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

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Estrogen signaling prevents diet-induced hepatic insulin resistance in male mice with obesity. Am J Physiol Endocrinol Metab 306: E1188–E1197, 2014. First published April 1, 2014; doi:10.1152/ajpendo.00579.2013.—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 with 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, which corresponded with dysregulation of insulin-stimulated HDL particles, an effect modulated by estrogen-signaling pathways (24). Reciprocally, pharmacological 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 the early changes in 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 increased cardiovascular risk (10, 36, 40). Liver, but not muscle insulin resistance, 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, pharmacological 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 the early changes with obesity that give rise to the risk of diabetes and cardiovascular disease.

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 alanine aminotransferase, is a 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, pharmacological 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 the early changes with obesity that give rise to the 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 knockout 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 the protective effects of estrogen signaling in males. We used mice with liver-specific ERα-knockout (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 compared with wild-type (WT) littermates for these studies are ERα flox/flox littermates. All mice were 12–14 wk old at the onset of diet (n ≥ 10/group) and 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. Four cohorts of LKO mice and their floxed/floxed WT controls were used for these studies.

Cohort 1 (before HFD) chow-fed. Mice fasted for 5 h and were then euthanized 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 wk (D08060104 60% fat from lard, 20% protein, and 20% carbohydrate from corn starch, 5.24 kcal/g; Research Diets) and then fasted for 5 h and euthanized. This cohort was included to obtain tissues that were not insulin treated for Figs. 2–4.

Cohort 3 (HFD/insulin clamp). Mice were fed HFD for 12 wk. 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 described previously (3). Mice were maintained on HFD for 1 wk of recovery and then fasted for 5 h before hyperinsulinemic euglycemic clamp study. This cohort was included to define insulin sensitivity and obtain insulin-treated tissues for Figs. 2–4.

Cohort 4 (HFD for 5 wk). Mice were fed HFD for 5 wk. After fasting for 5 h, mice underwent an intraperitoneal (ip) injection of insulin (0.75 units of regular human insulin diluted in 0.5 ml of saline) or saline and were euthanized 15 min 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-h-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 (2.5 μU·kg⁻¹·min⁻¹, Humulin R; Eli Lilly, Indianapolis, IN). Our clamp study design with an insulin infusion of 2.5 μU·kg⁻¹·min⁻¹ was optimized to define hepatic insulin action, because this insulin dose does not maximally suppress endogenous glucose production (EndoRγ; 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 euthanized and tissues flash-frozen. To obtain non-insulin-treated samples, a parallel set of experiments was performed where mice were fed a HFD for 12 wk, fasted for 5 h, and then euthanized (cohort 2).

Plasma processing and calculations. Insulin levels were determined by ELISA (no. EZRMJ-13K; Millipore, St. Charles, MO). [3-3H]glucose-specific activities were determined by liquid scintillation counting after plasma deproteination. Glucose disappearance rate (Ra) and EndoRγ 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/diacylglycerol content analysis. Liver lipid was extracted using Folch methodology as described (50). TG and diacylglycerol (DAG) were separated using TLC according to Zhu et al. (49). Total liver TG and DAG amount was quantified as reported previously (49).

Protein immunoblot. Antibodies for Akt and p-Akt Ser473 were from Cell Signaling Technology (Beverly, MA), antibodies for ERα (sc-7207), diacylglycerol acyltransferase (DGAT1; sc-31680), DGAT2 (sc-66859), FoxO1 (sc-11350), and β-actin (sc-47778) were from Santa Cruz Biotechnology (Santa Cruz, CA), and the antibody for apolipoprotein B (apoB)-100 was from Lifespan Biosciences (LS-c20729). Anti-microsomal triglyceride transfer protein (MTP) antibody was provided by Dr. Larry Swift (41). Primary antibody was incubated at 4°C overnight with the dilution recommended in the manufacturer’s database. Anti-mouse or anti-rabbit antibody was incubated with the dilution of 1:15,000 at room temperature for 1 h. 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 Image J processing program.

Liver glycogen quantification. Liver glycogen extraction was performed as established by Chan and Exton (6), with minor modifications. Briefly, 50–100 mg of liver tissue was homogenized in 0.03 N HCl, using 0.5 mm of zinc oxide beads (no. ZrOB05; Next Advance) in a Bullet-Blender. Homogenized tissues were incubated at 80°C for 10 min, and homogenate was blotted onto chromatography paper strips (no. 0303614; Whatman). Strips were washed three times in 70% ethanol for 40 min, rinsed in acetone, and then dried overnight. Dried strips were digested using 0.1 mg/ml amylglucosidase (no. A7420; Sigma) in 0.04 M sodium acetate for 3 h in a 37°C shaking water bath.

Glucose in glycogen was quantified by enzymatic assay. Twenty milliliters/well of a 96-well plate of glucose-enriched digest solution was loaded (no. 12565501; Fisher) with 250 μl of enzyme solution. The plate was incubated for 15 min at room temperature. Enzyme solution contained 70 mg of ATP (no. 100008; MP Biomedical), 24 ml of 200 mM Tris·HCl, 500 μl of 500 mM MgCl₂, 50 mg of β-NADP (no. 10128058001; Roche), 50 μl of hexokinase (no. 11426362001; Roche), and 125 μl of glucose-6-phosphate dehydrogenase (no. 10737232001; Roche). Plate absorbance was read at 340 nm to quantify glucose in digest solution. Oyster glycogen (Sigma no. G8751) and glucose (Sigma...
Table 1. HFD-induced obesity and insulin resistance for LKO and fl/fl mice

<table>
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<tr>
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<th>Chow fed</th>
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<tr>
<td><strong>Body weight, g</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>12-wk study</td>
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<tr>
<td><strong>%Adiposity</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>12-wk study</td>
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<tr>
<td><strong>%Muscle</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</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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<td><strong>Fasting insulin, ng/ml</strong></td>
<td>0.43 ± 0.07</td>
<td>3.38 ± 0.71*</td>
<td>0.47 ± 0.11</td>
<td>4.52 ± 1.02*</td>
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<tr>
<td><strong>Clamp insulin, ng/ml</strong></td>
<td>5.35 ± 0.6*</td>
<td>2.29 ± 0.11*</td>
<td>7.94 ± 1.59#</td>
<td>7.41 ± 0.29**</td>
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<tr>
<td><strong>Clamp C-peptide, ng/ml</strong></td>
<td>2.9 ± 0.9</td>
<td>67.0 ± 9.5</td>
<td>79.0 ± 2.4</td>
<td>79.0 ± 2.4</td>
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<tr>
<td>12-wk study</td>
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<tr>
<td><strong>Serum estradiol, pg/ml</strong></td>
<td>16.7 ± 1.0</td>
<td>16.7 ± 1.0</td>
<td>0.46 ± 0.22**</td>
<td>0.46 ± 0.22**</td>
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<td>5-wk study</td>
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<tr>
<td><strong>Body weight, g</strong></td>
<td>33.5 ± 1.2</td>
<td>3.35 ± 1.2</td>
<td>32.2 ± 1.1</td>
<td>32.2 ± 1.1</td>
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<td>5-wk study</td>
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<tr>
<td><strong>%Adiposity</strong></td>
<td>25.9 ± 1.1</td>
<td>74.3 ± 2.8</td>
<td>23.7 ± 1.6</td>
<td>75.6 ± 1.7</td>
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<tr>
<td><strong>%Muscle</strong></td>
<td>74.3 ± 2.8</td>
<td>74.3 ± 2.8</td>
<td>23.7 ± 1.6</td>
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<td>5-wk study</td>
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Values are means ± SE. HFD, high-fat diet; LKO, liver ERα-knockout. *P < 0.05, comparison between “before HFD” and “after HFD”; **P < 0.05, comparison of fl/fl-HFD with LKO-HFD; ***P < 0.005, comparison of fl/fl-HFD with LKO-HFD.
To determine the importance of ERα toward whole body insulin sensitivity, we performed hyperinsulinemic euglycemic clamps in LKO mice and WT littermates (Fig. 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 with the WT littermates (Fig. 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 compared with the WT littermates (P < 0.01; Fig. 1E). These results demonstrated that the 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 the clamp was optimized to define liver glucose metabolism (2.5 mU·kg⁻¹·min⁻¹; reviewed in Ref. 3). EndoRa was suppressed by hyperinsulinemia during the clamp in fl/fl littermates (Fig. 2A). In contrast, insulin failed to suppress EndoRa in male LKO mice, demonstrating hepatic insulin resistance (Fig. 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 wk and euthanized after a 5-h fast. In fl/fl mice, insulin during the clamp induced an increase in p-Akt Ser⁴⁷³ (Fig. 2, B and C). In LKO mice, insulin failed to induce p-Akt Ser⁴⁷³. In the insulin-sensitive liver, p-Akt Ser⁴⁷³ phosphorylates the transcription factor forkhead box protein O1 (FoxO1), resulting in nuclear exclusion of FoxO1 (46). Nuclear levels of FoxO1 were increased in LKO mice compared with fl/fl (Fig. 2, D and E). Insulin signaling through Akt and FoxO1 is an important pathway for the regulation of hepatic glucose and 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 compared with fl/fl controls (9.6 ± 2.1 vs. 17.5 ± 3.2 μg/mg, P < 0.05; Fig. 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; Fig. 2F). This impaired hepatic glycogen metabolism likely contributes to the impaired suppression of 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 Rd in the clamp period,
which was similar for fl/fl and LKO mice during the clamp (Fig. 4A). In muscle, insulin induced phosphorylation of Akt Ser^473^ to a similar degree in WT and LKO mice (Fig. 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 euthanized after a 5-h fast (liver TG: 15.3 ± 6.3 μg/mg for fl/fl and 16.1 ± 4.7 μg/mg for LKO; liver DAG: 0.31 ± 0.05 μg/mg for fl/fl and 0.29 ± 0.1 μg/mg for LKO). Hepatic steatosis developed in both WT and LKO mice following 12 wk of HFD, and this steatosis was more pronounced in LKO mice (Fig. 4A). Similarly, liver DAG content was also increased in HFD-fed LKO mice (Fig. 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 dephosphorylate 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 (Fig. 4, A, C, and D).

The acyl-CoA-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); also reviewed in Ref. 48). Insulin increases DGAT2 to promote TG synthesis and reduce hepatic free fatty acid 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 upregulate DGAT2 (Fig. 4, E and F). Reciprocally, DGAT1 is increased in fasting and reduced with hyperinsulinemia (27). Fasting levels of DGAT1 were increased in LKO mice compared with fl/fl, consistent with fatty liver in the LKO mice (Fig. 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 VLDL (10). In the liver, the enzyme MTP adds TG onto the nascent apoB-100 particle as it is translocated into the lumen of the endoplasmic reticulum for secretion as a VLDL (31). We observed that during fasting, liver apoB-100 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 (Fig. 5, A–D). Insulin reduces hepatic apoB-100 levels. Insulin suppressed expression of apoB-100 levels. Insulin suppressed expression of apoB-100 in both LKO mice and their fl/fl littermates (Fig. 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 the clamp in both groups of mice (Fig. 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 wk of HFD feeding. We next aimed to define the importance of liver ERα signaling at an intermediate duration of HFD feeding for 5 wk compared with 12 wk in the previous study. There were no differences in body weight or adiposity at 5 wk of HFD feeding (n = 9–10/group; Fig. 6A). To define acute insulin signaling, we administered intraperitoneal insulin or saline and euthanized mice 15 min later. We used this approach because insulin downregulates 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 Ser^473^ (Fig. 6, B and C). In LKO mice,

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**Fig. 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 (Ins)-stimulated phosphorylation from muscle extracts of Akt Ser^473^ was similar for LKO and fl/fl mice; n = 4–6/group for bar graphs. **P < 0.005. Differences between groups were determined by t-test.
baseline p-Akt Ser473 was reduced, and insulin failed to induce p-Akt Ser473. This finding is similar to the results of our 12-wk study for liver. Total Akt levels were unchanged in the liver between LKO and fl/fl. In muscle, for the fl/fl mouse, insulin also induced robust induction of p-Akt Ser473. In LKO mice, baseline p-Akt Ser473 was increased, and insulin had diminished capacity to induce p-Akt 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 wk of HFD feeding. Overall, this study confirms the importance of liver estrogen signaling in protecting the liver from insulin resistance and also protecting against 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 ERα-knockout (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 glycojen 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.

Fig. 4. Liver ERα signaling protects against triglyceride (TG) and diacylglycerol (DAG) accumulation with HFD feeding in male LKO mice. A and B: liver TG and DAG levels were increased in LKO mice compared with their littersmates. C and D: acyl-CoA carboxylase (ACC) phosphorylation (p-ACC) was decreased by insulin in LKO mice, shown by Western blotting from whole liver extracts. E–G: Western blotting of diacylglycerol acyltransferase (DGAT)1/2 impaired insulin regulation of DGAT levels in liver from LKO mice; n = 4–6/group for bar graphs. *P < 0.05 by t-test; ***P < 0.005 by t-test.
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⁻¹·min⁻¹ 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 wk 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, i.e., 5 wk. In these mice, there were no differences 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 knockout on muscle, as insulin-mediated glucose disposal (Rd) was not altered. However, we did see that muscle insulin signaling was impaired with an intraperitoneal injection of insulin in our cohort of mice fed HFD for 5 wk. 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 p-Akt was 4.2-fold in fl/fl mice at 5 wk but just 2.8-fold at 12 wk) and possibly because the duration of insulin infusion downregulated p-Akt in the clamp study. In the clamp study, we did see elevated C-peptide levels in LKO mice, which is also consistent with muscle insulin resistance. Thus, there is likely 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 Refs. 30 and 35). However, we were unable to measure sex hormone-binding globulin, 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 in 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 to avoid the counterregulatory changes that occur during the GTT. We additionally verified significant liver insulin resistance by loss of liver ERα with a second model, i.e., ip insulin vs. ip saline after 5 wk of HFD feeding (Fig. 6). Collectively, by coupling tissue-specific knockout of ERα with techniques optimized to study liver

**Fig. 5.** Increased fasting plasma TG level in LKO mice. A and B: Western blotting shows that liver apolipoprotein B (apoB)-100 protein levels were suppressed during insulin clamps for LKO and fl/fl mice. A and C: microsomal TG transfer protein (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 mice. E: cholesterol levels were reduced with insulin for LKO mice and their fl/fl controls; n = 4–6/group for bar graphs. *P < 0.05 by t-test; **P < 0.005 by t-test; #P < 0.05 by genotype by 2-way ANOVA.
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 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, the absence of liver ERα signaling leads to lipid accumulation with overnutrition (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 fatty acids and DAG. This coordinate synthesis of fatty acids with induction of DGAT2 minimizes the 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 with age- or BMI-matched men (14, 22). Our laboratory 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 can then be targeted 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.

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DISCLOSURES

The authors disclose no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS


REFERENCES

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