THE ROLE OF HEPATIC ESTROGEN RECEPTOR ALPHA IN CONTROL OF INSULIN SIGNALING PATHWAY AND GLUCOSE HOMEOSTASIS

A Thesis

by

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ABSTRACT

Estrogen has been reported to regulate various physiological processes such as cell growth, reproduction, development, and differentiation. Estrogen has also been shown to be connected with metabolic diseases by regulating glucose and lipid metabolism. The effects of estrogens are mediated mostly by estrogen receptors, estrogen receptor-α (ERα) and estrogen receptor-β (ERβ). Estrogens favor glucose homeostasis primarily through ERα, and ERα is the major ER isoform expressed in the liver. However, how ERα precisely regulates glucose metabolism in the liver remains unclear.

This study is aiming to explore the role of hepatic estrogen receptor alpha (ERα) in insulin signaling pathway to regulate glucose homeostasis under both physiological and pathological conditions. To determine the specific role of ERα in the liver, we use Cre-loxP recombination system to generate liver-specific ERα knockout mice ($ER\alpha^{LivKO}$). ERα flox mice ($ER\alpha^{F/F}$) were used as control wild-type mice. These mice were fed with a high-fat diet (HFD) for 12 weeks at the age of 5-6 weeks. Mice fed with a chow diet (CD) served as a control group. In the present studies, we found that in CD fed mice, hepatic ERα deletion led to impaired glucose tolerance and insulin signaling as evidenced by glucose tolerance tests and western blot in both male and female mice. In HFD fed group, HFD treatment impaired glucose homeostasis and induced inflammatory response as evidenced by glucose or pyruvate tolerance tests and quantification of gene expression. In HFD fed male mice, we did not observe significant differences in body weight, glucose tolerance, or mRNA expression of IRS between WT and $ER\alpha^{LivKO}$ mice. This may due to HFD treatment decreases ERα expression in WT male mice, loss of ERα protection in HFD fed male mice could be the reason. On the contrary, mice metabolic studies and histology studies showed hepatic ERα deficiency exacerbated insulin resistance and promoted lipid deposition in the liver from HFD fed female mice. In summary, we conclude that hepatic ERα plays an important role in mediating glucose and lipid homeostasis by participating in insulin signaling pathway under both healthy and pathological conditions.

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NOMENCLATURE

T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
PCK1	Phosphoenolpyruvate Carboxykinase 1
G6PC	Glucose-6-Phosphatase
PI3K	Phosphoinositide 3-Kinase
PDK1	3-Phosphatidylinositol-Dependent Kinase-1
NEFAs	Non-Esterified Fatty Acids
PIP2	Phosphorylate Phosphatidylinositol 4,5-Bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-Trisphosphate
CD	Chow Diet
HFD	High-Fat Diet
GTT	Glucose Tolerance Test
ITT	Insulin Tolerance Test
PTT	Pyruvate Tolerance Test
WT	Wild-type
ER	Estrogen Receptor
ERα	Estrogen Receptor-α
HGP	Hepatic Glucose Production
AKT	Protein Kinase B
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IR	Insulin Resistance

IRS	Insulin Receptor Substrate
MCP-1	Monocyte Chemotactic Protein-1
TNF-α	Tumor Necrosis Factor-α
RT-PCR	Reverse Transcription Polymerase Chain Reaction

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1. INTRODUCTION AND LITERATURE REVIEW

1.1 Type 2 Diabetes Mellitus

Diabetes mellitus has been one of the main threats to human public health in the 21st century. In the past two decades, the number of people diagnosed with diabetes worldwide has an explosive increase [1]. Globalization has been accompanied by changes in human environment, human behavior, and lifestyle, which results in an increased incidence of both obesity and diabetes [2].

There are two main forms of diabetes [3]. Type 1 diabetes (T1DM) is mainly caused by autoimmune-mediated destruction of pancreatic β-cell islets, leading to absolute insulin deficiency. Patients with type 1 diabetes must take exogenous insulin to survive to prevent the occurrence of ketoacidosis [2]. The incidence of type 1 diabetes is much lower than that of type 2 diabetes (T2DM), which accounts for more than 90% of global diabetes cases. Type 2 diabetes is characterized by insulin resistance and/or abnormal insulin secretion, either of which can be dominant. Exogenous insulin is not an absolute requirement for patients with type 2 diabetes, but if diet alone or oral hypoglycemic drugs cannot control blood sugar levels, insulin may be necessary [2].

1.1.1 Epidemiology of Type 2 Diabetes Mellitus

The number of people with diabetes has more than doubled during the past 20 years. One of the most worrying features of this rapid increase is the emergence of type 2 diabetes in children and young adults [4]. T2DM has been a major global public health threat. In 2010, global health expenditure for diabetes was estimated to be 12% of all global health expenditures. In the United States, the direct medical cost of diabetes was $176 billion in 2012 [4]. The International Diabetes Federation estimates that in 2013, there were 382 million adults aged 20-70 years old suffering from T2DM in the world. It is estimated that this number will increase to nearly 600 million by 2035 [5], the largest increases will come from people living in low- and middle-income countries. Asia is the center of the global diabetes epidemic due to rapid economic development, urbanization and nutritional transformation [6].

The most important risk factor for T2DM is increased obesity, which is reflected by higher BMI

levels. In the United States, the BMI of Asian descent is much lower than that of whites, and the risk of diabetes is increased by 30-50% [7]. Such ethnic variations could be attributed to different fat distributions and percentages of body fat [8]. Also, specific dietary components include lower intake of whole grains, green leafy vegetables, nuts, and coffee; intake of more refined grains, red and processed meats, and sugar-sweetened beverages is associated with an increased risk of T2DM [9]. In addition to diet, risk factors for T2DM also include cigarette smoking and physical activity, such as sedentary behavior [10]. Both short sleep (≤ 5 hours per night) and long sleep (≥ 9 hours per night) could increase the risk of T2DM development [11]. In humans, the prevalence of early insulin resistance, glucose intolerance, and T2DM is slightly higher in the early stage of men than in women [12].

In summary, T2DM has been one of the great challenges of healthcare in the 21st century. Improving the health care of people suffering from diabetes or diabetic complications in a preventive way is of great significance.

1.1.2 Pathophysiology of Type 2 Diabetes Mellitus

Type 2 diabetes is a heterogeneous disease characterized by chronic hyperglycemia caused by interactions between genetics, lifestyle and environment [12]. Reduced insulin secretion and decreased insulin sensitivity are the main underlying cause of profound postprandial hyperglycemia observed in patients with type 2 diabetes [13]. Both insulin resistance and β-cell dysfunction occur early in the pathogenesis of T2DM, insulin resistance is the earliest detectable abnormality in individuals who are likely to develop T2DM [14], with normal glucose tolerance gradually developing into glucose intolerance, leading to abnormal blood glucose levels and eventually lead to morbidity (nephropathy, neuropathy, retinopathy and increased risk of cardiovascular disease) and mortality [15].

One of the main risk factors for insulin resistance and subsequent T2DM is obesity [16]. Excess adiposity, assessed by a high BMI, is the strongest risk factor for T2DM [17] and is associated with many metabolic abnormalities that result in insulin resistance [18]. In T2DM, at a set insulin level, insulin resistance increases glucose production in the liver and decreased glucose uptake in muscle and adipose tissue [8]. When insulin cannot function normally in insulin-sensitive tissues, insulin

resistance occurs in the liver, muscle, and adipose tissue. Insulin resistance leads to β-cell stress [19], excessive secretion of islet amyloid polypeptide (IAPP) [20], reactive oxygen stress (ROS) [21], and activation of inflammatory response [19].

Abnormalities in β-cell function are essential for determining the risk and development of type 2 diabetes. Insulin secretion is impaired by dysfunction of pancreatic islet β-cells, in which β-cells are unable to secrete sufficient amounts of insulin to maintain normal glucose levels [22]. Impaired β-cell function is associated with epigenetic modifications [23] and microRNA patterns [24]. A variety of factors cause β-cell failure, including ageing [25], genetic abnormalities [26], resistance or lack of incretin hormone (glucagon-like peptide 1 (GLP1) and gastric inhibitory polypeptide (GIP)) [27], lipotoxicity [28], and glucotoxicity [29].

1.1.3 Mechanisms of Insulin Resistance

Most patients with type 2 diabetes have elevated plasma insulin levels [30]. The resistance of liver, fat and muscle to insulin is a major characteristic feature of T2DM and is the central pathophysiological event in the development of type 2 diabetes [31]. Pathological insulin resistance develops through complex interactions of obesity, heredity, and lifestyle, such as lack of exercise and overnutrition [32], which can cooperate to disrupt the balance in glucose and lipid metabolism. Insulin resistance places stress on β-cells, leading to β-cells dysfunction and a subsequent progressive decrease in insulin secretion [33].

The adipokines secreted by adipocytes that inhibit insulin sensitivity include TNFα, IL-6, and retinol-binding protein 4 [34]. Decreased insulin secretion impairs lipid metabolism in adipose tissue, leading to increased lipolysis and elevated levels of non-esterified fatty acids (NEFA) [35]. The accumulation of NEFA impairs IRS/PI3-kinase signaling, inducing translocation of GLUT4 to cytoplasm. Consistently, high levels of FFAs induced by overfeeding or metabolic stresses activate mTOR, JNK, and IKKβ. JNK activity is increased in liver, white adipose tissue, and skeletal muscle under insulin resistance state [31], which can increase serine and threonine phosphorylation of IRS1 and IRS2, leading to ubiquitination and degradation of IRS, thereby impairing insulin signaling [36].

1.1.4 Prevention of Type 2 Diabetes Mellitus

Although individual susceptibility to T2DM has heredity bias, strong evidence showed that many cases of T2D can be prevented by modifying lifestyles focusing on increasing physical activity and adopting a healthy diet.[37]. Lack of physical activity such as a sedentary lifestyle is a key behavioral risk factor for T2DM [38]. Increasing the amount of exercise is an essential component of all effective lifestyle-based prevention trials for T2DM. The Finnish Diabetes Prevention Study demonstrated that an increase in duration and intensity of exercise or even leisure-time physical activity (LTPA) is associated with decreased incidence of type 2 diabetes. Severe, structured LTPA reduced the incidence of T2D and prevented the evolution from impaired glucose tolerance to T2D in part by losing weight, which is a solid determinant of improved insulin sensitivity. Walking and low-intensity physical exercise also have benefits. Compliance with current findings, physical exercise may greatly reduce the incidence of type 2 diabetes and should be widely encouraged, especially in high-risk groups [39].

Diet is another important aspect of T2DM prevention. A reduction in total fat and calorie intake is beneficial to prevent people at high risk of type 2 diabetes with overweight [40]. Low glycemic index (GI) and high fiber foods have been shown to reduce HbA_{1C} and fasting plasma glucose in patients with type 2 diabetes. The quality and type of consumed fat are critical [9]. A higher intake of saturated fatty acids and cholesterol is associated with a higher risk for cardiovascular disease and trans fatty acids should be avoided, while the replacement of saturated fat with omega-6 polyunsaturated fatty acids (PUFA) is associated with a reduction in the risk of diabetes [41]. The use of meal replacements and high-protein diets also showed a reduction in HbA_{1C} [42].

Rich antioxidants, vitamins and unsaturated fatty acids in the Mediterranean diet can improve neurovascular health and reduce oxidative stress and chronic inflammation [43]. An observational study suggested that adherence to the Mediterranean diet in a group of middle-aged and elderly Puerto Ricans is associated with higher cognitive function in patients with type 2 diabetes, which is further maintained by control of glycemia [44].

Efforts are being made to implement lifestyle interventions in primary care and community settings [45]. Lifestyle interventions are safe, cost-effective, and effective in different age, gender,

racial and ethnic groups, independent of obesity and hyperglycemia [46]. Nevertheless, when lifestyle intervention is not feasible, pharmacological therapy can be considered as a strategy to prevent T2DM. For example, metformin reduced the incidence of T2DM by 31% during an average follow-up period of 2.8 years among populations having a high risk for diabetes in the United States [46].

1.2 Insulin Regulation of Glucose Homeostasis

Glucose homeostasis is mainly controlled by the liver, adipose tissue, and skeletal muscle. After a meal, most glucose disposal occurs in the skeletal muscle, and fasting plasma glucose levels mainly depend on the glucose output of the liver [47]. Glucose homeostasis is essential for maintaining the life of mammals. Following intake, glucose is absorbed and plasma levels elevate. This is a potent stimulation of insulin secretion by pancreatic β-cells. Insulin increases glucose disposal by peripheral tissues, and promotes the uptake of glucose and conversion to glycogen or triglycerides in muscle or adipose tissue, respectively. Insulin also stimulates glycogen synthesis and lipid synthesis in the liver. All these processes lead to the decrease in blood glucose levels and stop the stimulation of insulin secretion [48].

1.2.1 Insulin sensitivity

In normal individuals, insulin secretion from pancreatic β-cells is the response to increased plasma glucose levels [49]. This increase in circulating insulin levels directly regulates glucose production in the liver and indirectly regulates gluconeogenesis by acting on adipose tissue, skeletal muscle, and brain [50]. Insulin sensitivity in target tissues is physiologically regulated by circulating factors, including plasma lipids, circulating hormones [51], and adipokines [34]. The crosstalk between signaling pathways of these factors and the insulin signaling pathway constantly mediate insulin sensitivity [52].

1.2.2 Mechanisms of Insulin Action

Gluconeogenesis is the main driving force of liver glucose production in patients with type 2 diabetes [53]. Insulin inhibits the secretion of glucagon in pancreatic α cells, indirectly reducing hepatic glucose production (HGP) by blocking hepatic glucagon signaling [54]. Insulin also has

inhibitory effects on lipolysis, and decreases the plasma levels of non-esterified fatty acids (NEFAs) derived from adipose tissue [55]. A reduction of FFAs delivery to the liver has been shown to decrease hepatic glucose output [56]. Insulin signaling pathway mediates gluconeogenesis through the transcription activity of gluconeogenic genes [50]. Insulin also mediates about 75% of glucose clearance in skeletal muscle. Insulin signaling pathways that regulate glucose homeostasis include insulin receptor (IR), insulin receptor substrate (IRS), phosphoinositide 3-kinase (PI3K) and AKT kinase. In the muscle, activation of insulin signaling pathway leads to the translocation of glucose transporter 4 (GLUT4) from cytoplasm to the cell membrane, promoting the uptake of glucose into the cell [57]. Insulin also regulates hepatic gluconeogenesis by mediating the transcription activity of genes involved in the control of gluconeogenesis, including phosphoenolpyruvate carboxykinase 1 (PCK1) and glucose 6-phosphatase (G6pc) [58].

1.2.3 Hepatic Insulin Signaling

Insulin signaling is essential for maintaining glucose homeostasis. In mice lacking hepatic insulin receptor, glucagon secretion or hepatic glucose production is not inhibited by insulin, thus highlighting the importance of insulin receptor in the liver [59]. Insulin receptor is composed of 4 subunits, 2 extracellular α subunits and 2 transmembrane β subunits, once insulin binds to α subunits, β subunits with kinase activity will be autophosphorylated and be activated [60]. Insulin receptor will phosphorylate and activate insulin receptor substrates (IRS), IRS1 and IRS2 are the main isoforms. IRS proteins also play an essential role in regulating hepatic glucose production. Double knockout of IRS1 and IRS2 causes severe hyperglycemia, hyperinsulinemia, and induces expression of gluconeogenic genes, such as Pck1 and G6pc [61]. The activation of IRS proteins leads to the recruitment of lipid kinase PI3K to the plasma membrane. Once PI3K binds to IRS through P85 subunit, PI3K will phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3), which is an important second messenger of several growth factor receptors and mediators of PCK1 and G6pc expression levels [50].

The concentration of PIP3 increases, which stimulates the activity of 3-phosphatidylinositol-dependent kinase 1 (PDK1) [62] and recruits AKT into the plasma membrane. Insulin-stimulated PI3K-mediated phosphorylation of Akt at Ser473 by PDK1 can activate the kinase [63]. Akt kinase

controls multiple functions, including cell growth, survival, proliferation and metabolism. AKT can also phosphorylate many downstream proteins regulating the metabolism of insulin signaling [64].

1.3 Sex and Gender Differences in Pathophysiology of Type 2 Diabetes Mellitus

The sharp increase in the incidence of T2DM and associated complications is accompanied by more and more evidence of sex and gender differences in the clinic [65]. In humans, the prevalence of early insulin resistance and glucose intolerance is higher in men than in women [66]. However, women with estrogen deficiency after menopause show visceral obesity, insulin resistance and accelerated development of T2D [67]. Compared with age-matched men, premenopausal women exhibit enhanced insulin sensitivity and reduced incidence of T2D, but this advantage disappears after menopause, partly owing to a reduction in circulating 17β-estradiol (E2) [68]. Insulin sensitivity differs by gender, women are more resistant to insulin resistance induced by free fatty acids [69]. Women also tend to have elevated concentrations of postprandial insulin and C-peptide upon a meal test [70].

Compared with men, the mechanism by which women promote glucose homeostasis is unclear, but part of the reason may be the beneficial effects of circulating estrogen before menopause [71]. Sex hormones have a great influence on energy metabolism, body composition, blood vessel function and inflammatory response [65]. Modern personalized therapy has to consider differences in biological factors, such as genetic susceptibility, sex hormones, as well as behavioral and environmental differences between men and women [65]. The further characterization of these gender-specific differences in glucose homeostasis, insulin action, as well as the development of T2DM is essential to promote the development of diabetes treatments based on gender and will provide new ideas that can be used in clinical trials [71]. More research on the pathophysiological mechanisms of gender differences in T2DM and related complications may contribute to more understanding of gender and gender-specific risk factors and more personalized diabetes care in the future [65].

1.3.1 The Role of Estrogens in Pathogenesis of Metabolic Disease

Estrogen plays an important role in the physiology of reproduction, cardiovascular, and central nervous system. Estrogen mediates food intake, energy expenditure and lipid distribution in the hypothalamic nucleus. Estrogen has also been reported to regulate insulin production, promote insulin sensitivity and prevent inflammation. It was reported that estrogen deficiency can exacerbate metabolic dysfunction, inducing obesity, type 2 diabetes and certain cancers [72].

Estrogen signaling has beneficial effects on lipid metabolism by activating genes involved in lipolysis, such as hormone-sensitive lipase (HSL) and subsequent induction of lipolysis and inhibiting lipogenesis in the liver mainly by reducing the activity of lipoprotein lipase (LPL). Estrogen also promotes fatty acids oxidation in the muscle, thereby limiting the delivery of fatty acids to the liver. However, lack of estrogen induces the accumulation of triglycerides in the liver [73]. Proinflammatory cytokines such as IL-6 and TNFα are inhibited by high levels of E2, while postmenopausal women are more susceptible to chronic inflammation [74].

1.3.2 Estrogen Receptor α Regulates Metabolic Homeostasis in Different Tissues

The biological effects of estrogen are mainly mediated by estrogen receptors (ER), ERα and ERβ [75]. Estrogens regulate glucose homeostasis primarily through ERα. Estrogen enters the plasma membrane, then interacts with intracellular ERα by binding to DNA sequences [76]. ERα is able to translocate into the nucleus and induce the transcription activity of different genes by binding to DNA [77]. Estrogen receptor-α belongs to a large family of transcription factors activated by binding with estrogen. It structurally contains activation function domains (AF-1 and AF-2), a DNA-binding domain (DBD) responsible for interaction with estrogen response element (ERE), and a ligand-binding domain (LBD) for the 17 β-estradiol [78].

The ablation of ERα in the ventromedial hypothalamic nucleus (VMN) leads to an increase in food consumption, and a decrease in energy expenditure due to impaired thermogenic responses to feeding, indicating ERα plays an important role in regulating central energy homeostasis. [79]. Both female and male ERα knockout mice exhibit increased adipose tissue mass, aggravated insulin resistance and glucose intolerance, as well as adipocyte hyperplasia and hypertrophy [80].

The lack of ERα results in pancreatic islet dysfunction and subsequent hyperinsulinemia [81]. E2 treatment increased insulin production and improved insulin resistance. However, the protective effects of E2 were blocked in ERα knockout female mice [82].

ERα is the predominant ER isoform in hepatocytes [83]. ERα regulates the effect of E2 on the inhibition of hepatic glucose production (HGP) in the liver. E2 has been shown to reduce HGP, gluconeogenesis, and expression levels of gluconeogenic genes [68]. However, how ERα precisely regulates glucose metabolism in the liver remains to be elucidated.

2. INSULIN SENSITIVITY AND GLUCOSE TOLERANCE IS IMPAIRED IN HFD-INDUCED OBESITY MICE

2.1 Introduction

The rodent model of HFD-induced obesity has been widely used to study obesity and T2DM in humans. Obesity is the critical risk factor for insulin resistance and the development of T2DM [84] and other metabolic syndromes such as dyslipidemia and hypertension [85]. Overnutrition intake contributes to chronic inflammation, which regulates metabolic homeostasis [31]. Inflammation contributes to insulin resistance under obesity and diabetes states. Insulin resistance itself can also promote inflammation by impeding the anti-inflammatory effect of insulin [86]. Interactions between obesity, insulin resistance and β-cell dysfunction result in human T2DM [16].

In obese individuals, adipose tissue releases increased amounts of non-esterified fatty acids (NEFAs), glycerol, pro-inflammatory cytokines that contribute to the development of insulin resistance [87]. Elevated NEFA levels induce insulin resistance and impair β-cell function, preventing the expected compensatory β-cell response. Prolonged exposure to a high concentration of NEFAs is associated with impaired insulin secretion stimulated by glucose and reduced insulin biosynthesis [84].

In addition to metabolites derived from adipose tissue, the release of products from macrophages, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) also increases in obesity [88]. TNF-α and IL-6 stimulate both the c-Jun amino-terminal kinase (JNK) and the IB kinase-β (IKK-β)/nuclear factor-κB (NF-κB) pathways, inducing inflammatory response that may cause insulin resistance [84]. TNFα impairs insulin signaling by inhibiting the phosphorylation of insulin receptor (IR) at tyrosine residues and induces serine phosphorylation of insulin receptor substrate-1 (IRS-1), thereby weakening the association of PI3K to IRS-1 and the subsequent activation of Akt. The effects of IL-6 on inhibition of insulin signaling transduction were demonstrated in hepatocytes and in mice [86]. Here, we use the HFD-induced obesity rodent model to determine if the expression of hepatic ERα is different by HFD feeding

from CD feeding.

2.2 Methods

Animal Experiments

C57BL/6J mice for both genders from the Jackson Laboratory were fed with either a Chow Diet (CD) or a High Fat Diet (HFD) at the age of 5-6 weeks old for 12 weeks. CD contains 4% fat of total kcal, while composition of HFD is 60% fat, 20% protein and 20% carbohydrate of total kcal. Mice were housed at constant temperature under a 12-h light/dark cycle with free access to water and food. After the feeding period, mice were anesthetized with isoflurane and sacrificed for tissue samples as well as plasma collection. The animal experiments and protocols were approved by the Texas AM University.

Glucose Tolerance Test

Mice were received 2 g/kg body weight D-glucose via i.p injection after fasted for overnight (approximately 16 h). Blood glucose levels were measured from tail vain with a glucometer (Bayer, Whippany, NJ) at 15, 30, 60, 90, and 120 min after glucose administration. Glucose tolerance test (GTT) measures the ability of mice to clear the exogenous glucose load.

Pyruvate Tolerance Test

Mice were received 2 g/kg body weight pyruvate sodium via i.p injection after fasted for overnight (approximately 16 h). Blood glucose levels were measured from tail vain with a glucometer (Bayer, Whippany, NJ) at 15, 30, 60, 90, and 120 min after pyruvate administration. The pyruvate tolerance test (PTT) is used to elicit a glycemic excursion that will reflect the hepatic gluconeogenesis.

Insulin Tolerance Test

Mice were received 1 U/kg body weight insulin via i.p injection after fasted for approximately 4 h. Blood glucose levels were measured from tail vain with a glucometer (Bayer, Whippany, NJ) at 15, 30, 45, and 60 min after insulin administration. Insulin tolerance test (ITT) were used to determine the ability of mice to clear endogenous glucose after giving an injection of insulin.

Quantitative Real-Time PCR

Total RNAs were extracted with TRIzol reagent (Invitrogen Life Technologies). The cDNAs

were synthesized using iScript^{TM} Reverse Transcription Supermix (Bio-Rad). Quantitative real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The primers are listed in APPENDIX Table 1.

Statistical Analysis

All results are presented as mean ± SEM. P values were calculated using the Student-t test for the comparison of difference between two groups. $P < 0.05$ was considered statistically significant.

2.3 Results

HFD impairs glucose homeostasis in both male and female mice

We firstly investigated the effects of overnutrition on glucose metabolism. C57BL/6J male and female mice at 5-6 weeks of age were fed ad libitum with a chow diet (CD) or a high-fat diet (HFD) for 12 weeks. Compared to age- and gender-matched mice that were fed with a CD, mice in HFD group exhibited higher fasting blood glucose levels after fasted for approximately 16 h. HFD fed male mice showed 19%, while HFD fed female mice showed 15% higher blood glucose than CD fed mice (Figure 2.1 (A)). We also performed insulin and glucose tolerance tests on these mice. Comparing to CD fed mice, the plasma glucose levels of HFD fed mice kept higher after insulin ingestion during insulin tolerance test (Figure 2.1 (B)), which indicated that the tissues of HFD fed mice cannot respond to insulin properly. After receiving a solution of 2 g/kg body weight glucose, plasma glucose levels had a more profound increase in HFD group, which was observed in both male and female mice, suggesting HFD fed mice displayed impaired glucose tolerance (Figure 2.1 (C and D)). Consistent with metabolic study results, mRNA expression of IRS1 or IRS2 in the liver was markedly reduced in HFD fed mice (Figure 2.1 (E and F)), suggesting insulin signaling was impaired with overnutrition treatment. Interestingly, the expression of hepatic ERα was significantly downregulated in HFD fed male mice, while it was markedly upregulated in HFD fed female mice (Figure 2.1 (E and F)), sex and gender difference in hepatic ERα expression by HFD feeding remains to be explored.

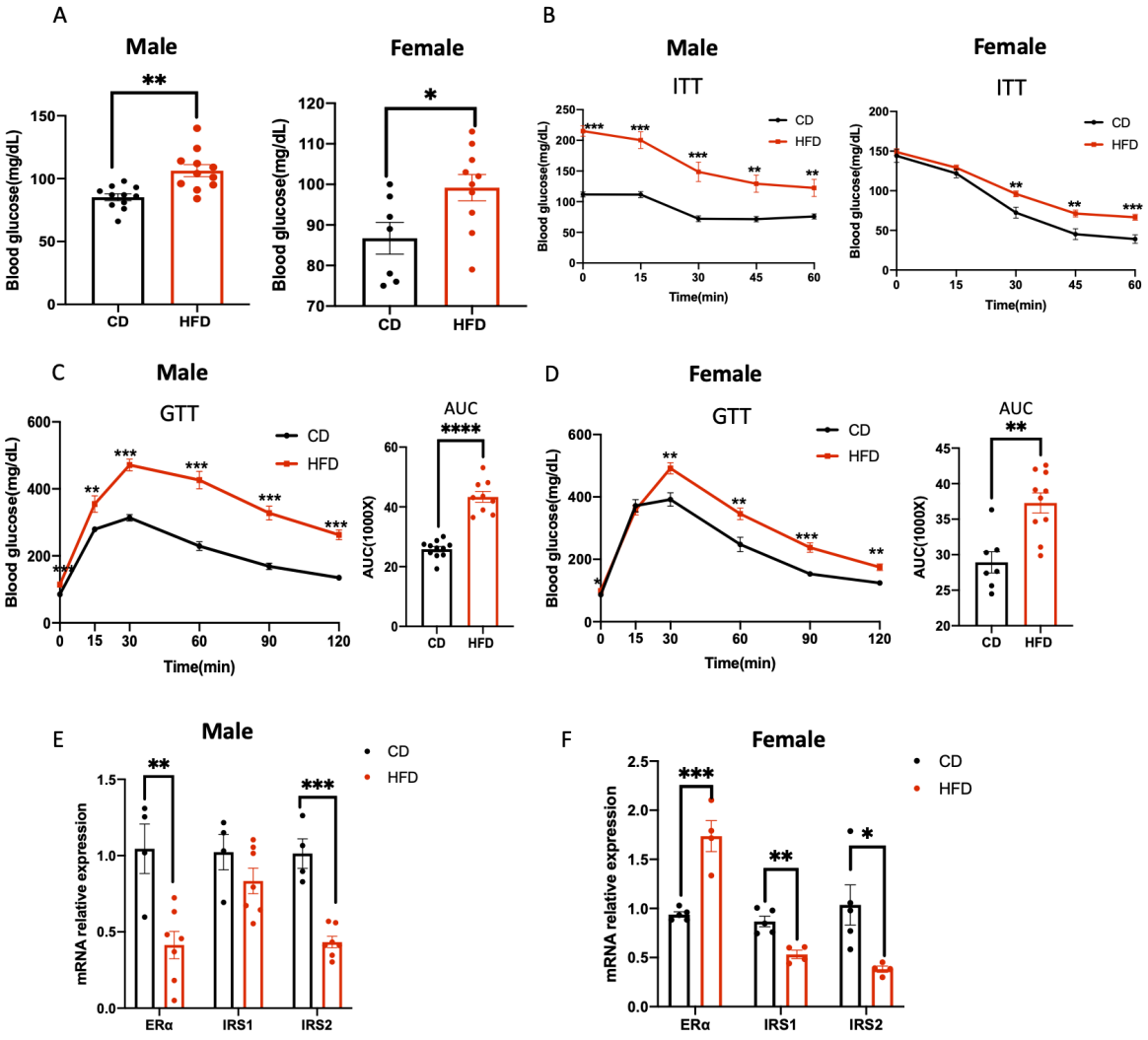

Figure 2.1: HFD impairs glucose homeostasis in both male and female mice.
(A) Fasting blood glucose levels of mice by CD or HFD feeding were measured after 16 h fasting. Left panel, male mice; right panel, female mice.
(B) Insulin was administered at 1 U/kg body weight of mice by intraperitoneal injection after 4 h fasting, and glucose levels were measured at indicated time points. Left panel, male mice; right panel, female mice.
(C and D) Glucose tolerance test and area under curve (AUC) of GTT in male and female mice, respectively. Glucose was administered at 2 g/kg body weight of mice by intraperitoneal injection after 16 h overnight fasting, and glucose levels were measured at indicated time points. All data are presented as mean ± SEM, n=6-10. *, P<0.05, **, P<0.01, ***, P<0.001 versus Vehicle.
(E and F) Relative mRNA levels of ERα, IRS1, and IRS2 in the liver were measured by real-time qPCR, n=4-5. *, P<0.05, **, P<0.01, ***, P<0.001 versus Vehicle.

HFD treatment induces hepatic inflammatory response in male mice.

To investigate the effects of overnutrition on the inflammatory response regarding gender difference. We also performed Real-time qPCR in the liver from male and female mice with CD or HFD treatment. mRNA expression levels of pro-inflammatory cytokines were measured to help link overnutrition treatment with inflammation. In the present study, we found that HFD treatment increased inflammatory responses in the liver from HFD fed male mice as evidenced by upregulated expression of IL-1β, IL-6 and MCP1 (Figure 2.2 (A)). On the contrary, no significant difference was observed in mRNA expression of these cytokines between CD fed and HFD fed female mice (Figure 2.2 (B)). These results indicated that there was a sex difference in inflammatory response induced by overnutrition.

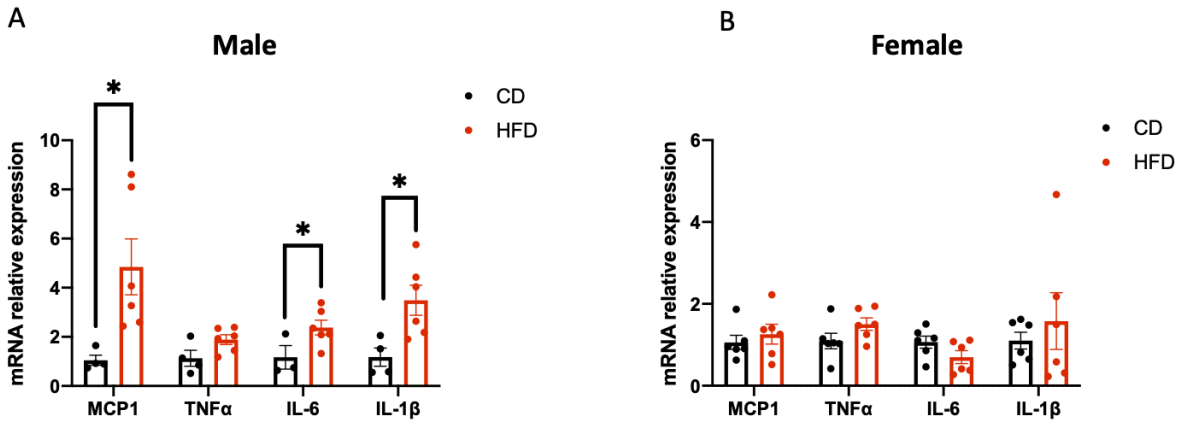

Figure 2.2: HFD treatment induces hepatic inflammatory response in male mice
(A) Relative mRNA levels of pro-inflammatory cytokines in the liver from male mice with CD or HFD treatment.
(B) Relative mRNA levels of pro-inflammatory cytokines in the liver from female mice with CD or HFD treatment. All data are presented as mean ± SEM, n=4-6. *, P<0.05 versus Vehicle.

2.4 Summary

Compared with CD fed mice, HFD fed C57BL/6J mice showed a more profound increase in body weight and higher fasting blood glucose levels. Concurrently, mice in the HFD group displayed impaired glucose tolerance and insulin sensitivity, along with reduced mRNA expression of hepatic IRS. These results demonstrated that HFD treatment induced hyperglycemia, glucose intolerance and insulin resistance in both male and female mice. Subsequent events downstream of insulin receptor substrate were also impaired with overnutrition treatment. Interestingly, mRNA expression of hepatic ERα was regulated differently in male and female mice, in which hepatic ERα was downregulated in male mice with HFD treatment, while increased in HFD fed female mice. What's more, sex and gender differences were also manifested in HFD-induced inflammation pathway, gene expression of pro-inflammatory cytokines were increased in the liver from HFD fed male mice. However, HFD fed female mice were resistant to overnutrition-induced inflammation response. Sex and gender differences in hepatic ERα expression and inflammatory response with overnutrition treatment remain to be elucidated. Also, the specific role of hepatic ERα in glucose and lipid homeostasis under physiological and HFD-induced pathological conditions will be discussed further in the following chapters.

3. HEPATIC ERα IS INVOLVED IN INSULIN SIGNALING TO REGULATE GLUCOSE HOMEOSTASIS

3.1 Introduction

During aging, there is a decline in E2 and subsequent ERα and ERβ activation, which provides evidence that reduction in the expression of ERα and ERβ may determine the decline in hippocampal function and cognition [89]. mRNA levels of ERα was reduced in isolated adipocytes from obese women compared to non-obese women [90]. ERα knockout mice for both genders exhibited impaired glucose tolerance (IGT), indicating that hypoglycemic effect of estrogen is partially mediated by ERα [80]. Researches on diabetic mice propose that estrogen regulates lipid metabolism exerting an anti-diabetic effect in the liver via ERα [91]. The role of ERα in regulating metabolic homeostasis has been demonstrated in a variety of studies from rodents and humans. Estrogen has been shown to regulate glucose homeostasis by promoting hepatic insulin sensitivity mainly via ERα [81], which might be due to the up-regulation of lipogenic genes. After 2 h of E2 treatment, an overrepresentation analysis revealed that 19 genetic categories including carboxylic acid metabolism, lipid metabolism, and amino acid metabolism significantly enriched the ERα promoter genes [83]. However, the signaling cascade from estrogen to estrogen receptor (ER) to the regulation of glucose metabolism remains unclear.

In the present work, we first determined the difference in the ability to clear exogenous glucose load and to produce hepatic glucose between WT and liver-specific ERα knockout mice for both genders by CD feeding. Second, we measured the expression of IRS in the liver and detected signaling events downstream of insulin receptor substrate in $ER\alpha^{LivKO}$ mice. Third, we determined difference in glucose tolerance and insulin sensitivity between WT and $ER\alpha^{LivKO}$ mice under a pathological state induced by HFD. Finally, we determined lipid profile differences between $ER\alpha^{LivKO}$ and WT mice by HFD treatment via liver histological analysis and determination of gene expression involved in lipid metabolism.

3.2 Methods

Animal Experiments

The transgenic mice carrying ERα floxed alleles ($ER\alpha^{F/F}$) were bred with the albumin-Cre mice to generate the liver-specific ERα knockout ($ER\alpha^{LivKO}$) mice as well as WT littermates ($ER\alpha^{F/F}$) mice used as control mice. Mice for both genders from the Jackson Laboratory were fed with either a Chow Diet (CD) or a High Fat Diet (HFD) at the age of 5-6 weeks old for 12 weeks. CD contains 4% fat of total kcal, while composition of HFD is 60% fat, 20% protein and 20% carbohydrate of total kcal. Mice were housed at constant temperature under a 12-h light/dark cycle with free access to water and food. During the 12-week feeding period, body weight of mice in both groups was monitored weekly. After the feeding period, mice were anesthetized with isoflurane and sacrificed for tissue samples as well as plasma collection. The animal experiments and protocols were approved by the Texas AM University.

Glucose Tolerance Test

Mice were received 2 g/kg body weight D-glucose via i.p injection after fasted for overnight (approximately 16 h). Blood glucose levels were measured from tail vain with a glucometer (Bayer, Whippany, NJ) at 15, 30, 60, 90, and 120 min after glucose administration. Glucose tolerance test (GTT) measures the ability of mice to clear the exogenous glucose load.

Pyruvate Tolerance Test

Mice were received 2 g/kg body weight pyruvate sodium via i.p injection after fasted for overnight (approximately 16 h). Blood glucose levels were measured from tail vain with a glucometer (Bayer, Whippany, NJ) at 15, 30, 60, 90, and 120 min after pyruvate administration. The pyruvate tolerance test (PTT) is used to elicit a glycemic excursion that will reflect the hepatic gluconeogenesis.

Insulin Tolerance Test

Mice were received 1 U/kg body weight insulin via i.p injection after fasted for approximately 4 h. Blood glucose levels were measured from tail vain with a glucometer (Bayer, Whippany, NJ) at 15, 30, 45, and 60 min after insulin administration. Insulin tolerance test (ITT) were used to determine the ability of mice to clear endogenous glucose after giving an injection of insulin.

Quantitative Real-Time PCR

Total RNAs were extracted with TRIzol reagent (Invitrogen Life Technologies). The cDNAs were synthesized using iScript^{TM} Reverse Transcription Supermix (Bio-Rad). Quantitative real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The primers are listed in APPENDIX Table 1.

Western Blot

Protein extracted from liver tissues and protein markers (cell signaling Technology) were subjected to sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis, and transferred onto a PVDF membrane for western blotting. Membranes were incubated with primary antibody specific to the protein of interest at a 1: 1000 dilution at 4 °C overnight. Subsequently, membranes were incubated with a 1:10000 dilution of goat anti-rabbit IgG, HRP-linked Antibody (CST 7074S) for 2 h at room temperature. The loading control is glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primary antibodies against pAKT-S473 (CST 4060S), pAKT-T308 (CST 13038S), Akt (CST 4691S) and GAPDH (CST 5174S) were purchased from Cell Signaling Technology (Danvers, MA, USA). The intensity of each band was analyzed by the ImageJ software (National Institutes of Health, USA). Antibodies information is listed in APENDIX Table 2.

Histopathological assay

Mouse liver tissue specimens were fixed in 4% formalin overnight, dehydrated, then waxed and embedded in paraffin. For H&E histology sections (4-5 μm) were sliced, baked in a 60°C oven for 3 h and stained with haematoxylin & eosin. Specimens were imaged with Leica Aperio scanscope slide scanner.

Statistical Analysis

All results are presented as mean ± SEM. P values were calculated using the Student-t test for the comparison of difference between two groups. $P < 0.05$ was considered statistically significant.

3.3 Results

Hepatic ERα deficiency leads to glucose intolerance in male and female mice by CD feeding

To examine the specific role of ERα in the liver, we generated liver-specific ERα knockout (*ER*α^{LivKO}) mice by breeding the transgenic mice carrying ERα floxed alleles (*ER*$\alpha^{F/F}$) with the albumin-Cre mice. ERα fl/fl (*ER*$\alpha^{F/F}$) and ERα fl/fl::Cre (*ER*α^{LivKO}) were selected and analyzed. With specific primers, PCR was used to genotype each animal. Liver-specific ERα deletion was confirmed by RT-qPCR, the mRNA levels of ERα in the liver was significantly reduced in *ER*α^{LivKO} mice of both male and female (Figure 3.2 (A and B)). At least 6 mice per genotype at the age of 6 to 8 weeks old were selected and analyzed metabolically. In chow-diet fed mice, glucose tolerance was significantly impaired in *ER*α^{LivKO} mice, which was observed in both male and female mice (Figure 3.1 (A and B)). Pyruvate tolerance test results showed that *ER*α^{LivKO} mice exhibited a higher rate of hepatic glucose production in response to pyruvate via intraperitoneal injection (Figure 3.1 (C and D)). Animal metabolic studies indicated that mice with hepatic ERα deficiency exhibited impaired glucose homeostasis compared to WT mice under physiological sate.

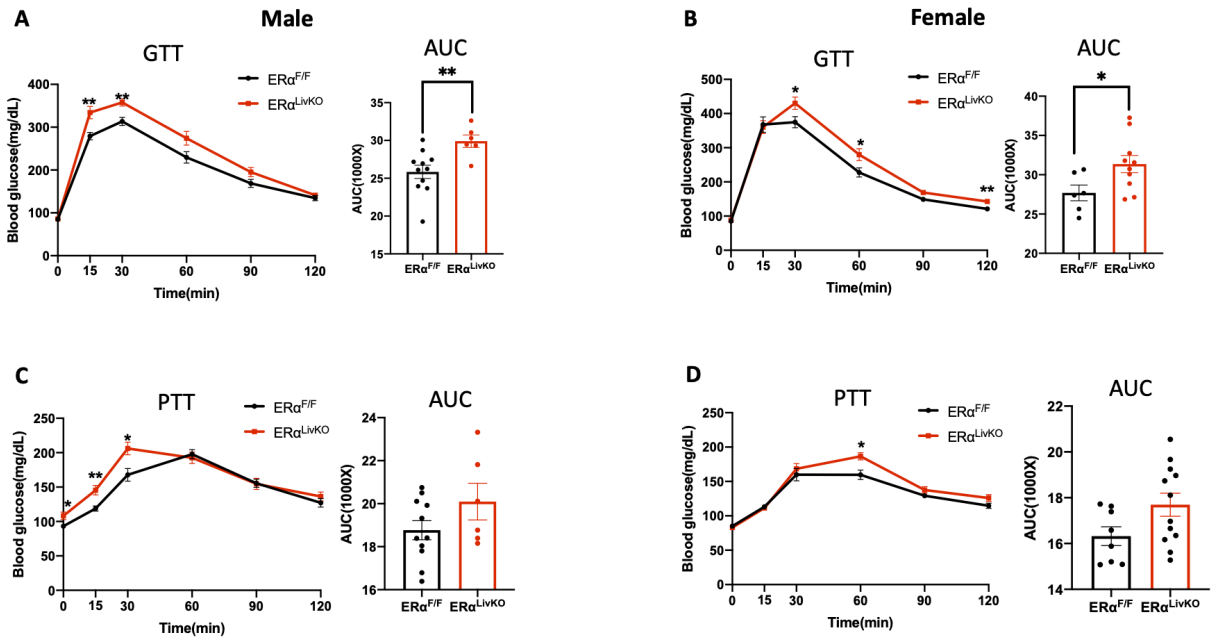

Figure 3.1: Hepatic ERα deficiency leads to glucose intolerance in CD fed mice for both genders. (A and B) Glucose tolerance test and area under curve (AUC) of GTT in male and female mice, respectively. Glucose was administered at 2 g/kg body weight of mice by intraperitoneal injection after 16 h overnight fasting, and glucose level was measured at indicated time points.
(C and D) Pyruvate tolerance test and area under curve (AUC) of PTT in male and female mice, respectively. Pyruvate was administered at 2 g/kg body weight of mice by intraperitoneal injection after 16 h overnight fasting, and glucose level was measured at indicated time points. All data are presented as mean ± SEM, *, P<0.05 and **, P<0.01, n=6-10.

Hepatic ERα deletion diminished events downstream of IRS signaling

Consistent with that, mRNA expression of IRS1 extracted from liver samples was significantly downregulated in *ER*α^{LivKO} mice (Figure 3.2 (A and B)), which led us to detect the expression of proteins downstream of IRS. To further elucidate the role of hepatic ERα in insulin signaling pathway, we detected the protein expression of phosphorylated AKT (pAKT), which is a downstream molecule of insulin. Mice were received 2 U insulin via intravenous injection, after 5 minutes mice were anesthetized and liver samples were excised. Western blot results showed that the protein expression of pAKT at Ser473 or Thr308 residue was markedly reduced in livers from *ER*α^{LivKO} mice of both sexes (Figure 3.2 (C and D)). These results suggested deletion of hepatic ERα impairs insulin signaling pathway by diminishing insulin-induced AKT phosphorylation.

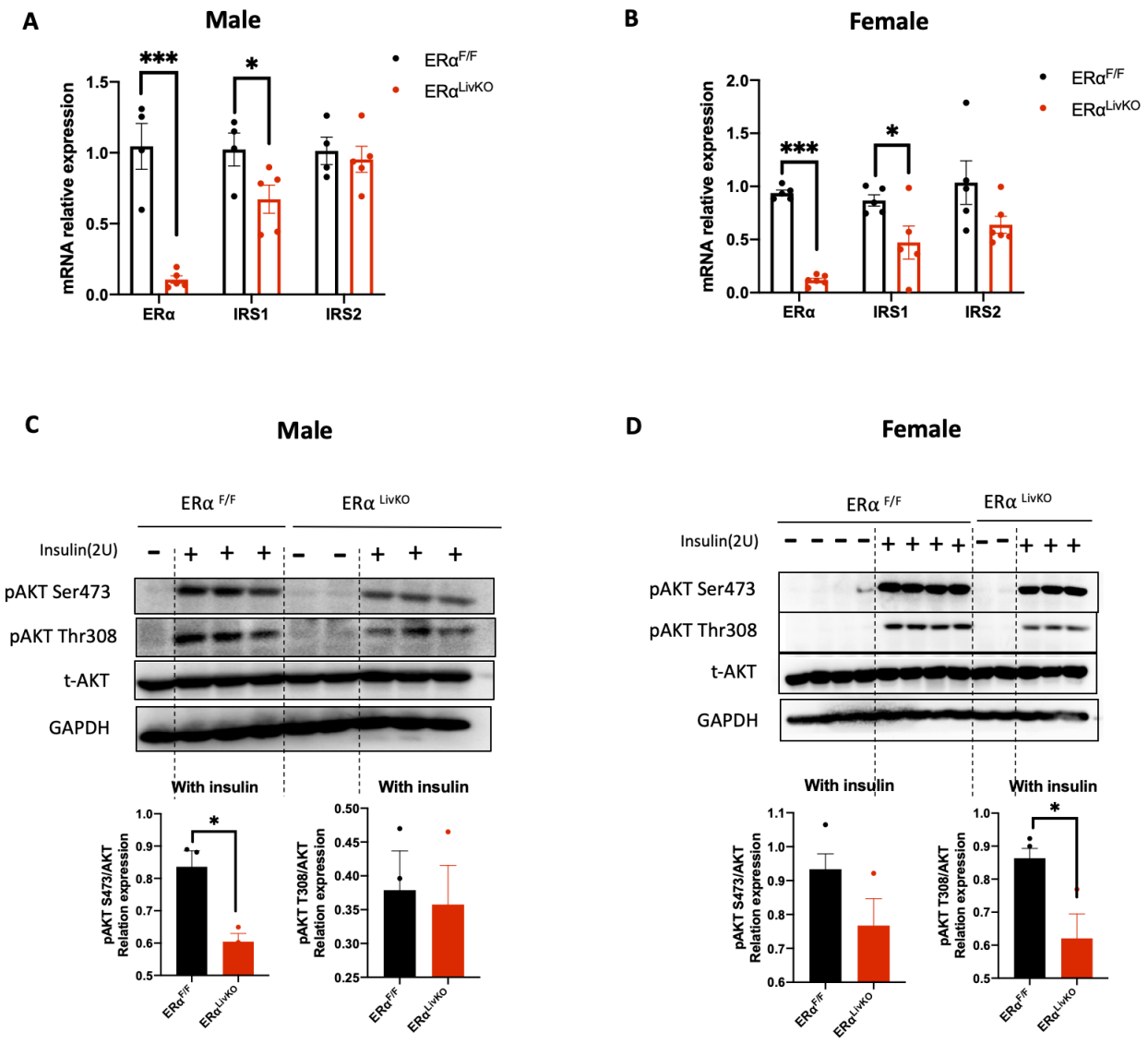

Figure 3.2: Hepatic ERα deletion diminished events downstream of IRS signaling.
(A and B) Relative mRNA levels of ERα, IRS1, and IRS2 in the liver were measured by real-time qPCR, n=4-5.
(C and D) Protein levels involved in insulin signaling were measured after 5 min intravenous injection of 2U insulin by Western Blots and relative intensity in the liver from male and female mice, respectively. p-, phosphorylated; t-, total. All data are presented as mean ± SEM, *, P<0.05 and **, P<0.01, n=6-10.

Hepatic ERα deletion exacerbates glucose intolerance in HFD fed female mice

To determine the effects of overnutrition on glucose and lipid metabolism in mice with hepatic ERα deficiency, we fed WT and *ER*α^{LivKO} mice with a high-fat diet for 12 weeks. During the feeding period, we monitored their body weight weekly. In HFD fed male mice, there was no obvious difference in body weight increase between WT and *ER*α^{LivKO} mice (Figure 3.3 (A)). Before they were sacrificedwe measured their body composition. No significant difference was observed in fat mass and lean mass ratio between WT and *ER*α^{LivKO} mice (Figure 3.3 (C)). In addition, HFD fed *ER*α^{LivKO} mice did not display obvious glucose intolerance compared to control mice (Figure 3.3 (E)). On the contrary, in HFD fed female mice, *ER*α^{LivKO} mice showed profound increases in body weight starting from 7 weeks of feeding (Figure 3.3 (B)). Consistent with that, fat mass ratio was markedly higher while lean mass ratio was significantly lower in *ER*α^{LivKO} mice compared to control mice (Figure 3.3 (D)). What's more, HFD fed female mice with hepatic ERα deletion exhibited more severe glucose intolerance (Figure 3.3 (F)).

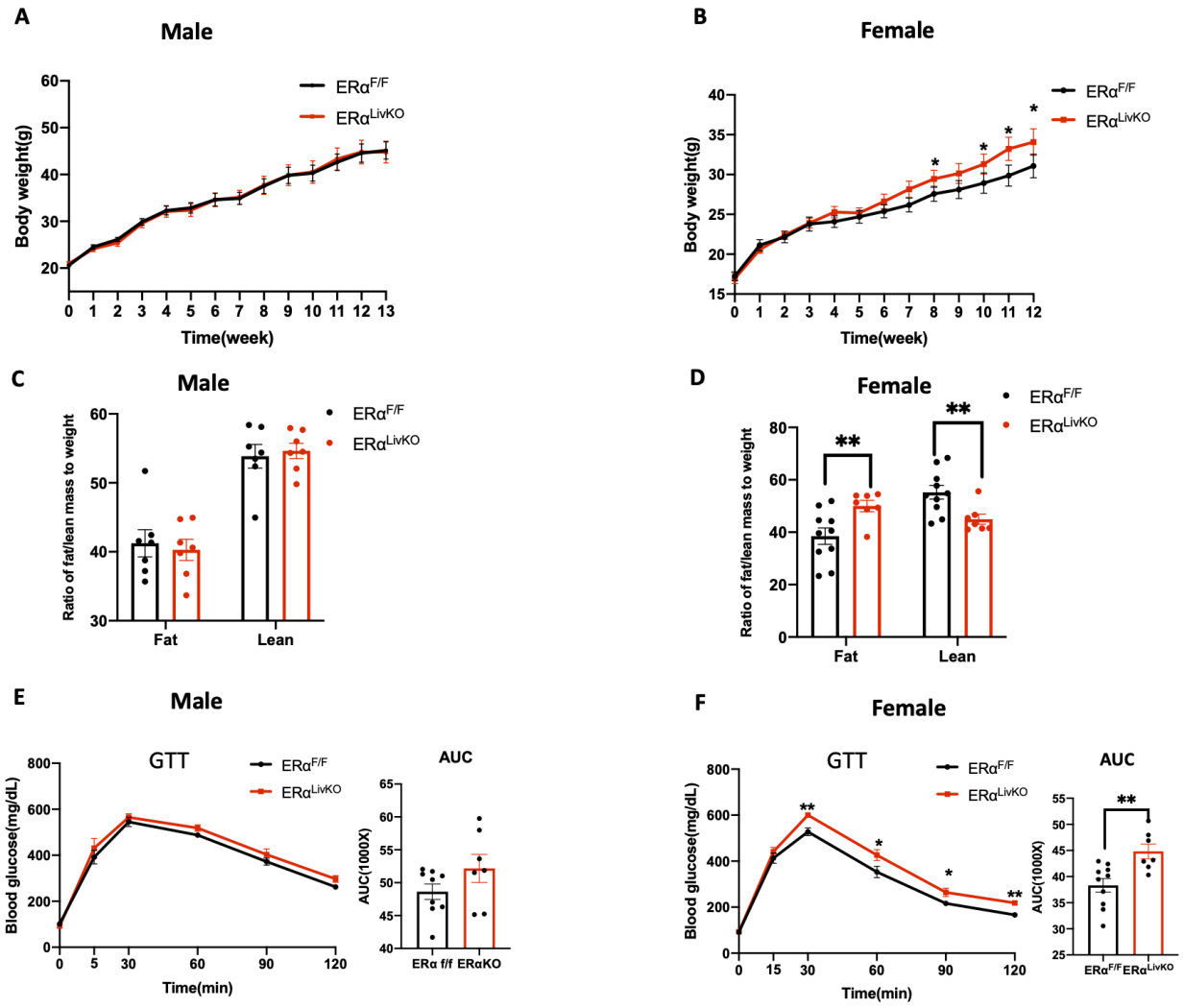

Figure 3.3: Hepatic ERα deletion exacerbates glucose intolerance in HFD fed female mice. (A and B) Body weight was monitored weekly in HFD fed male and female mice, respectively. (C and D) Body composition was measured in HFD fed male and female mice, respectively. (E and F) Glucose tolerance test and area under curve (AUC) of GTT in HFD fed male and female mice, respectively. Glucose was administered at 2 g/kg body weight of mice by intraperitoneal injection after 16 h overnight fasting, and glucose level was measured at indicated time points. All data are presented as mean ± SEM, *, P<0.05 and **, P<0.01, n=6-10.

Hepatic ERα deletion exacerbates insulin resistance in HFD fed female mice

HFD fed *ER*α^{LivKO} mice had higher blood glucose levels than WT mice in ITT, which was more obvious in female mice (Figure 3.4 (A and B)), suggesting insulin resistance was exacerbated in female mice with hepatic ERα deletion under HFD-induced pathological state. *ER*α^{LivKO} mice had higher blood glucose levels than WT mice in pyruvate tolerance test, indicating *ER*α^{LivKO} mice had a higher rate of gluconeogenesis than control mice upon pyruvate injection (Figure 3.4 (C and D)). What's more, mRNA expression of IRS1 in the liver was markedly reduced in *ER*α^{LivKO} female mice but not in male mice with HFD treatment. Taken together, these results suggested ablation of hepatic ERα exacerbates insulin resistance and glucose intolerance in HFD fed female mice. This difference may be explained by different expressions of hepatic ERα in male and female mice with HFD feeding. HFD feeding decreased expression of hepatic ERα in male mice. Male mice with overnutrition treatment lost the protection by ERα may be the reason for the diminished difference in glucose tolerance and expression of hepatic IRS1.

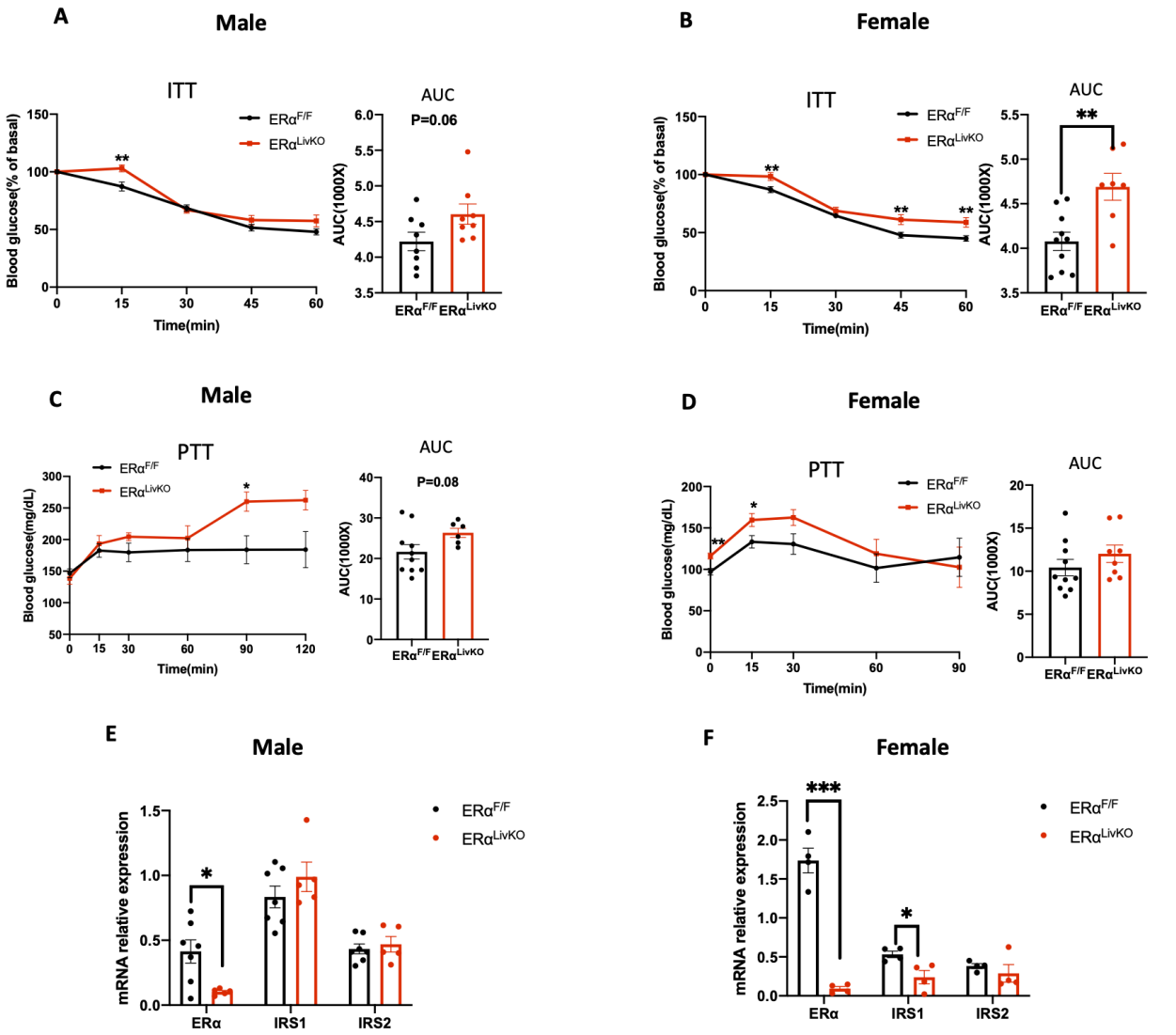

Figure 3.4: Ablation of hepatic ERα exacerbates insulin resistance in HFD fed female mice.
(A and B) Insulin tolerance test and area under curve (AUC) of ITT in HFD fed male and female mice, respectively. Insulin was administered at 1 U/kg body weight by intraperitoneal injection after 4 h fasting, and glucose level was measured at indicated time points.
(C and D) Pyruvate tolerance test and area under curve (AUC) of PTT in male and female mice, respectively. Pyruvate was administered at 2 g/kg body weight of mice by intraperitoneal injection after 16 h overnight fasting, and glucose level was measured at indicated time points.
(E and F) Relative mRNA levels of ERα, IRS1, and IRS2 in the liver were measured by RT-qPCR, n=4-5. All data are presented as mean ± SEM, *, P<0.05 and **, P<0.01, n=6-10.

Hepatic ERα deletion promotes lipid deposition in the liver from HFD fed female

H&E Staining demonstrated female *ER*α^{LivKO} mice with overnutrition treatment had much more fat deposition and increased fat accumulation in the liver than *ER*$\alpha^{F/F}$ mice. However, no obvious difference in fat deposition between male *ER*α^{LivKO} mice and control mice (Figure 3.5 (A)). Consistent with liver histology results, mRNA expression levels of genes involved in fatty acids oxidation, such as CD36 and CPT1 was significantly downregulated in *ER*α^{LivKO} female mice, with no significant difference between male mice (Figure 3.5 (B)). These results indicated hepatic ERα ablation promotes lipid deposition and impairs lipid homeostasis in the liver from *ER*α^{LivKO} female mice under an overnutrition state.

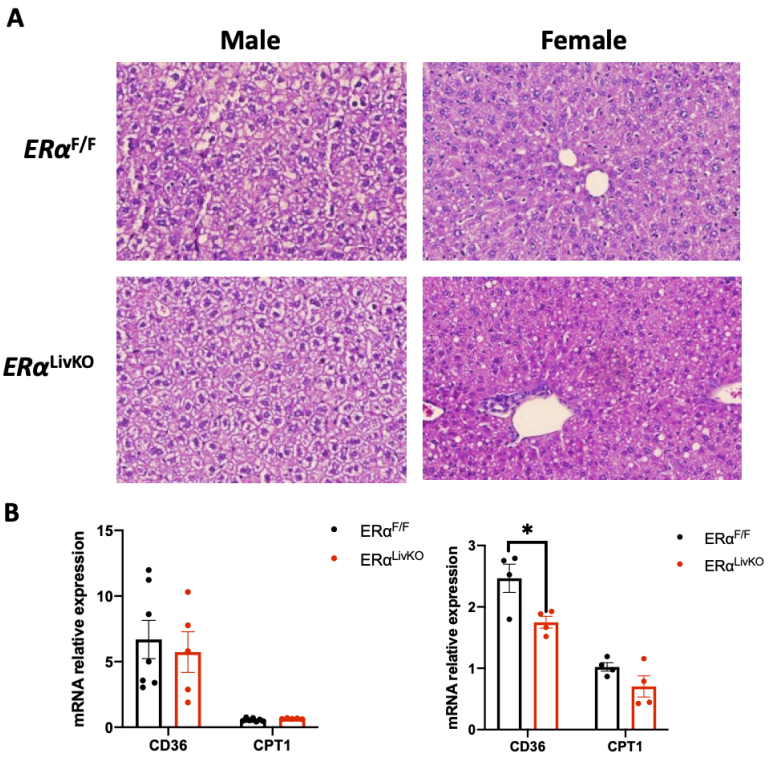

Figure 3.5: ERα deficiency promotes lipid deposition in the liver from HFD fed female mice. (A) Liver histology. H&E staining of liver from HFD fed WT and transgenic mice of both genders. (B) Relative mRNA levels of genes involved in fatty acids oxidation in the liver were measured by real-time qPCR. Left panel, male mice; right panel, female mice. All data are presented as mean ± SEM, *, P<0.05, n=4-7.

3.4 Summary

To investigate the role of hepatic ERα in regulating glucose homeostasis under both healthy and overnutrition states, we use Cre-loxP system to generate liver-specific ERα knockout mice, these mice were fed with a high-fat diet for 12 weeks. During the feeding period, we performed glucose tolerance tests to measure the ability of mice to clear exogenous glucose load upon glucose administration, and pyruvate tolerance tests to measure the ability to exert hepatic gluconeogenesis. In chow-diet fed mice, male and female *ER*α^{LivKO} mice exhibited similar phenotypes, which are impaired glucose tolerance and a higher rate of gluconeogenesis. mRNA expression levels of IRS1 was markedly reduced in *ER*α^{LivKO} mice. After they were received 2 U insulin via intravenous injection, western blot demonstrated protein expression of pAKT at Ser473 or Thr308 was obviously downregulated in *ER*α^{LivKO} mice. These results indicated impaired events downstream of IRS, especially impaired insulin-induced AKT phosphorylation in mice with hepatic ERα deficiency under physiological condition.

Under HFD fed state, there was a gender difference in phenotypes induced by hepatic ERα deletion between male and female mice. In HFD fed male mice, no significant differences were observed in body weight increase and body composition between *ER*α^{LivKO} and control mice. And *ER*α^{LivKO} mice did not show severe glucose intolerance compared to WT mice. However, in HFD female mice, *ER*α^{LivKO} displayed a more profound increase in body weight and more fat mass than *ER*$\alpha^{F/F}$ mice. *ER*α^{LivKO} female mice also showed markedly impaired glucose tolerance and impaired insulin sensitivity compared to WT mice. In addition, *ER*$\alpha^{F/F}$ mice of both sexes had higher blood glucose levels than WT mice in response to pyruvate administration. H&E staining demonstrated *ER*α^{LivKO} female mice had more lipid accumulation and deposition in the liver than *ER*$\alpha^{F/F}$ mice by HFD feeding. Also, gene expression involved in fatty acids oxidation was significantly downregulated in *ER*α^{LivKO} female mice but not in male mice. Taken together, these results suggested hepatic ERα deletion induced impaired glucose tolerance and reduced insulin sensitivity in both male and female mice by CD feeding. Under HFD-induced pathological state, ablation of hepatic ERα exacerbates glucose intolerance and insulin resistance in female mice. This difference might since male mice lost ERα protection by HFD feeding, which diminished the

difference between *ER*α^{LivKO} and control mice.

4. SUMMARY AND CONCLUSIONS

In this study, we firstly found that expression of hepatic α was different in male and female mice with overnutrition treatment and HFD fed female mice were more resistant to inflammation response compared to HFD fed male mice. Then to investigate the specific role of hepatic estrogen receptor α in maintaining glucose homeostasis under healthy and pathological states, we determined glucose tolerance, insulin response, and expression levels of genes involved in glucose and lipid metabolism in $ER\alpha^{LivKO}$ mice, $ER\alpha^{F/F}$ mice were used as control mice. We conclude that under physiological condition, $ER\alpha^{LivKO}$ mice exhibited impaired glucose tolerance and diminished insulin signaling as evidenced by downregulated mRNA expression of hepatic IRS1 and reduced protein levels of pAKT at Ser473 and Thr308 compared to $ER\alpha^{F/F}$ mice.

Under HFD-induced pathological state, $ER\alpha^{LivKO}$ female mice had a faster increase in body weight and more body fat mass distribution than WT mice. Hepatic ERα deficiency exacerbated glucose intolerance and insulin resistance in female mice. Hepatic insulin signaling was blocked in $ER\alpha^{LivKO}$ female mice as evidenced by reduced mRNA expression of IRS1 in the liver. However, these phenotypes were not observed in male mice. Both male and female mice deficient in hepatic ERα had higher blood glucose levels when treated with pyruvate, indicating mice with hepatic ERα deletion produced more hepatic glucose at a set time. Histology study in the liver from mice by HFD feeding demonstrated more lipid was accumulated in $ER\alpha^{LivKO}$ female mice. Consistent with that, mRNA expression of fatty acid oxidation genes, like CD36 and CPT1 was downregulated in $ER\alpha^{LivKO}$ female mice but not in male mice. Taken together, these results indicated that under HFD-induced pathological state, there was a gender difference in phenotypes induced by ablation of hepatic ERα. Reduced mRNA expression levels of ERα in male mice by overnutrition treatment might be the reason, in which HFD fed male mice lost the protection effects of ERα and difference between WT and $ER\alpha^{LivKO}$ mice was diminished with HFD feeding.

Overall, by CD feeding, ERα deletion in the liver induced glucose intolerance and impaired hepatic insulin signaling in both male and female mice. Ablation of hepatic ERα exacerbated glucose intolerance, insulin resistance and lipid accumulation in female mice by HFD feeding,

while the difference was diminished in HFD fed male mice. These results indicated that hepatic ERα plays an important role in mediating glucose and lipid metabolism in the liver.

People are increasingly aware of the gender difference in disease prevention, diagnosis and treatment, which will have more and more influence on clinical trials. Prospective research on the gender difference helps explore new methods and provide personalized treatments to improve the healthcare of patients in the future [92].

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APPENDIX A

TABLES

Table A.1: Mouse Primer List

Gene name	Forward 5'-3'	Reverse 5'-3'
TNFα	gagaaagtcaacctcctctctg	gaagactcctcccaggtatatg
IL-1β	tgttctttgaagttgacggaccc	tcatctcggagcctgtagtgc
MCP1	caggtgtcccaaagaagctgtag	gggtcagcacagacctctctct
IRS1	cccgttcggtgccaaatagc	gccactggtgaggtatccacatagc
IRS2	acttcccagggtcccactgctg	ggctttggaggtgccacgatag
CD36	gatgacgtggcaaagaacag	tcctcggggtcctgagttat
ACC1	cctccgtcagctcagataca	tttactaggtgcaagccagaca
CPT1	ccatgaagccctcaaacagatc	atcacacccaccaccacgata
SREBP1	ggagccatggattgcacatt	ggcccgggaagtcactgt
Cyclophilin	actgaatggctggatggcaag	tgcccgcaagtcaaaagaaat

Table A.2: Antibody List

REAGENT or RESOURCE	SOURCE	IDENTIFIER
GAPDH rabbit monoclonal antibody	Cell signaling technology	Cat#5174S
Phospho-Akt (Ser473) Rabbit mAb	Cell signaling technology	Cat#4060S
Phospho-Akt (Thr308) Rabbit mAb	Cell signaling technology	Cat#13038S
Akt Rabbit mAb	Cell signaling technology	Cat#4691S