAATATGTGGGGACAA; PPARA_R: AATCTTGCAGCTCCGATCAC

PPARA_F: TGTCGAATATGTGGGGACAA; PPARA_R: AATCTTGCAGCTCCGATCAC

SCD1_F: TTCTTACACGACCACCACCA; SCD1_R: CCGAAGAGGCAGGTGTAGAG

SHP_F: CTCATGGCCTCTACCCTCAA; SHP_R: GGTCACCTCAGCAAAAGCAT

SOCS3_F: AGCTCCAAAAGCGAGTACCA; SOCS3_R: AGCTGTCGCGGATAAGAAAG

SREBP1_F: CCCTGTGTGTACTGGCCTTT; SREBP1_R: TTGCGATGTCTCCAGAAGTG

UCP1_F: GGGCCCTTGTAAACAACAAA; UCP1_R: GTCGGTCCTTCCTTGGTGTA

UCP2_F: GCGTTCTGGGTACCATCCTA; UCP2_R: GCTCTGAGCCCTTGGTGTAG

UCP3_F: ATGAGTTTTGCCTCCATTCG; UCP3_R: GGCGTATCATGGCTTGAAAT

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