Estrogen signaling prevents diet-induced hepatic insulin resistance in male mice with obesity.

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Abstract

The development of insulin resistance in the liver is a key event that drives dyslipidemia, and predicts diabetes and cardiovascular risk with obesity. Clinical data show that estrogen signaling in males helps prevent adiposity and insulin resistance, which may be mediated through estrogen receptor α (ERα). The tissues and pathways that mediate the benefits of estrogen signaling in males with obesity are not well defined. In female mice, ERα signaling in the liver helps to correct pathway-selective insulin resistance with estrogen treatment after ovariectomy. We assessed the importance of liver estrogen signaling in males using liver ERα knockout (LKO) mice fed a high-fat diet (HFD). We found that the LKO male mice had decreased insulin sensitivity compared to their wild-type floxed (fl/fl) littermates during hyperinsulinemic-euglycemic clamps. Insulin failed to suppress endogenous glucose production in LKO mice, indicating liver insulin resistance. Insulin promoted glucose disappearance in LKO and fl/fl mice similarly. In the liver, insulin failed to induce phosphorylation of AKT-Ser473 and exclude FOXO1 from the nucleus in LKO mice, a pathway important for liver glucose and lipid metabolism. Liver triglycerides and diacylglycerides were also increased in LKO mice, which corresponded with dysregulation of insulin stimulated ACC phosphorylation and DGAT1/2 protein levels. Our studies demonstrate that estrogen signaling through ERα in the liver helps prevent whole-body and hepatic insulin resistance associated with HFD-feeding in males. Augmenting hepatic estrogen signaling through ERα may lessen the impact of obesity on diabetes and cardiovascular risk in males.
Keywords: estrogen, estrogen receptor alpha, sex-differences, males, insulin resistance, liver lipid metabolism, and hyperinsulinemic-euglycemic clamp.

Abbreviations:

ACC: Acyl-coA carboxylase
DAG: diacylglycerol
DGAT: Acyl-CoA:Diacylglycerol acyltransferase
FoxO1: forkhead box protein O1
fl/fl: floxed/floxed controls (wild-type)
GIR: glucose infusion rate
GTT: glucose tolerance testing
HFD: high-fat diet
LKO: liver estrogen receptor α knock-out
TG: triglyceride
MTP: microsomal triglyceride transfer protein
SREBP1c: Sterol Regulatory Element-Binding Protein 1C
**Introduction**

Estrogen and estrogen signaling are well studied with regard to metabolism in females, in part because females are protected against insulin resistance and cardiovascular disease compared males of the same age or body-mass-index (14, 15). This relative protection is diminished in postmenopausal women (15, 26, 45). Males also express estrogen receptor in many tissues, and aromatase can metabolize androgens to generate estrogen metabolites locally. An increasing body of evidence suggests that estrogens have important biological functions in males with regard to the control of metabolism and adiposity (12, 13, 21, 23, 29, 34, 37), although the pathways that mediate estrogen’s protective effects in males are not well-defined.

A recent clinical study in humans demonstrated that the aromatization of testosterone to estradiol is responsible for many of the physiologic effects that had previously been attributed to testosterone, such as increasing libido and prevention of visceral adiposity (12). Many of the effects of estrogen in males may be attributed to estrogen receptor alpha (ERα) mediated signaling. For example, polymorphisms in ERα are associated with increased risk of type-2 diabetes and cardiovascular disease in males (13, 21, 29). Furthermore, males with disruptive mutations in either the estrogen receptor alpha or aromatase genes have impaired glucose tolerance, hyperglycemia and hyperinsulinemia (23, 34, 37). The metabolic syndrome associated with aromatase deficiency is also improved by estrogen treatment (23, 34). Collectively, these human studies suggest that estrogen signaling through ERα may be metabolically protective in males as well as for females in the setting of obesity.
The onset of liver insulin resistance, even in the setting of normal blood glucose, is an important contributor to the development of diabetes and obesity-associated cardiovascular risk. Numerous studies have shown that liver insulin resistance, or even an elevated serum ALT, is significant predictor of type-2 diabetes, independent of BMI or muscle insulin sensitivity (4, 17, 43). The failure of insulin to suppress hepatic glucose production contributes to impaired fasting glucose in humans. Metabolomic data suggest the presence of liver insulin resistance up to a decade before the diagnosis of diabetes (32, 44). Liver insulin resistance is the major driver of obesity-associated dyslipidemia including elevated triglycerides in VLDL and low cholesterol levels in HDL, and increased cardiovascular risk (10, 36, 40). Liver, but not muscle insulin resistance contributes to impairments in the composition of HDL particles, an effect modulated by estrogen signaling pathways (24). Reciprocally, pharmacologic strategies that target estrogen signaling to the liver improve metabolic syndrome in animal models (11, 19). Thus, clinical and basic science evidence supports that understanding pathways influencing the development of liver insulin resistance will likely be important toward understanding the early changes with obesity that give rise to risk of diabetes and cardiovascular disease.

The use of mouse models has begun to establish tissues and pathways by which estrogen signaling may protect males from complications of obesity. Global loss of estrogen signaling through knockout of ERα leads to obesity, increased inflammation and hyperlipidemia in male mice (16, 28, 33). Estrogen signaling through ERα in adipose promotes adipose triglyceride (TG) storage in males, as demonstrated in an adipose-specific knock-out of ERα in mice (7). In humans, liver fat accumulation is a major
contributor toward both dyslipidemia and impaired glucose metabolism in males (10), but
the role of estrogen signaling in the liver in males is not well established. In females,
estrogen signaling through ERα has prominent signaling functions in the liver. Global
ERα knockout and ovariectomy in mice result in increased expression of lipogenesis
genes in the liver and development of steatosis (33, 49). In female liver ERα knockout
mice, estrogen limits liver fat accumulation and promotes insulin signaling under high-fat
diet (HFD)-feeding (49).

Given the importance of liver estrogen signaling in minimizing liver fat and
improving insulin sensitivity in females, we postulated that estrogen signaling in the liver
might mediate part of protective effects of estrogen signaling in males. We used mice
with liver specific knockout of ERα (LKO) to investigate the importance of liver ERα
toward the metabolic adaptation to HFD-feeding in males. In our study, we used the
hyperinsulinemic-euglycemic clamp technique to determine whole body insulin
resistance. Our clamp study design was optimized to define insulin action in the liver. We
demonstrate that male LKO mice develop hepatic insulin resistance and accumulate
hepatic triglycerides and diacylglycerols, which are associated with impaired liver insulin
signaling. Muscle glucose uptake in response to hyperinsulinemia is unchanged. These
studies demonstrate an important role of hepatic estrogen signaling to prevent the
metabolic complications of obesity in males.
MATERIALS AND METHODS

Animals and experimental design: Mice with liver-specific deletion of ERα (LKO) were generated as reported before (8, 9, 49). LKO mice are compared to wild-type littermates for these studies are ERα flox/flox littermates. All mice were 12-14 weeks old at the onset of diet (n ≥ 10 per group) were housed at 22±1 °C in a 12:12 h light:dark cycle. The Institutional Animal Care and Use Committee at Vanderbilt University approved the protocols. There were four cohorts of LKO mice and their floxed/floxed wild type controls (WT) used for these studies:

Cohort 1 (Before HFD): Chow-fed mice fasted for 5-hours then sacrificed to define genotype effects in the absence of HFD-feeding. There were no differences in body weight or body composition in chow-fed mice.

Cohort 2 (HFD no-insulin): Mice were fed HFD for 12 weeks (Research Diets, D08060104 60% fat from lard, 20% protein and 20% carbohydrate from corn starch, 5.24 kcal/g), then fasted for 5 hours and sacrificed. This cohort was included to obtain tissues that were not insulin-treated for figures 2-4.

Cohort 3 (HFD-insulin clamp): Mice were fed HFD for 12 weeks. At week 11, catheters were implanted by the Vanderbilt Mouse Metabolic Phenotyping Center in the left common carotid artery and right jugular vein for sampling and infusions as previously described (3). Mice were maintained on HFD for 1 week of recovery, and then fasted 5 hours before hyperinsulinemic-euglycemic clamp study. This cohort was included to define insulin sensitivity and obtain insulin-treated tissues for figures 2-4.

Cohort 4: (HFD-5 weeks): Mice were fed HFD for 5 weeks. After fasting for 5 hours, mice underwent intraperitoneal (IP) injection of insulin (0.75 units regular human insulin...
diluted in 0.5 ml of saline) or saline, and were sacrificed 15 minutes later. This study was included to define the importance of liver estrogen signaling at an intermediate time point of HFD feeding, and to define acute insulin signaling in liver, muscle and adipose tissues.

Hyperinsulinemic-euglycemic clamps: Five days after catheter placement, hyperinsulinemic-euglycemic clamps were performed in unrestrained 5-hour-fasted mice (cohort 3). A primed (5.4 μCi) continuous (0.135 μCi/min) infusion of 3-3H-glucose was initiated at t=-90 min. This basal period was followed by hyperinsulinemia started at t=0 (2.5mU/kg/min; Humulin R; Eli Lilly, Indianapolis, IN). Our clamp study design with insulin infusion of 2.5 mU/kg/min was optimized to define hepatic insulin action, because this insulin dose does not maximally suppress EndoRa (an index of hepatic glucose production) (2). At t=0 min the infusion rate was increased to 0.27 μCi/min. Euglycemia (~150 mg/dl) was maintained by measuring blood glucose every 10 min starting at t=0 min and adjusting the infusion of 50% dextrose as necessary. Mice received saline-washed erythrocytes from donors to prevent a fall in hematocrit. At t=120 min, mice were sacrificed and tissues were flash frozen. To obtain non-insulin treated samples, a parallel set of experiments was performed where mice were fed a HFD for 12 weeks, fasted 5 hours, and then sacrificed (Cohort 2).

Plasma processing and calculations: Insulin levels were determined by ELISA (Millipore #EZRMI-13K, St. Charles, MO). 3-3H-glucose specific activities were determined by liquid scintillation counting after plasma deproteination. Glucose disappearance rate (Rd) and endogenous glucose production (EndoRa) were determined
and insulin sensitivity index was calculated as described (1, 3, 38). Serum estradiol and testosterone values were measured by the Vanderbilt Hormone Assay Core Facility.

Liver TG/DAG content analysis: Liver lipid was extracted using Folch methodology as described (50). TG and DAG were separated using TLC according to Zhu et al (49). Total liver TG and DAG amount was quantified as previously reported (49).

Protein immunoblots: Antibodies for AKT and pAKTSer473 were from Cell Signaling (Beverly, MA); and antibodies for ERα (sc-7207) DGAT1 (sc-31680), DGAT2 (sc-66859), FoxO1 (sc-11350) and β-Actin (sc-47778) were from Santa Cruz Biotech (Santa Cruz, CA); and antibody for ApoB100 was from Lifespan Biosciences (LS-c20729). Anti-MTP antibody was provided by Dr. Larry Swift (41). Primary antibody was incubated at 4 °C overnight with the dilution recommend in manufacturer’s database. Anti-mouse or anti-rabbit antibody was incubated with the dilution of 1:15000 at room temperature for 1 hour. Nuclear extracts were prepared according to the manufacturer’s instructions (NE-PER, Thermo Scientific). Imaging and densitometry were performed using the Odyssey imaging system (Li-COR, Lincoln, NE) and ImageJ processing program.

Liver glycogen quantification: Liver glycogen extraction was performed as established by Chan and Exton, with minor modifications (6): Briefly, 50-100 mg of liver tissues were homogenized in 0.03N HCl using 0.5 mm zinc oxide beads (Next Advance # ZrOB05) in a Bullet-Blender™. Homogenized tissues were incubated at 80°C for 10 min and...
homogenate was blotted onto chromatography paper strips (Whatman # 0303614). Strips were washed three times in 70% ethanol for 40 minutes, rinsed in acetone, and then dried overnight. Dried strips were digested using 0.1 mg/mL amyloglucosidase (Sigma # A7420) in 0.04M sodium acetate for 3hr in a 37°C shaking water bath.

Glucose in glycogen was quantified by enzymatic assay. 20 μL of glucose-enriched digest solution were loaded per well of a 96-well plate (Fisher # 12565501) with 250 μL of enzyme solution. The plate was incubated for 15 minutes at room temperature. Enzyme solution contained 70 mg ATP (MP Biomedical # 100008), 24 mL of 200 mM Tris HCl, 500 μL of 500mM MgCl₂, 50 mg β-NADP (Roche # 10128058001), 50 μL of hexokinase (Roche # 11426362001), and 125 μL of glucose-6-phosphate dehydrogenase (Roche # 10737232001). Plate absorbance was read at 340 nm to quantify glucose in digest solution. Oyster glycogen (Sigma # G8751) and glucose (Sigma # G6918) were used as standard controls for the extraction and quantification procedures, respectively.

**Statistical analysis:** Data are presented as means ± SD. Differences between groups were determined by 2-way ANOVA followed by Bonferroni post tests, or by student t test as appropriate. Significance was considered as p < 0.05. The specific statistical test used are indicated in the figure legends.
RESULTS:

Male liver estrogen receptor α knockout (LKO) mice exhibit liver and whole-body insulin resistance compared to their wild-type (fl/fl) littermates after high-fat diet (HFD) feeding. To define the importance of liver estrogen signaling for glucose and lipid metabolism in males, 12-week old male LKO mice and their fl/fl littermates were fed a HFD for 12 weeks. The fl/fl littermates for these studies are ERα flox/flox littermates. In LKO mice, liver ERα expression was reduced >90% from whole-liver extracts, but unchanged in muscle (Figure 1A). The HFD-feeding caused increased body weight and adiposity in fl/fl and LKO male mice, with the LKO mice gaining more adiposity (Table 1). Fasting glucose and insulin levels were not different between fl/fl and LKO mice at baseline on chow diet (Table 1). HFD-feeding caused an increase in fasting glucose and insulin levels for both groups, with no significant differences between groups, however LKO mice tended to have higher insulin values (Table 1).

To determine the importance of ERα toward whole-body insulin sensitivity, we performed hyperinsulinemic-euglycemic clamps in LKO mice and WT littermates (Figure 1B). In the setting of hyperinsulinemia, a greater glucose infusion rate (GIR) indicates greater insulin sensitivity. Similarly, a lower GIR indicates greater insulin resistance. During the clamp, the GIR was lower for LKO mice compared to the WT littermates (Figure 1, C and D), indicating that male LKO mice were more insulin resistant than WT littermates. Fasting insulin, clamp-phase insulin and clamp-phase c-peptide levels are shown in Table 1. Since insulin infusion does not suppress endogenous insulin production in mice, clamp-phase plasma insulin and c-peptide levels were significantly higher in LKO mice, consistent with whole-body insulin resistance (Table
1. We calculated an insulin sensitivity index by normalizing the GIR to plasma insulin concentrations during the clamp period. The insulin sensitivity index was significantly lower in LKO mice than in fl/fl littermates (Figure 1E, p < 0.01). These results demonstrated that absence of liver estrogen signaling in males worsens whole-body insulin resistance induced by HFD-feeding.

Hepatic insulin signaling is impaired in male LKO mice. We used 3-3H-glucose as a tracer to assess liver glucose metabolism during the clamp studies. The dose of insulin infusion during clamp was optimized to define liver glucose metabolism (2.5 mU/kg/min, reviewed in (3)). Endogenous hepatic glucose production (EndoRa) was suppressed by hyperinsulinemia during the clamp in fl/fl littermates (Figure 2A). In contrast, insulin failed to suppress EndoRa in male LKO mice, demonstrating hepatic insulin resistance (Figure 2A). To obtain non-insulin treated samples so that we could define insulin-signaling pathways, we repeated a cohort of LKO and fl/fl mice that were fed HFD for 12 weeks and sacrificed after a 5-hour fast. In fl/fl mice, insulin during the clamp induced an increase in phosphorylation of AKT Ser473 (Figure 2B-C). In LKO mice, insulin failed to induce phosphorylation of AKT Ser473. In the insulin-sensitive liver, phosphorylated AKT Ser473 phosphorylates the transcription factor Forkhead box protein O1 (FoxO1), resulting in nuclear exclusion of FoxO1. Nuclear levels of FoxO1 were increased in LKO mice compared to fl/fl (Figure 2 D-E). Insulin signaling through AKT and FoxO1 is an important pathway for the regulation of hepatic glucose and triglyceride (TG) metabolism (46).
**LKO male mice have altered liver glycogen metabolism.** Diabetes is characterized by impaired glycogen synthesis and metabolism (18, 20). In our study, LKO male mice had significantly reduced fasting liver glycogen when compared to fl/fl controls (9.6 ± 2.1 vs. 17.5 ± 3.2 μg/mg, p<0.05, Figure 2F). Insulin-stimulated glycogen content was also impaired in LKO mice (17.4 ± 1.0 vs. 30.68 ± 4.9 μg/mg, p < 0.05, Figure 2F). This impaired hepatic glycogen metabolism likely contributes to the impaired suppression of hepatic glucose production (endoRa) that we observed in the clamp study.

**Muscle glucose disposal is not impaired in LKO mice.** An index of muscle insulin action is the glucose disappearance rate (Rd) in the clamp period, which was similar for fl/fl and LKO mice during the clamp (Figure 3A). In muscle, insulin induced phosphorylation of AKTSer473 to a similar degree in WT and LKO mice (Figure 3, B and C). Collectively, tracer analysis and tissue specific insulin signaling suggest that hepatic insulin resistance caused the whole-body insulin resistance in LKO mice.

**Liver ERα signaling protects against liver TG and DAG accumulation with HFD-feeding.** To define the effects of hepatic estrogen signaling on liver fat accumulation with HFD-feeding, we measured liver TG and DAG content in both chow-fed and HFD-fed mice. There were no differences in hepatic TG or DAG content in a cohort of mice that was chow-fed and sacrificed after a 5-hour fast (liver TG 15.3±6.3 for fl/fl and 16.1±4.7 μg/mg for LKO, and liver DAG 0.31±0.05 for fl/fl and 0.29±0.1 μg/mg for LKO). Hepatic steatosis developed in both WT and LKO mice following 12 weeks of HFD, and this steatosis was more pronounced in LKO mice (Table 1 and Figure 4A). Similarly,
liver DAG content was also increased in HFD-fed LKO mice (Table 1 and Figure 4B). These results demonstrate that liver estrogen signaling protects against liver TG and DAG accumulation in male mice with HFD-feeding.

Estrogen signaling protects against dyslipidemia in males after HFD-feeding. In the liver, insulin signaling promotes dephosphorylation of Acyl-coA carboxylase (ACC), increasing fatty acid synthesis. Our previous work showed that liver estrogen signaling limited liver lipid accumulation, in part by preventing insulin-mediated dephosphorylation of ACC, which promoted fatty acid oxidation, rather than synthesis (49). We compared ACC phosphorylation during fasting and hyperinsulinemia and found that in fl/fl animals with intact liver estrogen signaling, insulin did not de-phosphorylate ACC, consistent with our previous results seen in females. In contrast, insulin did dephosphorylate ACC in LKO mice, which would promote fatty acid synthesis, and is consistent with increased liver fat content (Figure 4, A, C and D).

The Acyl-CoA:Diacylglycerol acyltransferase (DGAT) enzymes catalyze TG synthesis by transferring fatty acid to DAG. DGAT1 and DGAT2 are from different gene families, and have distinct, often reciprocal regulation in the liver (5) (and reviewed in (48). Insulin increases DGAT2 to promote TG synthesis and reduce hepatic FFA and DAG levels, which may help to prevent hepatic insulin resistance (27, 47). In fl/fl mice we saw appropriate insulin-stimulation of DGAT2 protein levels; whereas in LKO mice insulin failed to up regulate DGAT2 (Figure 4, E-F). Reciprocally, DGAT1 is increased in fasting and reduced with hyperinsulinemia (27). Fasting levels of DGAT1 were increased in LKO mice compared to fl/fl, consistent with fatty liver in the LKO mice.
(Figure 4, E and G). There were no differences in either mature or processed forms of Sterol Regulatory Element-Binding Protein 1c (data not shown). Collectively, these data suggest that ERα signaling is involved in both fasting and insulin regulation of DGAT1/2, which likely contributes to limiting liver fat accumulation.

Increased fasting plasma TG level in LKO mice. Increased liver fat content is a major driver of increased production of very low-density lipoprotein (VLDL) (10). In the liver, the enzyme microsomal triglyceride transfer protein (MTP) adds TG onto the nascent apoB100 particle as it is translocated into the lumen of the endoplasmic reticulum for secretion as VLDL (31). We observed that during fasting, liver apoB100 and MTP protein levels were significantly higher in LKO mice than in fl/fl littermates, and were correlated with increased levels of plasma TG in LKO mice (Figure 5, A-D). Insulin reduces hepatic apoB100 levels. Insulin suppressed expression of apoB100 in both LKO mice and their fl/fl littermates (Figure 5, A and B). There was no significant difference in plasma cholesterol levels between LKO and fl/fl mice during fasting, and the fasting plasma cholesterol levels were suppressed by hyperinsulinemia during clamp in both groups of mice (Figure 5E). These findings suggest that liver estrogen signaling in males prevents liver fat accumulation, resulting in improvements in both dyslipidemia and glucose metabolism.

LKO male mice have impaired insulin signaling to liver and muscle at 5 week of HFD-feeding. We next aimed to define the importance of liver ERα signaling at an intermediate duration of HFD-feeding, 5 weeks compared to 12 weeks in the previous
study. There were no differences in body weight or adiposity at 5 weeks of HFD-feeding (Figure 6A, n=9-10 per group). To define acute insulin signaling, we administered intraperitoneal insulin or saline and sacrificed mice 15 minutes later. We used this approach because insulin down regulates its own signaling with chronic administration. We assessed insulin signaling to AKT in liver, muscle and adipose tissues. In the liver, we found that fl/fl mice responded to insulin and had a robust induction of phosphorylated AKT Ser473 (Figure 6 B, C). In LKO mice, baseline pAKT Ser473 was reduced, and insulin failed to induce pAKT Ser473. This finding is similar to the results of our 12-week study for liver. Total AKT levels were unchanged in the liver between LKO and fl/fl. In muscle, fl/fl mice insulin also induced robust induction of pAKT Ser473. In LKO mice, baseline pAKT Ser473 was increased and insulin had diminished capacity to induce pAKT Ser473. In adipose, baseline phosphorylation of AKT Ser473 was decreased in LKO mice, and the insulin induction of AKT was more robust. These results confirm that loss of liver ERα signaling impairs insulin signaling to AKT in the liver following 5 weeks of HFD-feeding. Overall, this study confirms the importance of liver estrogen signaling in protecting from liver and whole-body insulin resistance with HFD-feeding in male mice.
DISCUSSION:

Accumulating evidence supports the importance of estrogen and estrogen signaling in males with regard to metabolic control. A recent clinical study found that many of the benefits attributed to testosterone in males actually require aromatization of testosterone to estrogen (12). The target tissues responsible for estrogen’s beneficial effects in males are not yet clear. Insulin resistance in the liver is a major contributor to both impaired glucose metabolism and dyslipidemia with obesity. Because of the prominent role of hepatic estrogen signaling in limiting the adverse effects of high-fat feeding in females (16, 19, 49), we hypothesized that liver estrogen signaling may also limit the adverse effects of high-fat feeding in males. We discovered that male mice with liver estrogen receptor α knock out (LKO) developed insulin resistance and impaired insulin signaling in liver. This liver insulin resistance was associated with: 1) increased levels of proteins involved in fatty acid and TG synthesis in the liver, 2) decreased liver glycogen synthesis, and 3) increased hepatic glucose production during hyperinsulinemia. These results demonstrate that some of the protective metabolic effects of estrogen in males are mediated through hepatic ERα signaling.

We demonstrate that the liver is an important target of estrogen signaling in males for preventing whole-body insulin resistance using mice with liver-specific ERα knockout and hyperinsulinemic-euglycemic clamp techniques. Our clamp study design with insulin infusion of 2.5 mU/kg/min was optimized to define hepatic insulin action (2). In LKO mice, insulin failed to suppress hepatic glucose production (endoRa) in LKO mice, a measurement of hepatic insulin action. Correspondingly, we saw impaired insulin-stimulated phosphorylation of AKT in the liver of LKO mice. The LKO mice in
the cohort that was fed HFD for 12-weeks had a slightly higher adiposity than fl/fl mice, which would bias toward finding insulin resistance in the LKO group. To address this, we repeated a cohort of LKO and fl/fl mice on an intermediate duration of HFD-feeding, 5 weeks. In these mice there were no difference in adiposity, in fact the LKO mice were slightly leaner. The LKO mice still had a significant impairment in insulin signaling to AKT in the liver, confirming the finding that estrogen signaling through liver ERα promotes liver insulin sensitivity with HFD-feeding.

In the clamp study, there was minimal apparent effect of liver ERα knock-out on muscle, as insulin-mediated glucose disposal (Rd) was not altered. We did, however, see that muscle insulin signaling was impaired with an intraperitoneal injection of insulin in our cohort of mice fed HFD-for 5 weeks. The difference in these two studies is likely in part because the longer duration of HFD-feeding diminished the muscle insulin response in both groups (insulin stimulated pAKT was 4.2-fold in fl/fl mice at 5 weeks, but just 2.8-fold at 12 weeks); and possibly because the duration of insulin infusion down-regulated pAKT in the clamp study. In the clamp study, we did see elevated c-peptide levels in LKO mice, which also is consistent with muscle insulin resistance. Thus, there likely is a muscle phenotype induced by loss of hepatic ERα. The low total testosterone levels seen in the LKO mice may contribute to impaired insulin action in muscle, as testosterone is known to promote muscle glucose uptake (Table 1 and (30, 35)). We were unable to measure sex-hormone binding globulin, however, which may have been reduced in the LKO mice. There were no differences in serum estradiol. One published report did not show a major phenotype in older mice lacking liver ERα, either males or females using intraperitoneal glucose tolerance testing (GTT) (25). Our clamp approach
provides an advantage over the GTT because blood glucose and insulin levels are held constant, helping avoid the counter-regulatory changes that occur during the GTT. We additionally verified significant liver insulin resistance by loss of liver ERα with a second model, IP insulin vs. IP saline after 5 weeks of HFD-feeding (Figure 6). Collectively, by coupling tissue-specific knockout of ERα with techniques optimized to study liver insulin action, we were able to show an important role of hepatic estrogen signaling in preventing HFD-induced insulin resistance in males.

Our results suggest that many of the protective effects of liver ERα signaling with regard to lipid metabolism in females are also present in males. Estrogen signaling in the liver likely arose to couple reproduction to with nutritional signals. In cycling female mice, the mRNA levels of lipogenic genes in liver change significantly during the 4-day-long estrus cycle (42). Furthermore, amino acids regulate liver ERα and contribute to both the metabolic and reproductive effects of estrogen (8). Reciprocally, absence of liver ERα signaling leads to lipid accumulation with over-nutrition (49). We show that in males liver estrogen signaling through ERα also limits liver TG and DAG accumulation with HFD-feeding. In fl/fl male mice, with intact liver estrogen signaling, the reduction in both liver TG and DAG likely improved liver insulin sensitivity.

Dysregulation of DGAT2 by insulin in LKO mice may contribute to the DAG accumulation and liver insulin resistance. DGAT2 is involved in the bulk of TG synthesis in the liver (39). Insulin increases fatty acid synthesis and concordantly increases DGAT2 protein levels, which promote the synthesis of TG from FA and DAG. This coordinate synthesis of fatty acids with induction of DGAT2 minimizes accumulation of lipotoxic DAG species. LKO mice had increased dephosphorylation of ACC (which promotes fatty
acid synthesis) with insulin treatment, but impaired insulin-induction of DGAT2. Liver estrogen signaling seems to temper the insulin-stimulated accumulation of lipotoxic lipids by decreasing ACC-mediated fatty acid synthesis and by increasing DGAT2-mediated conversion of fatty acids and DAGs to a more innocuous TG molecule.

Studies in humans have shown that estrogen signaling protects from obesity-related complications in males (12, 13, 21, 23, 29, 34, 37). Our data suggest that estrogen signaling through ERα in the liver likely contributes to this protection. Women are relatively protected against cardiovascular disease compared to age or BMI-matched men (14, 22). Our lab and others have shown that liver estrogen signaling protects against complications of high-fat feeding in females. Our studies highlight the opportunity to define pathways that confer cardiovascular protection for females, which then can be targeted in order to lessen obesity complications in males. Strategies to augment hepatic estrogen signaling pathways in males may be a promising approach to reduce liver fat and improve insulin signaling associated with obesity.
GRANTS:
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DISCLOSURES:
The authors disclose no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS
L.Z, M.M., C.E, and B.P. performed the experiments; L.Z. and J.S. analyzed the data. L.Z., M.M., and J.S. prepared the figures. L.Z. and J.S. drafted the manuscript. L.Z, M.M., C.E., and B.P. approved the final version of the manuscript.
Table 1. High fat diet (HFD) induced obesity and insulin resistance for liver ERα knock out (LKO) and fl/fl mice.

<table>
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<th>fl/fl (Chow-fed)</th>
<th>fl/fl (After HFD)</th>
<th>LKO (Chow-fed)</th>
<th>LKO (After HFD)</th>
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<td><strong>Body weight (g) 12 wk study</strong></td>
<td>22.5 ± 2.6</td>
<td>35.2 ± 3.7*</td>
<td>23.1 ± 3.2</td>
<td>37.8 ± 4.3*</td>
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<td><strong>% Adiposity 12 wk study</strong></td>
<td>10.8 ± 3.9</td>
<td>23.5 ± 4.1*</td>
<td>9.2 ± 2.3</td>
<td>28.6 ± 3.9*#</td>
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<td><strong>% Muscle 12 wk study</strong></td>
<td>76.2 ± 4.5</td>
<td>64.7 ± 7.6*</td>
<td>79.6 ± 5.8</td>
<td>61.7 ± 6.4*</td>
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<tr>
<td><strong>Fasting Glucose (mg/dl) 12 wk study</strong></td>
<td>98.2 ± 11.7</td>
<td>144.8 ± 21.3*</td>
<td>105.9 ± 13.2</td>
<td>132.8 ± 20.5*</td>
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<tr>
<td><strong>Fasting Insulin (ng/ml) 12 wk study</strong></td>
<td>0.43 ± 0.07</td>
<td>3.38 ± 0.71*</td>
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<td>4.52 ± 1.02*</td>
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<td><strong>Clamp Insulin (ng/ml) 12 wk study</strong></td>
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<td>7.94 ± 1.59#</td>
<td>7.41 ± 0.29**</td>
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<td><strong>Clamp c-peptide (ng/ml) 12 wk study</strong></td>
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<td><strong>Serum estradiol (pg/ml)</strong></td>
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<td>79.0 ± 2.4</td>
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<td><strong>Serum total testosterone (ng/ml)</strong></td>
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<td>0.46 ± 0.22**</td>
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<tr>
<td><strong>Body weight (g) 5 wk study</strong></td>
<td>33.5 ± 1.2</td>
<td>32.2 ± 1.1</td>
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<td><strong>% Adiposity 5 wk study</strong></td>
<td>25.9 ± 1.1</td>
<td>23.7 ± 1.6</td>
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<tr>
<td><strong>% Muscle 5 wk study</strong></td>
<td>74.3 ± 2.8</td>
<td>75.6 ± 1.7</td>
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</table>

*, p < 0.05, comparison between “Before HFD” and “After HFD.”
#
*, p<0.05, comparison fl/fl-HFD with LKO-HFD.
**, p < 0.005, comparison fl/fl-HFD with LKO-HFD
FIGURE LEGENDS:

Figure 1. Liver estrogen signaling prevents insulin resistance in males with HFD-feeding. A. Western blots show that estrogen receptor α (ERα) protein levels are decreased in LKO mouse liver, but not in muscle. B. Schematic of the hyperinsulinemic-euglycemic clamp study design. C. Euglycemia was maintained at ~150 mg/dl during the hyperinsulinemic clamp. D. The glucose infusion rate (GIR) to maintain euglycemia was lower for LKO mice than for fl/fl mice. E. Insulin sensitivity index was lower in LKO mice. n= 6-8 per group. Asterisks indicates p < 0.05. Differences between groups were determined by t-test.

Figure 2. Hepatic insulin signaling is impaired in LKO mice. A. Insulin failed to suppress hepatic glucose production (EndoRa). B and C. Western blots from whole liver extracts show that AKT473 phosphorylation by insulin was decreased in LKO mice. D and E. Western blots show increased FoxO1 from nuclear extracts of liver from LKO mice. F. Liver glycogen levels were lower in LKO mice during fasting and hyperinsulinemia. n= 4-6 per group for bar graphs. * indicates p < 0.05 by t-test. ** indicates p < 0.005 by t-test. # indicates p<0.005 by genotype by 2-way ANOVA.

Figure 3. Muscle insulin action is not impaired in LKO mice. A. Insulin-stimulated glucose disappearance rate (Rd) was not different between LKO and fl/fl mice. B and C. Insulin-stimulated phosphorylation from muscle extracts of AKT-Ser473 was similar for LKO and fl/fl mice. n= 4-6 per group for bar graphs. ** indicates p < 0.005. Differences between groups were determined by t-test.
Figure 4. Liver ERα signaling protects against TG and DAG accumulation with HFD-feeding in male LKO mice. A and B. Liver TG and DAG levels were increased in LKO mice compared to their littermates. C-D. ACC phosphorylation was decreased by insulin in LKO mice shown by Western blotting from whole liver extracts. E-G. Western blotting of DGAT1/2 impaired insulin-regulation of DGAT levels in liver from LKO mice. n = 4-6 per group for bar graphs. * indicates p < 0.05 by t-test. ** indicates p < 0.005 by t-test.

Figure 5. Increased fasting plasma TG level in LKO mice. A-B. Western blotting shows that liver apoB100 protein levels were suppressed during insulin clamps for LKO and fl/fl mice. A and C. MTP levels were increased in LKO mice. D. Fasting plasma TG was increased in LKO mice, but insulin reduced serum TG in both LKO and fl/fl. E. Cholesterol levels were reduced with insulin for LKO mice and their fl/fl controls. n = 4-6 per group for bar graphs. * indicates p < 0.05 by t-test. ** indicates p < 0.005 by t-test. # indicates p<0.05 by genotype by 2-way ANOVA.

Figure 6. Hepatic insulin signaling is impaired in LKO mice with 5 weeks of HFD-feeding. A. There were no differences in adiposity or lean mass after 5 weeks of HFD-feeding between LKO and fl/fl male mice. B-E: After a 5-h fast, mice underwent an intraperitoneal (IP) insulin or saline injection. Western blotting of pSER473-AKT and total AKT show insulin resistance in liver and muscle, but not adipose tissue. n= 5-6 per
group for bar graphs. * indicates p < 0.05 by t-test. ** indicates p < 0.005 by t-test. # indicates p<0.05 by genotype by 2-way ANOVA.
References


and Hong Y. Heart Disease and Stroke Statistics--2009 Update: A Report From the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. 


47. Yamaguchi K, Yang L, McCall S, Huang J, Yu XX, Pandey SK, Bhanot S, Monia BP, Li YX, and Diehl AM. Inhibiting triglyceride synthesis improves hepatic


Figure 1

A

<table>
<thead>
<tr>
<th>fl/fl</th>
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<tr>
<td>Liver ERα</td>
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<td>Liver Actin</td>
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<td>Muscle ERα</td>
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<td>Muscle Actin</td>
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B

- Insulin 2.5mU/kg/min
- 50% Dextrose
- Donor erythrocytes
- $^{3-3H}$-glucose constant infusion

C

Blood glucose (mg/dl)

D

GIR (mg/kg/min)

E

Insulin Sensitivity Index (mg.ml[kg.min.mU]$^{-1}$)

*
Figure 2 (Liver)

A

Endo Ra (mg/kg/min)

Insulin

-  +  -  +

fl / fl Male  LKO Male

B

fl / fl  LKO

Ins pAKT473 Total AKT Actin

C

Liver pAKT473/total AKT

Insulin

-  +  -  +

fl / fl Male  LKO Male

D

fl / fl  LKO

Nuclear FoxO1  Nuclear Histone

E

Nuclear FoxO1 / Histone

WT  LKO

F

Liver glycogen (µg/mg tissue)

Insulin

-  +  -  +

fl / fl Male  LKO Male
Figure 3 (Muscle)

A

B

C

Rd (mg/kg/min)

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<tr>
<td>pAKT473</td>
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<td>Male</td>
<td>Male</td>
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<td>**</td>
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</table>
Figure 5

A) Western blots for ApoB100, MTP, and Actin in liver extracts from Male fl/fl and Male LKO mice under insulin-deprived (-) and insulin-repleted (+) conditions.

B) Bar graphs showing the ratio of Liver ApoB100/Actin in Male fl/fl and Male LKO mice under insulin-deprived (-) and insulin-repleted (+) conditions. The bars are presented with error bars indicating the standard error of the mean (SEM).

C) Bar graphs showing the ratio of Liver MTP/Actin in Male fl/fl and Male LKO mice under insulin-deprived (-) and insulin-repleted (+) conditions. The bars are presented with error bars indicating the SEM.

D) Bar graphs showing the plasma triglyceride (TG) levels (mg/dl) in Male fl/fl and Male LKO mice under insulin-deprived (-) and insulin-repleted (+) conditions. The bars are presented with error bars indicating the SEM.

E) Bar graphs showing the plasma cholesterol levels (mg/dl) in Male fl/fl and Male LKO mice under insulin-deprived (-) and insulin-repleted (+) conditions. The bars are presented with error bars indicating the SEM.
Figure 6

A

% Total Body Mass

fl / fl lean
LKO lean
fl / fl Adipose
LKO adipose

B

Insulin

Liver
p-Akt
Akt

Muscle
p-Akt
Akt

Adipose
p-Akt
Akt

C

Liver pAKT-Ser473 / total AKT

Insulin

fl / fl Male
LKO Male

D

Muscle pAKT-Ser473 / total AKT

Insulin

fl / fl Male
LKO Male

E

Adipose pAKT-Ser473 / total AKT

Insulin

fl / fl Male
LKO Male