Role of Estrogens in the Regulation of Liver Lipid Metabolism

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Abstract Before menopause, women are protected from atherosclerotic heart disease associated with obesity relative to men. Sex hormones have been proposed as a mechanism that differentiates this risk. In this review, we discuss the literature around how the endogenous sex hormones and hormone treatment approaches after menopause regulate fatty acid, triglyceride, and cholesterol metabolism to influence cardiovascular risk.

The important regulatory functions of estrogen signaling pathways with regard to lipid metabolism have been in part obscured by clinical trials with hormone treatment of women after menopause, due to different formulations, routes of delivery, and pairings with progestins. Oral hormone treatment with several estrogen preparations increases VLDL triglyceride production. Progestins oppose this effect by stimulating VLDL clearance in both humans and animals. Transdermal estradiol preparations do not increase VLDL production or serum triglycerides.

Many aspects of sex differences in atherosclerotic heart disease risk are influenced by the distributed actions of estrogens in the muscle, adipose, and liver. In humans, 17β-estradiol (E2) is the predominant circulating estrogen and signals through estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and G-protein-coupled estrogen receptor (GPER). Over 1000 human liver genes display a sex bias in their expression, and the top biological pathways are in lipid metabolism and

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genes related to cardiovascular disease. Many of these genes display variation depending on estrus cycling in the mouse. Future directions will likely rely on targeting estrogens to specific tissues or specific aspects of the signaling pathways in order to recapitulate the protective physiology of premenopause therapeutically after menopause.

Introduction

Before menopause, women are protected from atherosclerotic heart disease associated with obesity relative to men. Women have a nearly decade-long delay in first myocardial infarction compared to men (Freedman et al. 2004; Lloyd-Jones et al. 2009). Furthermore, at any given age, women have about half the risk of cardiovascular disease relative to men (Roger et al. 2011; Wilmot et al. 2015). Sex hormones have been proposed as a mechanism that differentiates the differential risk of cardiovascular disease in men versus women. In women, the ovaries produce estrogens and progesterone, which are the predominant female sex hormones. Therefore, naturally cycling estrogen has been proposed to be protective against atherosclerotic cardiovascular disease. This view is supported by the increase in cardiovascular disease risk in women seen after menopause, which involves a natural decline in ovarian hormone production. Estrogens have effects in many organ systems that contribute to cardiovascular risk vs. protection, including regulation of liver lipid metabolism and serum lipoprotein levels. The liver is an important site where fatty acid, triglyceride, and cholesterol metabolism are coordinated to meet metabolic needs in normal physiology, and this coordinated metabolism goes awry with obesity. In this chapter, we review the complex effects of estrogens on regulation of plasma lipids and liver lipid metabolism in humans and animal models.

Many aspects of sex differences in atherosclerotic heart disease risk are influenced by the distributed actions of estrogens in muscle, adipose, and liver. In response to obesity, both men and women have increased free fatty acid (FA) release into blood. In response to increased FA delivery to the liver with obesity, circulating FA are packaged into triglyceride (TG)-rich very-low-density lipoprotein (VLDL) particles by the liver. Obesity is associated with increased production of VLDL-TG particles by the liver to a greater degree in men than in women (Reaven and Bernstein 1978; Mittendorfer et al. 2003). This is, in part, due to enhanced FA clearance by muscle (Frias et al. 2001; Clegg et al. 2017; Ribas et al. 2016), resulting in less FA delivery to the liver to drive VLDL-TG production. It is also known that in response to FA delivery to the liver that women secrete VLDL particles that are more TG rich (Magkos et al. 2007b), which would help the liver export liver TG and prevent liver fat accumulation with obesity. Production of more TG-rich VLDL is matched with accelerated VLDL-TG clearance rates in women (Matthan et al. 2008), which collectively contribute to lower plasma VLDL-TG levels with obesity in women. Estrogens exert regulatory control in nearly every step of these control points of lipid metabolism.

Mechanisms of Estrogen Signaling in the Liver

In humans, 17β-estradiol (E2) is the predominant circulating estrogen and is made by the ovaries and circulates as an endocrine hormone transported in plasma by sex hormone-binding globulin, where it passively diffuses across the cell membrane into target tissues. Tissues may also make estrogens locally from androgenic precursors where it acts in a paracrine manner. This is established in breast cancer cells and in the male reproductive tract but is less clear in tissues such as the liver (Pasqualini et al. 1996; Qian et al. 2001). The uterus is the classic estrogen-responsive target tissue since estrogen increases proliferation of the uterine lining. Thus, uterine mass can serve as a proxy for total body estrogen levels in animal studies. Many other tissues are responsive to estrogen action in vivo. In a transgenic mouse model designed to detect estrogen signaling, the liver was actually the most responsive to E2 (Ciana et al. 2003).

Estrogens can mediate their biologic effects in the liver through a number of mechanisms. The classic mechanism of E2 action involves E2 binding to the steroid nuclear hormone receptors, estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ). ERα and ERβ have the classic features of steroid hormone receptors – an activation function 1 (AF1) domain, a ligand-binding domain, a DNA-binding domain, and an activation function 2 (AF2) domain (Osborne and Schiff 2005). When unbound to ligand, ERα and ERβ are retained in the cytosol by association to heat shock protein 90 (Hsp90) complexes. Estrogens binding to either ERα or ERβ cause a conformational change that promotes dissociation from Hsp90, dimerization, and translocation into the nucleus. Once in the nucleus, ERα and ERβ bind to genomic locations based on sequence recognition of the DNA-binding domain (Osborne and Schiff 2005). These genomic sequences are commonly referred to as estrogen response elements (EREs) and are often characterized by an inverted repeat separated by three nucleotides (5′ AGGTCAnnnTGACCT 3′). These EREs are commonly found in the promoter or enhancer regions of liver genes whose transcription is regulated by estrogens. Over 1,000 human liver genes display a sex bias in their expression (Zhang et al. 2011). The top biological pathways are in lipid metabolism and genes related to CHD (Zhang et al. 2011). Genetic analysis of 100 mouse strains revealed important diet and sex interactions in the development of insulin resistance. Some of the most prominent genes discovered were involved in TG metabolism and FA oxidation and were sexually dimorphic (Parks et al. 2015). Additionally, chromatin immunoprecipitation assay revealed 43 of the lipid genes are transcriptionally regulated by ERα (Gao et al. 2008). In the mouse, scores of liver genes involved in TG and cholesterol metabolism vary with the 4-day estrous cycle of the mouse in an ERα-dependent manner (Villa et al. 2012), demonstrating a tight coordination of liver lipid metabolism with reproductive needs.

In addition to binding to genomic locations with by recognition of EREs, ERα and ERβ can bind to genomic locations indirectly, via protein-protein binding

with other transcription factors (Osborne and Schiff 2005). For instance, ERα interacts with the c-rel subunit of NFκB, preventing NFκB from promoting IL-6 expression (Galien and Garcia 1997). ERα can either coactivate or corepress Fos-/Jun-mediated transcription depending on the presence of ligand (Paech et al. 1997). In the liver ERα serves as a co-regulator to repress IL-1 beta gene transcription (Galien and Garcia 1997). Thus, ERα and ERβ can promote or inhibit gene transcription depending on the transcriptional machinery available at a particular genomic location.

An additional aspect of ERα regulation of liver lipid metabolism occurs by modifying signaling and is transcriptionally independent (Park et al. 2011). Estrogens can also alter cell signaling via binding to receptors localized to the plasma membrane. ERα and ERβ have been shown to localize to the plasma membrane (Bjornstrom and Sjoberg 2005; Marino et al. 2006; Levin 2009). Membrane localization is achieved through palmitoylation of a serine residue and association with caveolin-1 (Cav-1) (Levin 2009). Membrane ERα and ERβ signal through the ERK 1/2 and the PI3K pathways (Bjornstrom and Sjoberg 2005; Levin 2009; Marino et al. 2006). After removal of ovarian hormones by ovariectomy, the benefits of the ERα agonist propylpyrazoletriol (PPT) with regard to liver lipid metabolism can largely be restored by membrane-localized ERα (Pedram et al. 2013).

In addition to membrane-localized ERα and ERβ, estrogens can signal through another cell surface receptor, G-protein-coupled estrogen receptor (GPER, also called Gpr30), which is expressed in multiple tissues including liver (Sharma et al. 2017; Nilsson et al. 2011; Owman et al. 1996). E2 binding to GPER initiates two signaling cascades – one results in increases in cyclic AMP (cAMP), and the other results in increases in intracellular Ca^{2+} (Nilsson et al. 2011). Some of the signaling mediated by GPER also involves activation of epidermal growth factor receptor (EGFR) (Nilsson et al. 2011). Although GPER is most well characterized for its ability to regulate cell signaling, GPER-activated cell signaling may also regulate gene expression since E2 treatment has been shown to alter gene expression in ERα/ERβ double-knockout mice (Lindberg et al. 2002). Whole-body deletion of GPER accelerates atherosclerosis and increases LDL cholesterol levels in mice fed an atherogenic diet (Meyer et al. 2014). It is unclear though whether the LDL cholesterol increases seen in GPER null mice are due to hepatic effects or due to indirect changes due to loss of GPER in other tissues. Thus, the current literature supports a robust role of estrogen signaling through ERα to impact glucose and TG metabolism, both membrane and transcriptional effects. The relative contributions of estrogen signaling through ERα, ERβ, or GPER with regard to liver gene expression and cardiovascular risk still warrant further investigation.

Hormone Treatment and the Risk of Cardiovascular Disease in Postmenopausal Women

The important regulatory functions of estrogen signaling pathways with regard to lipid metabolism have been in part obscured by the conflicting results of clinical trials with hormone treatment of women after menopause. These controversies arise in part from the different formulations of estrogen used for treatment of women after menopause, different routes of administration, and different pairings with progestins. The estrogen hypothesis suggests that the higher level of estrogen in women before menopause protects against cardiovascular disease. In support of this, postmenopausal women have increased risk of cardiovascular disease compared to premenopausal women (Colditz et al. 1987; Hu et al. 1999; Kannel et al. 1976; van der Schouw et al. 1996). A number of prospective studies conducted in the 1970s through the 1990s suggested that hormone treatment, typically with conjugated estrogens, improved risk of cardiovascular disease (Burch et al. 1974; Bush et al. 1983; Criqui et al. 1988; Croft and Hannaford 1989; Grady et al. 1992; Grodstein and Stampfer 1995; Hammond et al. 1979; Henderson et al. 1991; Hernandez Avila et al. 1990; Petitti et al. 1987; Stampfer et al. 1985; Sullivan et al. 1990; Wilson et al. 1985; Wolf et al. 1991). Prospective studies, while informative, are subject to various sources of bias. One potential source of bias in prospective studies of estrogen treatment has been labeled the "healthy woman" bias (Aguilar-Salinas et al. 2002), a form of selection bias. This source of bias is due to active seeking of medical care. According to this model, women more willing to be on hormone treatment are also more likely to monitor their health and take other medications to treat other diseases, thus enriching for a population that is healthier overall.

To more definitively determine the effect of hormone treatment on risk of cardiovascular disease, a number of randomized controlled trials were aimed to experimentally determine whether hormone therapy could prevent cardiovascular disease in postmenopausal women. Two of the largest randomized controlled trials, conducted in 1990s, were the Women's Health Initiative (WHI) and the Heart and Estrogen/Progestin Replacement Study (HERS) (Hulley et al. 1998; Manson et al. 2003). Hormone treatment consisted of conjugated estrogens and progestin if the women had an intact uterus or conjugated estrogens alone if the women had a prior hysterectomy. The WHI trial enrolled over 16,000 postmenopausal women and monitored cardiovascular disease outcomes over an average of 5.6 years. The HERS trial enrolled over 2,700 women and monitored cardiovascular disease outcomes over an average of 6.8 years (Grady et al. 2002). Despite the improvement in cholesterol and diabetes risk factors, hormone treatment did not improve cardiovascular disease in the HERS trial. In the WHI trial, hormone treatment actually worsened cardiovascular disease risk. The increased cardiovascular risk associated with hormone treatment in the WHI was the worst in women who had been assigned to hormone treatment over 10 years after the onset of menopause. This led to the development of the "timing hypothesis," which suggests that hormone treatment is

most beneficial if initiated soon after menopause and potentially harmful if initiated late (>10 year) in menopause.

The Early versus Late Intervention Trial with Estradiol (ELITE) study was designed to test the timing hypothesis (Hodis et al. 2016). The ELITE study enrolled over 600 postmenopausal women and randomized them to treatment with placebo or oral estradiol plus vaginal progesterone for 10 days per cycle. Women in the ELITE trial were stratified into two groups – one group of women were considered in early menopause if menopause occurred in the last 6 years, and the other group of women were considered in late menopause if menopause occurred at least 10 years prior to enrollment in the study. Women were followed 5 years, and carotid intima medial thickness (CIMT) and coronary artery calcium (CAC) score were used as indices of atherosclerosis. Estradiol treatment reduced the progression of CIMT in the early menopause group but failed to delay atherosclerosis in the late menopause group. This result supports timing hypothesis of estrogen treatment. Coronary atherosclerosis was approximated using CAC score, but this measure was added late to the ELITE trial. Oral estradiol did not alter coronary atherosclerosis in either the early or late menopause group. It is unclear whether the failure to detect a difference in coronary atherosclerosis was due to insufficient power or the ineffectiveness of oral estradiol to reduce coronary atherosclerosis. A post hoc analysis of recently postmenopausal women (age 50–59) in the WHI supported that treatment with conjugated estrogens reduced coronary atherosclerosis as measured by coronary calcium imaging (Manson et al. 2007). Interestingly, hormone treatment increased plasma TGs in ELITE, in agreement with the WHI and HERS trial. This may suggest that increases in TGs with hormone treatment may mitigate other improvements in plasma lipids with regard to risk of coronary heart disease. Further work will be needed to confirm whether treatment with estrogen formulations improve cardiovascular outcomes in addition to the improvements in CIMT in women beginning treatment soon after menopause and how this is balanced by a potential worsening of dyslipidemia with different hormone treatment approaches.

In addition to cardiovascular outcomes, two smaller, related randomized controlled trials found somewhat divergent results regarding hormone treatment on measures of coronary atherosclerosis. In the Women's Estrogen–Progestin Lipid-Lowering Hormone Atherosclerosis Regression Trial (WELL-HART), 226 postmenopausal women with an average age of 63.5 with known coronary disease were randomized to receive placebo, micronized E2, or micronized E2 and progesterone (Hodis et al. 2003). In the WELL-HART study, neither micronized E2 nor micronized E2 and progesterone prevented progression of coronary atherosclerosis. In the Estrogen in the Prevention of Atherosclerosis Trial (EPAT), 222 healthy postmenopausal women with an average age of 62.2 without preexisting coronary disease were randomized to receive micronized E2 or placebo (Hodis et al. 2001). Contrary to the WELL-HART study, micronized E2 treatment reduced the rate of subclinical atherosclerosis of the carotid artery in the EPAT study. The differences in the WELL-HART and EPAT studies raise several possibilities regarding hormone treatment and cardiovascular disease. Firstly, estrogen alone may be required to prevent cardiovascular disease. Secondly, hormone treatment may be effective in prevention

of atherosclerosis but may be ineffective at reversing established atherosclerosis in postmenopausal women. Thirdly, estrogen therapy may perhaps have more potent effects on preventing or reversing carotid artery atherosclerosis than on atherosclerosis in coronary arteries since the EPAT and ELITE trials both showed that estrogen therapy reduced progression of carotid atherosclerosis.

The cause of the TG-rich dyslipidemia with hormone treatment of postmenopausal women has been controversial. Normal cyclic variations in estrogen levels through the menstrual cycle do not impact VLDL-TG or VLDL-apoB kinetics or concentrations (Magkos et al. 2006). Neither cycling hormones nor hormone treatment with E2 alone or in combination alters FA concentration or fluxes to the liver to a greater degree than day-to-day variation (Jensen et al. 1994; Magkos et al. 2007a). By contrast, estrogens seem to have a significant impact on liver VLDL production that depends on the route of delivery. Oral delivery of micronized estradiol increased VLDL production rates by 80%, which was greater than conjugated estrogen, whereas transdermal estradiol had no effect on VLDL production rates in this study (Walsh et al. 1991). Another study with oral ethinyl estradiol increased VLDL-apoB production over 100% (Schaefer et al. 1983), which is a similar result found to earlier studies with conjugated equine estrogens (Glueck et al. 1975). That estrogens are the major driver of hypertriglyceridemia with oral hormone treatment of postmenopausal women is further supported in that progestins oppose the effect of estrogens, by stimulating VLDL clearance in both humans and animals (Kissebah et al. 1973; Kim and Kalkhoff 1975) and reviewed in (Magkos and Mittendorfer 2009).

Relative to oral estrogens, transdermal estradiol has less potent effects on lowering LDL cholesterol and increasing HDL cholesterol (Baksu et al. 2007; Chen et al. 2001; Sanada et al. 2004; Strandberg et al. 2003; Zegura et al. 2006). Additionally, transdermal estradiol treatment does not seem to increase plasma TGs when compared to oral estrogen formulations (Baksu et al. 2007; Chen et al. 2001; O'Sullivan et al. 1998; Sanada et al. 2004; Strandberg et al. 2003; Zegura et al. 2006). In fact, most studies demonstrate that transdermal estradiol actually reduces plasma TGs (Baksu et al. 2007; Chen et al. 2001; Zegura et al. 2006), but this was not consistently demonstrated in all trials. In a small study examining VLDL-TG kinetics, transdermal estradiol was shown to reduce plasma TG by increasing the rate of VLDL-TG clearance without affecting VLDL-TG production (Smith et al. 2014). Since transdermal estradiol seems to have less potent effects on plasma lipid metabolism compared to oral estrogen formulations, the liver is likely responsible for most of estrogen's effects on increasing VLDL-TGs in the blood after menopause.

Lack of Estrogen Signaling Promotes Liver TG Accumulation and Leads to Hepatic Insulin Resistance

The converse implication of estrogen-mediated reductions in FA delivery to the liver and estrogen-mediated increases in VLDL-TG export is that deficiency of estrogen, with antagonist, after menopause, or in experimental models, leads to liver

fat accumulation. Tamoxifen (TMX) is an antiestrogen drug used for the treatment of hormone-sensitive breast cancer. One side effect of TMX is the development of nonalcoholic fatty liver disease and steatohepatitis (Nishino et al. 2003; Murata et al. 2000; Oien et al. 1999). As women transition to menopause, the risk of nonalcoholic fatty liver disease increases (Ryu et al. 2015; Yang et al. 2014). Aging is a natural risk factor for NAFLD, which may confound the impact of menopause on risk of NAFLD. However, some younger women have their ovaries surgically removed for medical reasons. In women undergoing surgical menopause, the risk of NAFLD is increased nearly twofold (Matsuo et al. 2016). Furthermore, in postmenopausal women, hormone treatment reduced plasma levels of liver enzymes, a marker of liver damage in NAFLD (McKenzie et al. 2006). Thus, absence of ovarian hormones leads to an increased risk of NAFLD that is at least partially reversible with estrogen treatment in postmenopausal women.

The impact of reductions in estrogen signaling on liver lipid metabolism has been studied extensively in rodent models. Ovariectomy, or surgical removal of ovaries, in rodents leads to an accumulation of liver triglyceride content (Barrera et al. 2014; Cote et al. 2012; de Oliveira et al. 2016; Paquette et al. 2007; Rogers et al. 2009), similar to that seen in postmenopausal women. Additionally, a chemical model of menopause created by administration of 4-vinylcyclohexene diepoxide (VCD), which depletes primordial follicles, creates insulin resistance, fatty liver, and dyslipidemia (Romero-Aleshire et al. 2009). In addition to estrogen deficiency causing steatosis in rodent models, estrogen replacement reduces steatosis (Barrera et al. 2014; Bryzgalova et al. 2008; Villa et al. 2012; Camporez et al. 2013; Chambliss et al. 2016; Palmisano et al. 2016; Wang et al. 2015; Zhu et al. 2013; Kim et al. 2014).

Estrogens and the Physiologic Regulation of Liver Lipid Metabolism Through ER Alpha

The physiologic reason that estrogen regulates to liver TG metabolism may be connected to an evolutionarily conserved need to coordinate nutritional status with reproduction. Insects, birds, and fish all have increased transport of TG from the liver to facilitate egg development through estrogen-like pathways (Davis 1997). To define tissue-specific contributions of estrogen signaling, several groups have created hepatocyte-specific ERα knockout mice to define the effects of estrogen signaling specifically through liver ERα. Della Torre and colleagues demonstrated a requirement for liver ERα in mediating amino acid regulation of the reproductive cycle (Della Torre et al. 2011). Using a mouse model with ERα deficiency in hepatocytes, we demonstrated that the ability of estrogens to reduce liver steatosis is lost in with deletion of liver ERα, suggesting that estrogens are acting directly in the liver to reduce TG content through ERα (Palmisano et al. 2016; Zhu et al. 2013; Villa et al. 2012). As expected, loss of liver ERα results in increased expression of

lipid synthesis genes (Bryzgalova et al. 2006), loss of estrogen regulation of target genes (Palmisano et al. 2016; Zhu et al. 2013), and impaired estrogen regulation of other lipid metabolic target genes (Della Torre et al. 2016). One proposed mechanism for ERα regulation of lipid synthesis targets involves estrogen-ERα regulation of the nuclear receptor Small Heterodimer Partner (SHP), a target gene of ERα (Palmisano et al. 2016; Wang et al. 2015). Additionally, estrogen-ERα regulation of liver lipid metabolism has been proposed to act via microRNA mir-125b (Zhang et al. 2015). Additional work is needed to establish the clinical relevance of these proposed targets in humans and to develop therapies that can recapitulate the lipid-lowering effect of estrogen in the liver to reduce the clinical burden of NAFLD.

In female mice, estradiol treatment at the time of ovariectomy blocks the effects of hyperinsulinemia with regard to lipid metabolism, leading to a suppression of de novo lipogenesis and maintenance of hepatic VLDL production (Zhu et al. 2013). The net effect of E2 treatment was to reduce liver TG and diacylglycerol content and to improve insulin action with regard to glucose metabolism. In female mice lacking the liver ERα, E2 following ovariectomy limits adiposity but fails to improve insulin sensitivity, fails to limit liver DAG, and fails to prevent insulin suppression of VLDL production. E2 administration at the time of ovariectomy and high-fat diet feeding significantly decreases liver TG and DAG content compared to ovariectomy and sham mice by limiting ^{14}C-glycerol deposition into liver triglycerides and diacylglycerol, combined with maintaining the efficiency of triglyceride export from the liver in the setting of hyperinsulinemia (Camporez et al. 2013; Zhu et al. 2013).

The effects of liver estrogen signaling to improve liver insulin sensitivity, suppress lipogenesis, and promote VLDL output from the liver were independent of estrogen's ability to regulate body weight as E2-treated hepatocyte knockout mice after ovariectomy were lean due to intact estrogen signaling in CNS and other tissues, yet E2 treatment failed to regulate liver metabolism (Zhu et al. 2013). Additionally, paired feeding in mouse studies shows direct effects of E2 protecting from fatty liver that are independent on its effects in the CNS to reduce food intake (Bryzgalova et al. 2008).

Estrogen Signaling Limits Liver Fat Accumulation by Reducing de novo Lipogenesis in the Liver

The mechanisms by which estrogen signaling protects against hepatic steatosis include reductions in de novo lipogenesis, as reported by different laboratories. Using a combination of chromatin immunoprecipitation and tiled microarrays (ChIP-on-chip) approach, Gao et al. identified binding regions of ERα to DNA in intact chromatin in the liver (Gao et al. 2008). This analysis revealed 19 gene ontology (GO) categories including lipid biosynthesis (GO 0008610) and fatty acid metabolism (GO 0006520) which are significantly enriched for genes that had ERα recruited to their promoter after 2 h of estradiol treatment (Gao et al. 2008).

Conventional ChIP followed by qPCR shows binding of ERα to promoter regions of lipogenesis genes including STAT3 and SHP which are consistently increased after treatment with estradiol or ERα agonist (Gao et al. 2008). This report is consistent with their previous observation that E2 treatment promotes ERα binding to STAT3 promoter and STAT3-Tyr phosphorylation, which subsequently suppresses Fasn, Scd1, Acaa1, and Gpam expression in the liver in ob/ob mice (Gao et al. 2006). Estradiol treatment in female mice with intact ovary suppresses FASN and SCD-1 in the liver (Bryzgalova et al. 2008). The authors also reported that E2 treatment decreases HGP by suppressing G-6-P expression (Bryzgalova et al. 2008).

E2 treatment suppresses liver lipogenesis by maintaining ACC phosphorylation as has been reported in studies from our laboratory and others (Cole et al. 2010; Zhang et al. 2013; Zhu et al. 2013, 2014). This mechanism likely contributes to the correction of pathway-selective insulin resistance in the liver by E2 treatment. This is due to the observation that insulin suppresses ACC phosphorylation during hyper-insulinemic clamp to promote lipogenesis, which action is diminished after E2 treatment (Zhu et al. 2013, 2014). ACC phosphorylation is regulated by AMPKα phosphorylation in the liver (Tuazon et al. 2015). Estradiol induces signal transduction through ERα, which localizes to both the plasma membrane and nucleus. A study by Pedram et al. shows that activation of liver estrogen signaling by ERα agonist PPT promotes AMPK phosphorylation in WT mice and transgenic mice expressing only the ligand-binding domain of ERα exclusively at the plasma membrane but not in ERα knockout mice. This study shows that gene expression changes mediated by membrane-localized ERα result in important metabolic effects independent of nuclear ERα (Pedram et al. 2013). In this study, phosphorylation of AMPK by activation of ERα is associated with phosphorylation of ACC. In addition, activation of membrane ERα also counteracts insulin's action to promote the mRNA of lipogenesis gene Srebf1 (Pedram et al. 2013). Additionally, oral CE and the SERM and BZA also promote AMPK phosphorylation via ERα in the liver after ovariectomy (Kim et al. 2014). In this study by Kim et al., oral CE and BZA reduce hepatic FAS expression and FAS activity and are associated with a decrease in liver TG accumulation in female after OVX. This appears to be mediated in part by inducing CEACAM1 expression and phosphorylation, which triggers CEACAM1 binding to and downregulation of FAS activity in liver (Kim et al. 2014).

E2 treatment also likely promotes FA oxidation in liver. Levels of mRNA for CPT-1, a protein to transport fatty acid into mitochondrial for β-oxidation, are induced with E2 treatment. Increased oxygen consumption and liver ATP production associated with changes in UCP2 expression in the liver were reported in E2 treatment after ovariectomy, indicating increased fatty acid oxidation in the liver in those mice (Camporez et al. 2013). Additionally, E2 and CE increase production of FGF21 by the liver which may also increase hepatic FA oxidation (Kim et al. 2014).

Lipotoxicity due to the accumulation of lipid in hepatocytes leads to hepatic insulin resistance with regard to glucose metabolism. Insulin's action to suppress hepatic glucose production (HGP) during hyperinsulinemic-euglycemic clamp is blunted after ovariectomy female mice compared to sham controls after a short term of high-fat diet feeding, although liver TG and DAG are not significantly increased

with this duration of diet (Camporez et al. 2013). The blunted insulin action is associated with decreased insulin signaling, indicated by phosphorylation of AKT, in the liver (Camporez et al. 2013). Without challenged by high-fat feeding, HGP is blunted by 20% in global ERα KO mice compared to WT controls, which is also associated with diminished insulin signaling response during hyperinsulinemic-euglycemic clamp (Ribas et al. 2010). In unconscious mice with no differences in glucose disposal, decreased glucose infusion rate (GIR) during clamp is attributed to increased HGP in ERα-deficient mice compared to WT controls (Bryzgalova et al. 2006). Furthermore, we found that ovariectomy with high-fat diet feeding results in liver TG and DAG accumulation, decreased GIR and increased HGP, and diminished hepatic insulin signaling during hyperinsulinemia (Zhu et al. 2013).

Estrogen Regulation of Factors Indirectly Affecting Liver Lipid Metabolism

While estrogens have been proposed to directly regulate a number of pathways involved in cardiovascular disease directly, especially in the liver, estrogen activity in a number of other tissues may contribute indirectly to plasma lipid responses to estrogens and liver lipid metabolism by estrogens. One of the original hypotheses purported to explain male-female differences in cardiovascular disease relates to body fat distribution differences between men and women. With obesity, women have more subcutaneous fat, whereas men have more visceral fat. Additionally, body fat distribution changes in women from a more subcutaneous distribution to a more visceral distribution of fat with menopause (Svendsen et al. 1995). Since body fat distribution, as measured by waist-to-hip ratio, predicts risk of cardiovascular disease (Canoy et al. 2007; Yusuf et al. 2005), women may have lower risk of cardiovascular disease due to a more favorable body fat distribution. The hypothesis that body fat distribution contributes to risk of cardiovascular disease was put forth by Vague in 1947 (Vague 1947). Experimental evidence to prove this hypothesis would take many decades, but two large prospective studies confirmed that body fat distribution did indeed predict risk of future cardiovascular disease (Canoy et al. 2007; Yusuf et al. 2005). Exercise and weight loss can reduce waist-to-hip ratio and reduce risk of cardiovascular disease, but long-term weight loss in obese patients remains a clinical challenge due to weight regain. Pharmacologic agents that modify body fat distribution are not currently available. Furthermore, a pooled meta-analysis found that waist-to-hip ratio contributed to cardiovascular risk similarly between men and women (de Koning et al. 2007). Therefore, it is important to understand other factors that may explain how women have lower risk of cardiovascular disease relative to men.

Some of estrogen's protective effects in the liver are likely indirectly due to estrogen signaling adipose tissue to limit the release of serum FA in response to insulin, and in skeletal muscle to promote FA oxidation, thereby limiting FA deliv-

ery to the liver. In fasting, about 75% of the lipid that ends up in VLDL is from FA delivered to the liver by lipolysis and only ~4% from lipogenesis. With feeding, 43% still come from adipose, and 25% are from diet, either from chylomicrons or spillover from the plasma FA pool (Barrows and Parks 2006; Barrows et al. 2005). Thus, FA flux to the liver is a much more important driver of fatty liver and dyslipidemia than insulin-driven de novo lipogenesis (reviewed in Otero et al. 2014). In this regard, it is important that female humans and rodents are protected against FA-mediated insulin resistance in muscle and whole body in response to increased delivery of FA experimentally (Frias et al. 2001; Hevener et al. 2002). Female mice with muscle-specific ERα knockout have insulin resistance and muscle lipid accumulation due to abnormal mitochondrial function (Ribas et al. 2016).

In response to increased FA flux, the liver oxidizes or re-esterifies FA. Some of this FA results in diacylglycerol accumulation, which activates PKCs and causes impaired glucose metabolism (Jornayvaz et al. 2011). The FA that is made into TG is matched with increased VLDL-TG secretion during fasting. Beyond physiologically normal liver fat (~5%) however, VLDL export is maximized, and liver fat accumulation ensues (Fabbrini et al. 2009). Thus, the dyslipidemia and glucose abnormalities of obesity result in part from impaired coordination of the adipose, muscle, and liver with regard to the flux of FA, and estrogen signaling pathways exert regulatory control on several of these key steps as reviewed above.

Mouse-Human Differences in Estrogen-Regulated Liver Lipid Metabolism and Implications for Disease

Studies with hormone treatment after menopause suggest that estrogen treatment reduces plasma glucose and insulin levels. These reduced insulin and glucose levels ultimately were associated with lower incidence of impaired glucose tolerance and type-2 diabetes (Bonds et al. 2006; Espeland et al. 1998; Ferrara et al. 2001; Rossi et al. 2004; Zhang et al. 2002). Thus, estrogen lowers risk of type-2 diabetes, a negative risk factor for coronary heart disease. Despite improvements in a number of risk factors, hormone treatment in postmenopausal women had certain negative effects on risk of cardiovascular disease. Hormone treatment increased plasma TGs in the WHI, HERS, and ELITE trials along with a number of prospective studies (Hodis et al. 2003; Hsia et al. 2006; Hulley et al. 1998; Barrett-Connor et al. 1997; Trial 1995; Wiegratz et al. 1998; Walsh et al. 1991; Schaefer et al. 1983). Several studies have demonstrated that this increase in plasma TGs is due to increased VLDL production with estrogen treatment approaches (Walsh et al. 1991; Schaefer et al. 1983; Glueck et al. 1975). The progestin component of hormone treatment accelerates TG clearance and thus likely does not contribute to hypertriglyceridemia (Kissebah et al. 1973). Additionally, only oral CE increases serum TG, likely because of high first-pass metabolism, which is minimized by transdermal E2. Mechanisms responsible for this increase in VLDL-TG production with oral CE have been hampered

since mouse models do not recapitulate the increase in plasma TGs in response to E2 treatment (Bourassa et al. 1996; Camporez et al. 2013; Marsh et al. 1999; Zhu et al. 2013).

Cholesteryl ester transfer protein (CETP) is a 74 kD glycoprotein that is expressed in the liver and adipose and is secreted into the circulation where it shuttles TG and cholesteryl esters (CE) between lipoproteins. With obesity, TGs are elevated in VLDL. CETP shuttles these TGs into HDL particles, which destabilizes binding of the HDL scaffold protein ApoA1, leading to increased HDL clearance and low HDL cholesterol levels. Although CETP has been mostly studied in association with HDL cholesterol levels, the expression pattern of CETP suggests an important role of CETP the metabolic adaptation to obesity. CETP is highly expressed in the liver and adipose, tissues that mediate glucose and TG metabolism (Jiang et al. 1991). CETP activity varies as much as six- to eightfold in human studies (Tato et al. 1995; de Vries et al. 2005). Cholesterol feeding, insulin, and ovarian hormones, all lead to significant changes in CETP activity (Arii et al. 1997; Marotti et al. 1993; Johansson et al. 2012). Our recent work demonstrated that CETP is required for mice to increase plasma TGs in response to E2 treatment after ovariectomy (Palmisano et al. 2016). CETP is a secreted plasma protein that shuttles TG and cholesteryl ester between plasma lipoproteins. Mice naturally lack CETP. Transgenic expression of CETP in mice results a similar hypertriglyceridemic effect of estrogen in female mice (Palmisano et al. 2016). We demonstrated that CETP expression creates estrogen gain of function for several pathways involved in liver lipid metabolism. For instance, E2 treatment increases in the activity of protein disulfide isomerase (PDI), a protein involved in the lipidation of VLDL only in CETP mice. Additionally, we found that estrogen-mediated increases in VLDL-TG production were eliminated with hepatocyte-specific deletion of the nuclear receptor small heterodimer partner (SHP) but not ERα (Palmisano et al. 2016). Since increased plasma TGs are associated with increased risk of cardiovascular disease, the increase in plasma TGs caused by estrogen may mitigate some of the beneficial aspects of estrogen treatment on cardiovascular disease risk. A better understanding of the CETP-SHP-PDI pathway regulating estrogen-mediated increases in VLDL production may lead to therapeutic strategies that alleviate the hypertriglyceridemic effect of estrogen treatment approaches.

Selective Estrogen Delivery Approaches and Liver Lipid Metabolism

Experimental models have demonstrated therapeutic value of targeting estrogen toward specific tissues or toward specific aspects of estrogen signaling pathways. An estrogen dendrimer conjugate (EDC) comprised of estradiol (E2) molecules linked to a poly(amido)amine dendrimer selectively activates nonnuclear ER and in mice. This EDC compound does not have activity to promote uterus growth and

does not promote breast cancer growth but does blunt liver fat accumulation with obesity, suggesting that the nonnuclear effects of estrogen are critical with regard to regulation of hepatic fat content (Chambliss et al. 2016; Chambliss et al. 2010). Interesting targeting nonnuclear estrogen with EDC did not prevent atherosclerosis in apoE null mice, which estradiol did (Chambliss et al. 2016).

Treatment of female mice after ovariectomy with bazedoxifene (BZA) alone, or in combination with low-dose estradiol or conjugated estrogen, each prevent weight gain with high-fat feeding after ovariectomy (Kim et al. 2014). The BZA also prevents fatty liver accumulation after ovariectomy, but not to as great a degree as conjugated estrogens (Barrera et al. 2014; Kim et al. 2014). Interestingly compared to conjugated estrogens and E2, BZA is a much more potent inducer of several pathways which may mediate estrogen's protective effects with regard to liver fat metabolism, FGF15, and SHP (Kim et al. 2014).

Additionally, targeting estradiol to metabolic tissues by conjugating GLP-1 to estradiol prevents weight gain and glucose metabolism in obese mice. These actions are not mediated by the GLP-1 component alone. Like the EDC compound, GLP-1 conjugated estradiol does not promote growth of the uterus or MCF-7 breast cancer cells (Finan et al. 2012).

Estrogen Regulation of Liver Cholesterol Uptake and Reverse Cholesterol Transport

Estrogen is proposed to protect against atherosclerosis via its role in reverse cholesterol transport. Reverse cholesterol transport (RCT) is the process of cholesterol removal from peripheral tissues and delivered into the feces, either by direct excretion into bile or conversion to bile acids and subsequent secretion into bile (reviewed elsewhere Rosenson et al. 2012). Estrogen's role in the initial steps of the RCT pathway is controversial in humans. Two studies by the same group support that plasma from women has greater cholesterol efflux capacity (Badeau et al. 2009) or similar cholesterol efflux capacity relative to men (Badeau et al. 2013). In premenopausal women, the concentration of estrogen in plasma is not associated with cholesterol efflux capacity (Badeau et al. 2013). In premenopausal women, polycystic ovary syndrome (PCOS), which is a state of low estrogen, is associated with reduced cholesterol efflux capacity (Roe et al. 2014). The estrogen deficiency of menopause, however, increases the cholesterol efflux capacity of HDL relative to premenopausal women, likely because of increased VLDL-TG levels after menopause (El Khoudary et al. 2016). In postmenopausal women, hormone replacement therapy effectively increases cholesterol efflux capacity of HDL (Ulloa et al. 2002). Thus, estrogen has been shown to have some effects on cholesterol efflux capacity, but there is no consistent relationship between estrogen enhancing and impairing this initial step in RCT based on the literature in humans.

Estrogen signaling pathways have a more firmly established role promoting the later aspects of RCT through action in the liver. Liver estrogen signaling through ERα has been shown to regulate hepatic cholesterol uptake and the efflux capacity of HDL from macrophages during the proestrus period when estrogen levels are high (Della Torre et al. 2016). The role of sex and estrogen on later stages in RCT is not well studied in humans, since no available methodologies exist yet to quantify RCT in humans. Our recent work demonstrates that female mice have increased total body RCT compared to males fed a Western diet (Zhu, submitted). We also found that liver deletion of ERα impaired total body RCT in female mice, suggesting that liver ERα is required for females to enhance total body RCT (Zhu, submitted). E2 and PPT treatment of mice both promotes liver secretion of cholesterol into bile and is prevented by concurrent treatment with an ERα antagonist (Wang et al. 2004).

Regulation of Hepatic Cholesterol Biosynthesis by Estrogens

A functional estrogen-responsive element has been identified within the promoter of the HMG-CoA reductase gene, one of the first steps of liver cholesterol synthesis. In peripheral tissues such as breast ductal epithelium and uterine endometrium, E2 promotes HMG-CoA reductase gene promoter activity and transcription to increase cholesterol biosynthesis for cell proliferation (Di Croce et al. 1999). However, E2 treatment reduces free cholesterol content in hepatocytes (Semenkovich and Ostlund 1987). Animal studies show that HMG-CoA reductase protein levels are lower in female or E2-treated male rats than in adult untreated males, which is regulated through nuclear SREBP2 activity (De Marinis et al. 2008). In line with this observation, Pedram et al. reported that the ERα agonist PPT suppresses HMG-CoA reductase expression and hepatic cholesterol content accompanied by decreased expression of *srebf2* (Pedram et al. 2013).

Regulation of Hepatic Cholesterol Uptake by Estrogens

To maintain efficient HDL-mediated cellular cholesterol efflux from foam cells, cholesterol and cholesteryl esters in HDL particles are either removed by the liver through the scavenger receptor class B member I (SR-BI) pathway or transferred via cholesteryl ester transfer protein (CETP) to apoB-containing particles in the blood and subsequently cleared through LDLR or remnant receptor pathways. Estradiol promotes liver secretion of cholesterol into bile, and this is prevented by concurrent treatment with an ERα antagonist (Wang et al. 2004). In ovariectomized rats, estrogen deficiency downregulates expression of a number of enzymes involved in bile acid synthesis (Liao et al. 2015). In mice, natural variation in estrogen cycling is associated with changes in expression of bile acid synthesis gene expression

(Della Torre et al. 2016). Additionally, estrogen enhances synthesis of bile acids, which is also preventable with concurrent treatment with an ERα antagonist (Wang et al. 2006). Furthermore, E2 fails to promote bile secretion in mice lacking ERα (Wang et al. 2004), suggesting that estrogen acts through ERα to promote bile secretion. The mechanism of estrogen-mediated increases in bile acid synthesis gene expression seems to require liver ERα (Della Torre et al. 2016; Yamamoto et al. 2006).

LDLR

Impaired regulation of several steps in RCT can associate with increased CHD risk (Rader and Tall 2012), and hepatic LDLR and SR-BI play critical roles in RCT. The activity of low-density lipoprotein (LDL) receptors in the liver constitutes a major mechanism by which dietary and hormonal agents may regulate plasma cholesterol levels (Rudling et al. 1992). Ethinyl estradiol treatment promotes clearance of β-VLDL and LDL in human subjects with type III hyperlipidemia (Kushwaha et al. 1977). An in vitro study showed that estradiol increases cell surface LDLR activity in human hepatoma cells but not in human fibroblasts (Kushwaha et al. 1977). LDL bound to LDLR is stimulated by ethinyl estradiol in a dose- and time-dependent manner, and this binding is decreased by a pretreatment of LDL in the media (Kushwaha et al. 1977). In rats, pharmaceutical doses of estrogens stimulate hepatic LDLR mRNA and protein levels, which are accompanied by a markedly increased clearance of plasma LDL concomitant with a decrease in plasma cholesterol (Ma et al. 1986; Cooper et al. 1987; Windler et al. 1980; Di Croce et al. 1996).

Although the LDLR promoter does not contain a classical estrogen-responsive element, observations that LDLR mRNA is stimulated by estrogen in vivo and in human hepatoma cells suggest an alternative mechanism of estrogen-regulated expression of this gene. Using human hepatoma cells that transiently express functional ERα and LDLR promoter constructs, Li et al. demonstrated that E2 promotes LDLR promoter activity mediated by the Sp1 binding to the promoter (Li et al. 2001). Consistently, we observed that LDLR protein levels from liver tissue were decreased in hepatic ERα knockout female and male mice (unpublished data). Both tyrosine kinase (TK) and protein kinase C (PKC) signaling pathways are activated by E2 in vivo and in hepatoma cells (Marino et al. 1998; Marino et al. 2001). A LDLR promoter construct was transfected in human hepatoma cells overexpressing ERα, and the promoter activity was analyzed in the absence and presence of TK and PKC inhibitors (Distefano et al. 2002). This study demonstrated that basal transcription of LDLR gene depends on PKC activity, and TK activity is required for the induction of LDLR gene expression by E2 (Distefano et al. 2002).

Indirect effects of estrogens on hepatic LDLR expression have also been reported. Estrogen may promote LDLR through the action of growth hormone, which as supported by the observation that E2 fails to stimulate hepatic LDLR expression and to decrease plasma cholesterol when given to hypophysectomized rats (Steinberg et al. 1967; Rudling et al. 1992). Reduction of free cholesterol content in human hepatoma cells by estradiol treatment has been shown to be an important mechanism for maintaining LDLR activity by lowering intracellular cholesterol (Semenkovich and

Ostlund 1987). LDLR protein levels are increased by E2 in a dose-dependent manner in human hepatic HuH7 cells; however, LDLR mRNA does not increase unless E2 doses are high, suggesting a posttranslational regulation of LDLR by estrogen (Starr et al. 2015). Additionally, estrogen also likely promotes LDLR-mediated cholesterol uptake through modifying PCSK9 activity because E2 treatment fails to increase LDLR in PCSK9 knockout HuH7 cells (Starr et al. 2015).

SR-BI Stimulation of HDL cholesterol uptake in the liver promotes reverse cholesterol transport and reduces atherosclerosis in mice (Arai et al. 1999; Zhang et al. 2005; Rader et al. 2009). Several groups have found that estrogens upregulate mRNA expression of SR-BI, the HDL receptor, and promotes HDL cholesterol uptake in peripheral tissues (Lopez and McLean 2006; Fukata et al. 2014). Therefore, estrogen regulation of HDL uptake by the liver may contribute to the sex difference in cardiovascular risk. Stimulation of this pathway may be a potential therapeutic target for preventing cardiovascular disease.

Estrogens Protect Against Fatty Liver and Hepatic Insulin Resistance in Males

Males also express estrogen receptors in many tissues, and aromatase catalyzes the conversion of androgens to estrogens. In humans, loss of function mutations in ERα or aromatase genes associates with glucose intolerance, hyperglycemia, and hyperinsulinemia (Maffei et al. 2004; Rochira et al. 2007; Smith et al. 1994). Aromatization of testosterone to estradiol is responsible for the increase in libido and prevention of visceral adiposity associated with testosterone treatment in men (Finkelstein et al. 2013). Hepatic steatosis has been reported in ERα-deficient male mice but not in ERβ-deficient male mice (Ohlsson et al. 2000). The aromatase knockout (ArKO) mice exhibit a striking accumulation of lipid droplets in the liver accompanied by increased expression of lipogenic genes such as FASN and SCD-1 (Jones et al. 2000, 2001; Chow et al. 2011). Estradiol treatment or agonist specific to ERα in ArKO male mice suppresses FASN gene expression and reverses liver fat accumulation (Chow et al. 2011; Jones et al. 2000). Antiestrogen treatment of TMX in male mice induces TG accumulation in the liver by activation of fatty acid synthesis (Cole et al. 2010). In line with this, the incorporation of ^{3}H-oleate into TG in hepatocytes was increased after TMX treatment (Cole et al. 2010). In male rats, high-fat diet induced the fatty liver which is improved by E2 treatment and associated with the downregulation of lipogenesis by ACC phosphorylation with E2 (Zhang et al. 2013). In line with the observation for hepatocyte treated with TMX, E2 treatment decreased the incorporation of ^{14}C labeling from the lipid-containing phase when de novo lipogenesis in hepatocytes was determined using a ^{14}C-labeled acetate method (Zhang et al. 2013) The authors also reported similar changes in gene expression in men with liver steatosis (Zhang et al. 2013).

In male mice, deletion of hepatic ERα promotes liver TG and DAG content after high-fat diet feeding, which corresponds with dysregulation of insulin-stimulated ACC phosphorylation and DGAT1/2 protein expression (Zhu et al. 2013; Zhu et al. 2014). Our studies with E2 treatment of mice after OVX demonstrated that estrogen signaling through hepatic ERα helps prevent insulin resistance associated with high-fat diet feeding in males. Thus, augmenting hepatic estrogen signaling through ERα may lessen the impact of obesity on diabetes and cardiovascular risk in both sexes.

Conclusions and Future Directions

Efforts to understand how women are protected from cardiovascular disease relative to men have led to the discovery that estrogens regulate a number of steps in liver lipid metabolism. Treatment of postmenopausal women with various estrogen formulations does not wholly restore the protection from cardiovascular disease seen in premenopausal women. Estrogen therapy and hormone treatment approaches can protect against fatty liver, insulin resistance, and diabetes but do not conclusively protect from cardiovascular disease. The hypertriglyceridemic effect of exogenous oral estrogen therapy may mitigate some of the other cardioprotective benefits of estrogens. A deeper understanding of the mechanisms of estrogen signaling pathways will likely yield specific targets governing estrogen's effect on lipid metabolism. Future directions will likely rely on targeting estrogens to specific tissues or specific aspects of the signaling pathways in order to recapitulate the protective physiology of premenopause therapeutically after menopause. Furthermore, estrogen signaling pathways in the liver are protective against insulin resistance in males; thus the pathways identified in studying sex differences have potential therapeutic significance in both men and women with regard to obesity associated with cardiovascular risk.

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