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Activation of Estrogen Response Element-independent ERα signaling protects female mice from diet-induced obesity

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Abstract

17β-estradiol (E2) regulates central and peripheral mechanisms that control energy and glucose homeostasis predominantly through estrogen receptor α (ERα) acting via receptor binding to estrogen response elements (ERE). ERα signaling is also involved in mediating the effects of E2 on diet-induced obesity (DIO), although the roles of ERE-dependent and -independent ERα signaling in ameliorating the effects of DIO remain largely unknown. We hypothesize that ERE-dependent ERα signaling is necessary to ameliorate the effects of DIO. We addressed this question using ERαKO (KO) and ERαKIKO (KIKO) female mice; the latter expressing an ERα that lacks a functional ERE binding domain. Females were ovariectomized, fed low-fat (LFD) or high-fat (HFD) diet, and orally dosed with vehicle or estradiol benzoate (EB, 300 µg/kg). After 9 weeks, body composition, glucose and insulin tolerance, peptide hormone and inflammatory cytokine levels, and hypothalamic arcuate nucleus and liver gene expression were assessed. EB reduced body weight and body fat in WT, regardless of diet, and in HFD-fed KIKO, in part by reducing energy intake and feeding efficiency. EB reduced fasting glucose levels in KIKO mice fed both diets but augmented glucose tolerance only in HFD-fed KIKO. Plasma insulin and IL-6 were elevated in KIKO and KO compared to WT on a LFD. Expression of arcuate neuropeptide and receptor genes and liver fatty acid biosynthesis genes was altered by HFD and by EB through ERE-dependent and –independent mechanisms. Therefore, ERE-independent signaling mechanisms in both the brain and peripheral organs mediate, in part, the effects of E2 during DIO.
Abbreviations

Acc1, acetyl-CoA carboxylase 1; Acc2, acetyl-CoA carboxylase 2; Acly, ATP citrate lyase; Actb, beta-actin; Agrp, agouti-related peptide; AUC, area under the curve; ARC, arcuate nucleus; Cart, cocaine- and amphetamine-regulated transcript; DIO, diet-induced obesity; Dgat2, diglyceride acyltransferase; E2, 17β-estradiol; EB, estradiol benzoate; ERα/Esr1, estrogen receptor alpha; ERα KO (KO), ERα knockout; ERα KIKO (KIKO), ERα knockin/knockout; ERE, estrogen response element; Fas, fatty acid synthase; Fatp2, very long-chain acyl-CoA synthetase; Fatp5, bile acyl-CoA synthetase; Foxo1, forkhead box O1; G6pase, glucose 6-phosphatase; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Ghsr, growth hormone secretagogue receptor; Gq-mER, Gq-coupled membrane estrogen receptor; HFD, high-fat diet; Hprt, hypoxanthine-guanine phosphoribosyltransferase; IL-6, interleukin 6; Insr, insulin receptor; Lepr, leptin receptor; LFD, low-fat diet; MCP-1, monocyte chemoattractant protein 1; Npy, neuropeptide Y; Pepck, phosphoenolpyruvate carboxykinase; Pomp, proopiomelanocortin; Pgc1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Pparγ, peroxisome proliferator-activated receptor γ; 5ht2cr, serotonin receptor 2c; TNF-α, tumor necrosis factor-α; Ucp2, uncoupling protein 2
Introduction

Obesity is a growing global health concern that is due, in part, to changes in diet and lifestyle (1). Postmenopausal women are more susceptible to obesity and its associated diseases (e.g., cardiovascular disease, type II diabetes, and metabolic syndrome) than premenopausal women due, in part, to a reduction in circulating 17β-estradiol (E2) (2–5). During the menstrual cycle in humans and primates, feeding behavior varies with a peri-ovulatory nadir and a luteal phase peak in food consumption, illustrating E2’s effects (6–9). Consequently, the decrease in circulating estrogens due to menopause is associated with positive weight gain in humans and primates (10) subsequently an increased risk for these diseases (2–5).

Many of these effects of menopause can be ameliorated by hormone replacement therapy (11).

The primary receptor mediating E2’s effects on energy homeostasis is estrogen receptor α (ERα). ERα knockouts (KO) exhibit an obese phenotype with increased visceral adiposity, decreased energy expenditure, altered glucose homeostasis, and insulin resistance (12–14). ERα is also necessary for the attenuation of body weight gain after ovariectomy (OVX, surgical menopause) (13,15). Interestingly, the restoration of estrogen response element (ERE)–independent ERα signaling normalizes energy balance in KO females (16). Female mice expressing an ERα that lacks the ability to bind to ERE due to mutations in the DNA-binding domain, called ERα knockin/knockout (KIKO), do not become obese like their KO counterparts (16). Using the KO and KIKO mouse models, we have previously shown that ERE-independent ERα signaling is not sufficient to suppress post-ovariectomy body weight gain and fat accumulation or augment oxygen consumption and anorexigenic neuropeptide signaling in chow-fed females (17).

Peripheral expression of ERα in the liver, adipose tissue, skeletal muscle, and the pancreas mediates E2’s actions in metabolism and glucose homeostasis (18,19). E2 reduces insulin resistance induced by high-fat diet (HFD) and improves insulin signaling in skeletal muscles through an ERα-mediated mechanism, yet elevates inflammatory cytokines (IL-6, TNFα) in the plasma in HFD-fed OVX females (20). E2 replacement in HFD-fed OVX females also restores oxygen consumption and improves glucose
homeostasis and insulin sensitivity (21). In HFD-fed OVX females, E2 suppresses expression of a number of hepatic lipogenic and gluconeogenic genes including glucose 6-phosphatase (G6pase), stearoyl-CoA desaturase (Scd1), and peroxisome proliferator-activated receptor γ (Pparγ) (22). While ERα-mediated signaling mechanisms involved in these effects on liver function are not clearly understood, analysis of E2-induced regulation of the liver transcriptome suggests that the DNA binding domain of ERα is necessary to control liver gene expression (23). However, it is unknown if E2 requires a functional DNA-binding domain to reduce the effect of diet-induced obesity (DIO) in OVX female mice. Therefore, we hypothesize that ERE-dependent ERα signaling is necessary to ameliorate the effects of DIO on body weight, adiposity, glucose homeostasis, and gene expression in the arcuate nucleus (ARC) of the hypothalamus and the liver.

**Materials and Methods**

**Animals**

All animal treatments were in accordance with institutional guidelines based on National Institutes of Health standards and were performed with approval from the Rutgers University Institutional Animal Care and Use Committee. Female wild-type (WT C57BL/6J), ERαKO (KO), and ERαKIKO (KIKO) transgenic mice (provided by Dr. Ken Korach, National Institute of Environmental Health Sciences) (24,25) were selectively bred in-house and maintained under controlled temperature (23°C) and photoperiod conditions (12/12 h light/dark cycle) with food and water ad libitum. WT/KO heterozygous males and females were mated to produce ERαKO females. Nonclassical ERα knock-in heterozygous males (WT/KI) and WT/KO heterozygous females were crossed to generate ERαKIKO females. WT females were generated from both colonies and used with their KIKO and KO littermates. At weaning, females were tagged and ear-clipped for genotyping. Genotype was determined by PCR of extracted DNA using previously published protocols (24,25).
Drugs and diets

Estradiol benzoate (EB) and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) were purchased from Sigma-Aldrich. Ketamine, Marcaine®, and Rimadyl® were purchased from Henry Schein Animal Health. EB was dissolved in 100% ethanol (Sigma) prior to dissolving in sesame oil (Sigma). Diets were purchased from Research Diets: low-fat diet (10% kcal fat; D12450B) and high-fat diet (45% kcal fat; D12451).

Experimental design

Our study will use a post-menopausal mouse model with or without E2 replacement to mimic hormone replacement therapy in women. Adult females (8-10 weeks old) were OVX via a single ventral incision under isoflurane anesthesia [2% in O2:N2O (2:1)] delivered by face mask with a local injection of Marcaine® (2 mg/kg) followed by 48 h of pain management using an injection of Rimadyl® (4 mg/kg, every 24 h; Henry Schein). Females from each genotype were fed ad libitum a low-fat diet (LFD; n=20) or a high-fat diet (HFD; n=20) for 9 weeks. Half of each diet group was administered sesame oil or EB (300 µg/kg, in sesame oil) daily perorally via a peanut butter carrier [all groups (LFD-oil, LFD-EB, HFD-oil, HFD-EB): n=10, except for KIKO LFD-oil and HFD-EB: n=9] (26). Average age for each genotype was 9.9 ± 0.2 weeks (n=40) for WT, 8.2 ± 0.1 weeks (n=38) for KIKO, and 8.5 ± 0.1 weeks (n=40) for KO. We chose peroral E2 replacement for the DIO studies to reduce the stress-inducing effects of repeated injections and to maintain a constant systemic level of E2 in the blood (27). Females were housed in genotype-matched pairs, and body weight and food intake were measured weekly. At the end of 9 weeks, body composition was measured using an EchoMRI 3-in-1 Body Composition Analyzer (Echo Medical Systems). A glucose tolerance test (GTT) was performed on each female as follows. Females were fasted overnight (1700h-900h) in a new cage. At the start of the test and 30 min after application of a local anesthetic (Lidocaine®; Henry Schein) to the tail, mice were placed in Plexiglass restrainers and tails were nicked to collect a baseline (time=0) glucose reading using a glucometer (AlphaTRAK2). Immediately after baseline reading, females were injected IP with a bolus of glucose (2.0 g/kg body
weight) and placed back individually into clean cages with no food or water. Tail blood samples were collected at 15, 30, 60, 90, 120, and 180 min post-injection. After sufficient recovery (~3-4 d), an insulin tolerance test (ITT) was performed after a 5h (0900-1400h) fast in a similar manner as the GTT with an IP injection of insulin (0.75 units/kg). Tail blood samples were collected in individual cages at 15, 30, 60, 90, and 120 min post-injection. After each test, all mice were returned to their home cages with their original cage mate with *ad libitum* access to water and food. Experimenters were blind to the experimental treatment groups (28,29).

**Brain and body dissections**

After sufficient recovery from the ITT (~1 week), females were dosed at 0900h and decapitated after sedation with ketamine (100 µl of 100 mg/ml, IP) at 1000h. Food was removed at the last dosing. Trunk blood was collected in a K+ EDTA collection tube. Plasma was prepared for analysis of peptide hormone and inflammatory cytokine by adding the protease inhibitor AEBSF (1 mg/mL) to each collection tube. Samples were maintained on ice until centrifugation at 3,000 rpm for 10 min at 4°C. Plasma was stored at −80°C until analysis. Ghrelin (active), insulin, leptin, interleukin 6 (IL-6), monocyte chemoattractant protein 1 (MCP-1), and tumor necrosis factor α (TNFα) were determined by multiplex assay (#MMHMAG-44K; EMD Millipore). Total plasma E2 levels were measured using Mouse/Rat Estradiol ELISA kit (ES180S-100; Calbiotech).

Abdominal cavity was dissected for liver tissue (secondary lobe) and the uterus. Each uterus was weighed (wet weight). Liver tissue was fixed in RINalater (Life Technologies) and stored at −80°C. Liver RNA was extracted using a standard TRIzol® extraction (Life Technologies) coupled with NucleoSpin® RNA extraction and DNase-I kit (Macherey-Nagel). The brain was immediately extracted from the skull and rinsed in ice-cold Sorensen’s buffer for 30 sec. The brain was cut using a brain matrix (Ted Pella), into 1-mm thick coronal rostral and caudal blocks corresponding to Plates 42 to 47 and Plates 48 to 53, respectively, from The Mouse Brain in Stereotaxic Coordinates (30). Blocks of the basal hypothalamus...
(BH) were transferred to RQAlater (Life Technologies) and stored overnight at 4°C. The rostral and caudal parts of the ARC were dissected from slices using a dissecting microscope. Dissected tissue was stored at −80°C. Total RNA was extracted from the combined nucleus (rostral and caudal arcuate) using Ambion RNAqueous-Micro Kits (Life Technologies) according to the manufacturer’s protocol. Total RNA was also DNase I-treated, using the extraction kits, at 37°C for 30 min to minimize any genomic DNA contamination. Both liver and arcuate RNA quantity and quality were determined using a NanoDrop ND-2000 spectrophotometer (ThermoFisher) and an Agilent 2100 Bioanalyzer and RNA Nano Chips (Agilent Technologies). Only samples with RNA integrity number > 8 were used in the analysis of gene expression.

**Quantitative Real-Time PCR**

Complementary DNA (cDNA) was synthesized from 500 ng of total liver RNA and 200 ng of total ARC RNA using Superscript III reverse transcriptase (Life Technologies), 4 µl 5x buffer, 25 mM MgCl₂, 10 mM dNTP (Clontech Laboratories), 100 ng random hexamer primers (Promega), 40 U/µl Rnasin (Promega) and 100 mM DTT in DEPC-treated water (Bioexpress) in a total volume of 20 µl. Reverse transcription was conducted using the following protocol: 5 min at 25°C, 60 min at 50°C, and 15 min at 70°C. The cDNA was diluted to 1:20 with nuclease-free water (Bioexpress) for a final cDNA concentration of 1.25 ng/µl for liver and 0.5 ng/µl for ARC and stored at −20°C. Untreated liver and BH test tissue RNA was used for the calibrator and negative control (no reverse transcriptase) and processed simultaneously with the experimental samples.

All primers were designed to span exon-exon junctions and synthesized by Life Technologies, using Clone Manager 5 software (Sci Ed Software). See Supplemental Table 1 for a listing of all the primer sequences used for quantitative real-time PCR (qPCR). For qPCR, 4 µl of cDNA template were amplified using either PowerSYBR Green (Life Technologies) or Sso Advanced SYBR Green (BioRad) on CFX-Connect Real-time PCR instrument (BioRad). Standard curves for each primer pair were prepared using
serial dilutions of liver or BH cDNA in triplicate to determine the efficiency \[E=10^{(-1/m)} - 1, \ m=\text{slope}\] of each primer pair. All efficiencies, expressed as percent efficiency, were approximately equal at one doubling per cycle (90-110%). The relative mRNA expression was calculated using the \(\Delta\Delta C_q\) method with diluted (1:20) cDNA from liver or BH of an untreated male used as a calibrator. The amplification protocol for all the genes was as follows: initial denaturing at 95°C for 10 min (PowerSYBR) or 3 min (SsoAdvanced) followed by 40 cycles of amplification at 94°C for 10 sec (denaturing), 60°C for 45 sec (annealing), and completed with a dissociation step for melting point analysis with 60 cycles of 95°C for 10 sec, 65°C to 95°C (in increments of 0.5°C) for 5 sec, and 95°C for 5 sec. The geometric mean of the reference genes \(\beta\)-actin (\(\text{Actb}\)), hypoxanthine-guanine phosphoribosyltransferase (\(\text{Hprt}\)), and glyceraldehyde-3-phosphate dehydrogenase (\(\text{Gapdh}\)) was used to calculate \(\delta C_q\) values. Calibrator, negative controls, and water blank were added to each plate. Quantification values were generated only from samples showing a single product at the expected melting point. All gene expression data are expressed as an \(n\)-fold difference relative to the calibrator (31,32).

**Statistical analysis**

All data are expressed as mean ± SEM. All data were analyzed using a multifactorial (steroid, diet, genotype) ANOVA followed by a *post-hoc* Newman-Keuls test using Statistica 7.1 software (StatSoft). Cumulative weight gain, GTT, and ITT data were analyzed using repeated-measures, two-way ANOVA with a *post-hoc* Newman-Keuls test. All *post-hoc* tests compared differences within genotype between treatments for diet and steroid effects and compared differences across genotypes within the same diet*steroid treatment. All gene expression data were analyzed using two-way ANOVA with a *post-hoc* Newman-Keuls test within each genotype because expression was normalized to the LFD-Oil samples of each genotype. All ANOVA statistics are reported in Supplementary Tables 2-4. In all experiments, effects were considered significant at \(\alpha \leq 0.05\).
Results

Body weight and body composition

On the day of surgery, intact WT weighed 19.8 ± 0.1g (n=40), intact KIKO weighed 18.6 ± 0.2g (n=38), and intact KO weighed 20.6 ± 0.2g (n=40). KO weighed more than WT (P<.01) or KIKO (P<.0001). For cumulative body weight, EB suppressed post-OVX weight gain in both LFD-fed and HFD-fed WT (Figure 1A). The attenuation of body weight was significant by week 6 (P<.01) in the LFD-fed females and by week 4 (P<.01) in the HFD-fed females. In KIKO, EB suppressed post-OVX weight gain only in HFD-fed females (Figure 1B). The attenuation of body weight was significant initially by week 4 (P<.05) and then continuously from week 6 to week 9 (P<.05) in the HFD-fed females. In KO, EB did not suppress post-OVX weight gain in either LFD-fed or HFD-fed groups (Figure 1C).

After 9 weeks, oil-treated HFD-fed females from all genotypes weighed more than their LFD-fed counterparts (WT: P<.001; KIKO: P<.0001; KO: P<.0001; Figure 1D). EB suppressed body weight in WT and HFD-fed KIKO females compared to their oil-treated counterparts. LFD-oil WT (n=10) weighed 29.3 ± 1.5g and LFD-EB WT (n=10) weighed 24.4 ± 0.9g (P<.01). HFD-oil WT (n=10) weighed 38.2 ± 1.9g and HFD-EB WT (n=10) weighed 31.5 ± 2.3g (P<.01). LFD-oil KIKO (n=9) weighed 26.8 ± 1.2g and LFD-EB KIKO (n=10) weighed 26.1 ± 1.8g (ns). HFD-oil KIKO (n=10) weighed 39.1 ± 1.5g and HFD-EB KIKO (n=9) weighed 30.9 ± 1.7g (P<.001). LFD-oil KO (n=10) weighed 28.3 ± 1.6g and LFD-EB KO (n=10) weighed 26.9 ± 0.8g (ns). HFD-oil KO (n=10) weighed 39.7 ± 2.0g and HFD-EB KO (n=10) weighed 36.0 ± 1.7g (ns).

Body fat accumulation (% of body weight) was affected by both steroid treatment and diet. As previously reported (17), body fat accumulation after 9 weeks in the LFD-fed WT females was reduced by EB treatment (P<.0001; Figure 1E). EB also reduced fat accumulation in HFD-fed WT females (P<.0001), although between EB-treated females, fat accumulation was higher in HFD-fed than LFD WT females (P<.001). In KIKO, HFD increased body fat accumulation in oil-treated females (P<.001), which was abrogated by EB treatment (P<.01). Interestingly, percent body fat in LFD-fed KIKO was higher than in LFD-fed WT (P<.01). There was no effect of EB on fat mass in KO females, regardless of diet,
although HFD increased body fat accumulation in KO (oil: P<.001; EB: P<.0001). HFD-EB KO were also fatter than HFD-EB WT (P<.01).

Lean mass (% of body weight) was also affected by steroid and diet as well as genotype. As expected, HFD reduced lean mass in oil-treated (P<.0001) and EB-treated (P<.001) WT females (Figure 1F). Lean mass was elevated in EB-treated WT compared to oil-treated WT (LFD: P<.0001; HFD: P<.0001). Similar to WT, EB treatment increased lean mass but only in HFD-fed KIKO (P<.01). In KO females, HFD reduced lean mass, regardless of steroid treatment (oil: P<.05; EB: P<.001). KIKO and KO females have less lean mass compared to WT females when orally dosed with EB (P<.0001).

3.2. Food intake and efficacy of oral EB treatment

Cumulative amount of food consumed per pair (each cage, n=5/group) from week 2 to week 9 (Figure 2A), not including week 1 during recovery from surgery, was not altered by steroid or diet in any genotype. However, when analyzed as average weekly food intake (data not shown), HFD-fed WT, KIKO, and KO consumed more food than their LFD-fed counterparts except for EB-treated KIKO females. As previously reported (17), KIKO and KO consumed less food than WT except for HFD-fed, oil-treated females. A similar pattern was found when analyzed as average weekly energy intake (kCal) (Figure 2B). HFD increased energy intake in all genotypes. EB reduced weekly energy intake in HFD-fed KIKO and KO females (both P<.01) but not in HFD-fed WT females. Feeding efficiency (g gained/kCal consumed) is as an indirect measure of metabolism. In our study, EB reduced feeding efficiency in LFD- and HFD-fed WT females (both P<.05) and in LFD-fed KIKO (P<.05) and KO (P<.05) females (Figure 2C). HFD elevated feeding efficiency in EB-treated KIKO (P<.001) and KO (P<.01) compared to LFD-fed counterparts.

Uterine wet weight was also measured to confirm the hypertrophic actions of EB, an ERα-mediated process (33). Past studies in our lab have found that subcutaneous injection of EB (250 ng/dose, every other day) for 4 weeks significantly increased the uterine weight in WT females (17). In the current study, EB increased the uterine weight in both LFD- and HFD-fed WT (both P<.0001; Figure 2D) but did not
increase uterine weight in KIKO and KO females. Plasma E2 levels were higher in all the EB-treated females regardless of genotype or diet (Figure 2E).

Glucose and insulin tolerance

To determine the interactions of EB and HFD on glucose homeostasis, we conducted glucose and insulin tolerance tests on all females. For the GTT, all mice were fasted overnight (1700-0900h). Fasting glucose levels were suppressed by EB in all genotypes, regardless of diet, except for HFD-fed KO females (Figure 2F). EB-treated KO females fed a LFD (P<.01) or HFD (P<.0001) had elevated glucose levels compared to their WT and KIKO counterparts.

Glucose tolerance was determined over 180 minutes following an IP injection of glucose (2 g/kg). In WT females, EB augmented glucose tolerance in both LFD- and HFD-fed females (Figure 3A). In KIKO females, HFD reduced glucose clearance in oil-treated KIKO with EB augmenting glucose clearance in HFD-fed females (Figure 3B). In EB-treated KO females, HFD reduced glucose tolerance (Figure 3C). The effects of genotype, diet, and steroid on glucose tolerance are further illustrated in Figure 3D including an effect of genotype where EB-treated KIKO and KO females exhibited slower glucose clearance compared to EB-treated WT females (P<.01, P<.01, P<.001, and P<.0001, respectively).

Insulin tolerance was measured over 120 minutes after an IP injection of insulin. There were no effects of diet or steroid in WT or KIKO females (Figure 4, A and B). HFD decreased glucose clearance at 15 min in oil-treated WT females (P<.01) but did not affect insulin tolerance in KIKO. HFD reduced the response to insulin in oil-treated KO at all time points and in EB-treated KO at 0, 60, and 120 min (Figure 4C). The effects of diet on insulin tolerance are further illustrated in Figure 4D especially the effect of HFD in KO females.

Peripheral peptide hormones and inflammatory cytokines

To determine the effects of diet and EB on peptide hormones and inflammatory cytokines, we analyzed plasma samples using multiplex assays. Plasma insulin levels were elevated in LFD-EB and
HFD-Oil KIKO and KO females compared to WT (Figure 5A), with those groups expressing two to three times as much insulin. HFD elevated plasma leptin levels in oil-treated WT (P<.01), KIKO (P<.05), and KO (P<.05) females and in EB-treated KO (P<.05; Figure 5B). EB suppressed leptin in both LFD-fed (P<.01) and HFD-fed (P<.01) WT females, but had no effect in KIKO or KO. Ghrelin (active), an orexigenic peptide hormone, was suppressed by both EB (P<.01) and HFD (P<.0001) in WT and by HFD (P<.05) in KIKO (Figure 5C).

The selected inflammatory cytokines, MCP-1, TNFα, and IL-6, are all implicated in obesity (34). MCP-1 levels were suppressed by EB in the LFD-fed WT and KIKO (both P<.05). HFD nullified EB’s effect in WT and KIKO females (Figure 5D). MCP-1 levels in KO females were elevated compared to their counterparts in both WT and KIKO (P<.0001 for all comparisons). There was no effect of steroid or diet on TNFα levels in any genotype (Figure 5E). However, TNFα levels in KO were elevated compared to WT and KIKO in all groups (P<.01, P<.05, P<.05, P<.01, respectively). Plasma IL-6 levels were elevated by HFD only in WT, regardless of steroid (oil: P<.01; EB: P<.05), and were elevated in LFD-fed KIKO (oil: P<.001; EB: P<.05) and KO (oil: P<.001; EB: P<.05) females compared to WT females (Figure 5F).

Arcuate and liver gene expression

To determine the interactions of EB and HFD within each genotype on ARC gene expression, we analyzed expression of neuropeptides and receptors for hormones, fatty acids, and neurotransmitters involved in energy balance (35,36). For the anorectic neuropeptide proopiomelanocortin (Pomc), EB and HFD reduced expression only in WT (Figure 6A). There was no effect of diet or steroid in any genotype on cocaine- and amphetamine-regulated transcript (Cart) expression (data not shown). For orexigenic neuropeptide Y, HFD suppressed Npy expression in WT, KIKO, and KO, and EB augmented Npy expression in LFD-fed KO (Figure 6B). EB enhanced agouti-related peptide (Agrp) expression in WT while HFD suppressed Agrp in WT and KO females (Figure 6C).
EB augmented the expression of ghrelin’s receptor (Ghsr) only in WT, regardless of diet (Figure 6D). In both LFD-fed WT and KIKO, EB augmented leptin receptor (Lepr) expression and HFD nullified this effect (Figure 6E). EB augmented expression of insulin receptor (Insr) in WT, which was nullified by HFD (Figure 6F), and HFD augmented Insr expression in oil-treated KO. In all genotypes, HFD suppressed expression of the serotonin 5HT2c receptor (5ht2cr; Figure 6G), while augmenting the expression of the fatty acid receptor PPARγ (Pparγ) in all genotypes (Figure 6H). Expression of the ERα gene, Esr1, was suppressed by EB only in WT, with no effect of HFD as has been previously reported (37) (data not shown).

Previous studies have found that ERα DNA binding is required for E2 regulation of liver gene expression (23) including genes for receptors, signaling proteins, and enzymes that control glucose and triglyceride production, lipid biosynthesis, and fatty acid catabolism. In our study, Insr was augmented by EB in LFD-fed WT and by HFD in oil-treated WT (Figure 7A). Lepr expression was suppressed by HFD in WT, KIKO, and KO (Figure 7B). Expression of forkhead box O1 (Foxo1), a transcription factor that mediates hepatic gluconeogenesis by glucagon and insulin (38), was augmented in LFD-fed WT by EB and HFD (Figure 7C). Mitochondrial uncoupling protein 2 (Ucp2) expression was augmented by EB in WT, regardless of diet (Figure 7D). In KO, HFD augmented phosphoenolpyruvate carboxykinase (Pepck) expression (data not shown). There were no effects of steroid or diet on glucose 6-phosphatase (G6pc), diglyceride acyltransferase (Dgat2c), peroxisome proliferator-activated receptor gamma coactivator 1-α (Pgc1α), Pparγ, or Esr1 expression in the liver in any genotype (data not shown).

Genes involved in lipid biosynthesis and fatty acid catabolism including Acetyl-CoA carboxylase (Acc1, Acc2), ATP citrate lyase (Acly), very long-chain acyl-CoA synthetase (Fatp2), bile acyl-CoA synthetase (Fatp5), and fatty acid synthase (Fas) were analyzed. Expression of Acc1 was reduced by EB, regardless of diet, in WT and in LFD-fed KIKO (Figure 8A). Acc1 was also reduced by HFD in oil-treated KIKO and KO. In WT, Acc2 expression was suppressed by EB, regardless of diet (Figure 8B). Likewise, Acly expression was reduced by EB, regardless of diet, in WT females and by EB and HFD in
KIKO females (Figure 8C). Fatp2 expression was reduced by HFD (Figure 8D), and Fatp5 was suppressed by EB and HFD only in WT (Figure 8E). Fas was augmented by HFD in oil-treated WT and suppressed by EB in HFD-fed WT (Figure 8F).

**Discussion**

E2 regulates central and peripheral mechanisms that control energy and glucose homeostasis and ameliorate the effects of DIO in OVX females predominantly through ERα binding to ERE. In the current study, we characterized the role of ERE-independent ERα signaling in controlling energy homeostasis and reducing the impact of DIO in a mouse postmenopausal model. We found that E2 reduced the effects of OVX and DIO on weight gain, adiposity, feeding efficiency, and glucose homeostasis (fasting glucose and tolerance) in WT females. As we previously reported (17), E2 did not reduce body weight and adiposity in LFD-fed KIKO but surprisingly reduced these effects of DIO in KIKO females. These diet-dependent effects in KIKO suggest that ERE-independent ERα signaling is activated by a constituent of a diet high in fatty acids and/or during states of positive energy balance.

Our study indicates that the DNA-binding domain, which recognizes the ERE promoter sequence, is not solely necessary to ameliorate many of the effects of DIO in females. Another transgenic ERα KO mouse model that lacks the AF-2 ligand-binding domain of ERα is similar to KO mice in adiposity and glucose tolerance and is more susceptible to DIO, which produces insulin resistance when compared to WT (39). Consequently, E2 replacement in HFD-fed OVX females requires the AF-2 domain to ameliorate the effects of DIO on energy and glucose homeostasis. That study and our current study, when considered together, demonstrate that ERE-independent, but not ligand-independent, ERα signaling is protective against the deleterious effects of DIO in females.

Another recent study characterized the activation of membrane-initiated E2 signaling in OVX female mice using an estrogen dendrimer conjugate (EDC) (40). Unlike E2, the EDC did not ameliorate the
effects of HFD on glucose homeostasis or adiposity but did improve diet-induced hepatic steatosis, due to
the suppression of fatty acid and triglyceride synthesis genes in the liver. When considered with our
study, these data suggest that nuclear-initiated ERE-independent signaling is sufficient to control
adiposity and glucose homeostasis but membrane-initiated ERE-independent signaling is central to the
control of liver lipogenesis by E2. While the exact cellular pathways are unknown, these ERE-
independent mechanisms, which include both nuclear- and membrane-initiated pathways, act in the
hypothalamus, the liver, adipose tissue, and other peripheral organs (18,19).

E2 signaling through ERα in the ARC is a central pathway for E2 to control feeding behavior (41)
although activation of the Gq-mER expressed in POMC and NPY neurons also controls feeding behavior
in rodents (42–44). In our study, weekly food and energy intake was reduced by EB in both HFD-fed
KIKO and KO indicating that another ER (e.g., Gq-mER) (43) may compensate for ERα in controlling
food intake in the KIKO and KO during DIO. Furthermore, our data suggest that a primary effect of
ovariectomy in the WT and LFD-fed KIKO and KO is a decrease in metabolism (as indicated by the
decrease in feeding efficiency), which is ameliorated by E2 replacement corroborating our previous study
(17).

The differential expression of ARC neuropeptides (Pomc, Npy, Agrp) across the genotypes did not
directly correlate with effects on food intake, which has been reported in mice (45), rats (46,47), and
guinea pigs (43,48). However, another study found that differences in arcuate orexigenic gene expression
between intact and OVX females is lost after long-term (~5 weeks) ovariectomy despite observable
effects on metabolism (e.g., increase in body weight) (49). Perhaps, the same compensatory mechanisms
underlying arcuate gene expression are functional in our long-term study. Nevertheless, an increase in
Agrp, which increases food intake by blocking α-melanocyte stimulating hormone at the melanocortin 3/4
receptors expressed in downstream hypothalamic neurons (50), is counterintuitive to E2’s well-
characterized suppression of food intake in most rodent models (43,51,52). Nevertheless, an increase in
Agrp mRNA expression does not necessarily lead to an increase in AgRP peptide release, especially since
E2 reduces the excitability of NPY/AgRP neurons via an upregulation of the M-current (KNCQ channels) (53) and reduces NPY release in the PVH (46).

The ARC nucleus is not the only brain region involved in E2’s control of feeding, energy expenditure, and activity. These brain regions include the ventromedial hypothalamus (VMH), the paraventricular hypothalamus, and the discreet hindbrain nuclei such as the nucleus tractus solitarius (NTS) (54–57). For example, specific deletion of ERα in VMH SF-1 neurons in female mice decreases energy expenditure, activity, and heat production (41), and specific ERα knockdown by RNAi in the VMH of female rats induces a phenotype defined by obesity, hyperphagia, glucose intolerance, and reduced activity (energy expenditure) that is resistant to E2’s actions (55). In the NTS, E2 via ERα also augments the control of food intake (meal size) by cholecystokinin, a satiety hormone from the small intestine (13,56). These key brain regions may compensate for the orexigenic profile of arcuate neuropeptide expression (EB suppressed Pomc and augmented Agrp) and may account for the lack of E2’s effect on feeding in WT females.

The lack of E2’s effects on food intake may also be due to the experimental design. In our study, females were housed in pairs to reduce the stress induced by single housing to which KIKO and KO females are especially sensitive (as observed previously; 17). Numerous other studies illustrating E2’s effect on food intake used either singly housed females and/or cages specifically designed to measure discreet feeding parameters (meal duration, frequency, size) (41,54,55,52,58). Although our paired-housing design reduced stress, it diminishes the sensitivity of our weekly food intake measurements. Furthermore, our study used daily doses of EB to maintain a continuous level of systemic E2 similar to hormone replacement therapy in postmenopausal women, while other studies have used cyclical (52), bi-daily (17,43,48), or short-term (12–24h) E2 treatment (45,59) to examine food intake and ARC neuropeptide expression.

In female rodents and premenopausal women, E2 controls the deposition of adipose tissue by increasing subcutaneous fat accumulation and decreasing visceral fat deposition, primarily through an
ERα-mediated mechanism (60). In OVX rodents and postmenopausal women, the loss of E2 produces an increase in abdominal or visceral adiposity. In our study, fat mass was reduced by EB in WT and HFD-fed KIKO, suggesting that ERE-independent ERα signaling can ameliorate, in part, the effects of DIO on adiposity. However, recent evidence suggests that another membrane ER, GPER1, controls adiposity in females during DIO and may underlay some of the effects on adiposity found in the KIKO females (61).

E2 controls glucose metabolism primarily through ERα acting in the liver, adipose tissue, and skeletal muscle (18,19). In our study, glucose clearance was reduced by HFD in both WT and KIKO but restored by EB. One of the potential mechanisms subject to E2’s actions is the regulation of glucose transporter type 4 (GLUT4) expression and activity in skeletal muscle. E2 augments GLUT4 expression and insulin-induced trafficking to the membrane (18,62,63), which may utilize both ERE-dependent and -independent signaling to regulate expression and activity. Although skeletal muscle GLUT4 expression is augmented by ERα activation in the extensor digitorum longus (63), the GLUT4 promoter lacks a consensus ERE, which suggests that this regulation is through ERE-independent mechanisms. Our data reveal that these ERE-independent mechanisms are key to the restoration of normal KIKO glucose clearance (uptake) after DIO. Furthermore, the loss of ERE-dependent signaling diminishes E2’s control of glucose transport into skeletal muscle and/or adipose tissue and slows glucose clearance as illustrated by an elevated AUC in the LFD- and HFD-fed KIKO females compared to WT.

HFD reduced insulin tolerance only in KO females, which along with elevated plasma insulin in KO (and KIKO) females indicates potential insulin resistance. The loss of ERE-dependent ERα signaling sensitizes females to the effects of DIO on insulin sensitivity. In fed animals, insulin signaling in the liver suppresses hepatic gluconeogenesis, in part, via the phosphorylation of FoxO1 and the subsequent inhibition of FoxO1-mediated transcription of Pepck and G6pase (38). In our study, Insr and Foxo1 expression in the WT liver was augmented by HFD and EB, the latter through ERE-dependent transcription. Because insulin receptor and FoxO1 oppose each other in hepatic gluconeogenesis, an
enhancement of \textit{Insr} would augment insulin actions in the liver and suppress gluconeogenesis, while a boost in \textit{Foxo1} would counteract insulin’s actions.

Elevated plasma IL-6 levels observed in HFD-fed WT (and all KIKO and KO groups) may abrogate this apparent interaction of EB and HFD on insulin signaling in the liver because acute IL-6 administration blocks the suppression of hepatic gluconeogenesis by insulin (64). Alternatively, elevated IL-6 without other inflammatory signals promote insulin production and glucose-stimulated insulin secretion from the pancreas (65–68). In our study, both WT and KIKO females expressed elevated IL-6 levels when fed a HFD without elevated TNF\(\alpha\) or MCP1. These females were also resistant to the effects of HFD on glucose and insulin tolerance, potentially by the actions of IL-6 on glucose-stimulated insulin secretion from the pancreas.

Low-grade inflammation is a hallmark of DIO, with most of the inflammatory cytokines being produced by the increasing deposition of adipose tissue. These cytokines are transported through cardiovascular circulation to other organs that control metabolic process including the liver, brain, and muscle (14,69,70). In a previous study, E2 reduced HFD-induced insulin resistance, improved insulin signaling in skeletal muscles, and increased plasma levels of inflammatory cytokines (IL-6, TNF\(\alpha\)) in OVX females through an ER\(\alpha\)-mediated mechanism (20). Although EB had no effect on plasma IL-6 or TNF\(\alpha\) levels in our study, KO females exhibited elevated levels of these cytokines and MCP-1 compared to WT and KIKO, indicating that the loss of ER\(\alpha\) signaling sensitizes females to OVX- and DIO-induced inflammation. Presumably, the elevated levels of inflammatory cytokines in the KO are an underlying mechanism behind the disruption in glucose homeostasis and insulin sensitivity in this genotype (14). ERE-independent ER\(\alpha\) signaling is involved in the regulation of MCP-1 and IL-6 expression in MCF-7 cells through the response element for another transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF\(\kappa\)B) (34). Our data support this hypothesis with EB reducing plasma MCP-1 in LFD-fed WT and KIKO females, although abrogated by HFD.
ERα is expressed in the liver and activates both nuclear-initiated and membrane-initiated mechanisms (18,19). While the ERα-mediated signaling mechanisms involved in liver function are not fully understood, it is suggested that the DNA binding domain of ERα is necessary for E2’s regulation of liver gene expression (23). For example, E2, via ERα, controls the expression of signal transducer and activator of transcription 3 (STAT3) in the liver to improve insulin sensitivity and regulate lipogenic genes (Pparγ, Fas, Acc1), which are augmented by OVX in mice (71–73). E2 protects against fatty liver and associated insulin resistance, in part, by reducing lipid synthesis concurrently with activation of lipid oxidation in HFD-fed OVX females (21,72,74). E2 also alters expression of a number of hepatic lipogenic and gluconeogenic genes including G6pase and Pparγ in HFD-fed OVX females (22) and attenuates the increase in Acc1 and Fas due to OVX, reducing hepatic steatosis (75). While E2 had no effect on G6pase and Pparγ in the liver in our study, EB did reduce the expression of lipid biosynthesis genes (Acc1, Acc2, Acly, Fatp2, Fatp5) in WT females, thus controlling lipid production and deposition in the liver during DIO. One such ERE-dependent mechanism is the regulation of a microRNA, miR-125b, by E2 in the liver via ERα, which subsequently targets Fas expression to reduce lipid deposition (76).

Two lipogenic genes (Acc1, Acly) were suppressed by E2 in LFD-fed KIKO, which indicates that ERE-independent ERα signaling impacts liver lipogenesis. In KIKO, HFD reduced the expression of Acc1 and Acly but not in WT, suggesting that HFD overwhelms ERE-independent ERα-mediated regulation of Acc1 and Acly expression. Another gene involved in liver metabolism and lipogenesis is uncoupling protein 2 (Ucp2), which was augmented by EB through ERE-dependent signaling in the WT liver, regardless of diet. An increase in Ucp2 expression in WT acts as a buffer against the production of reactive oxygen species by fatty acid beta-oxidation and subsequently promotes mitochondrial biogenesis (77,78), thereby reducing the effects of DIO on liver metabolism and lipid deposition.

In conclusion, the present study demonstrates that ERα mediates E2’s control of energy homeostasis through ERE-dependent and -independent mechanisms contingent upon the diet consumed in a postmenopausal mouse model. These mechanisms include regulation of hypothalamic and liver gene
expression, adiposity, energy intake, peptide hormone production, and glucose clearance. Thus, our study identifies several unique cellular and gene targets for mechanistic characterization of ERα signaling, both ERE-dependent and -independent, in these tissues. A prime example of ERα- and ERE-independent signaling is activation of the Gq-mER, which is expressed throughout the hypothalamus. Activation of the Gq-mER by its selective ligand, STX, reduces post-OVX body weight gain by modulating feeding behavior and controls thermoregulation and bone remodeling, all of which are disrupted during menopause (43). The identification of similar membrane-initiated E2 signaling in vivo is necessary to develop selective therapies to treat the dysregulation of energy and glucose homeostasis in menopausal women, which increases the risk for obesity, metabolic syndrome, and type 2 diabetes.

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Figure Legends

Figure 1. Cumulative body weight gain and body fat accumulation were reduced in WT and HFD-fed KIKO females by estradiol benzoate (EB). A: WT, B: KIKO, C: KO – Cumulative body weight gain (g). D: Week 9 body weights (g). E: Percent body fat (fat mass/body weight). F: Percent lean mass (lean mass/body weight). Data were analyzed by multifactorial ANOVA with post-hoc Newman-Keuls test. Sample sizes were 9-10 per group. For A-C: Letters denote comparisons between oil and EB within the same diet. For D-F: Capped lines denote comparisons within genotype between steroid (oil vs. EB) and diet (LFD vs. HFD) combinations, and underlined letters denote comparisons between WT and either KIKO or KO within the same treatment combination (a = P<.05; b = P<.01; c = P<.001; d = P<.0001).

Figure 2. EB reduced feeding efficiency and fasting glucose in WT and LFD-fed KIKO and KO females. A: Cumulative food intake (g). B: Average weekly energy intake (EI; kCal). C: Average weekly feeding efficiency (FE; g gained/kCal consumed). For A-C, all food intake data were per cage (n = 5). D: Uterine weights normalized to body weights (mg/g). E: Plasma 17β-estradiol (E2) levels (pg/ml). F: Fasting glucose levels (mg/dL). Data were analyzed by multifactorial ANOVA with post-hoc Newman-Keuls test. For D-F, sample sizes were 9-10 per group. Capped lines denote comparisons within genotype between steroid (oil vs. EB) and diet (LFD vs. HFD) combinations, and underlined letters denote comparisons between WT and either KIKO or KO within the same treatment combination (a = P<.05; b = P<.01; c = P<.001; d = P<.0001).

Figure 3. Glucose tolerance after glucose injection was increased by EB in WT and HFD-fed KIKO females. GTT from A. WT, B. KIKO, and C. KO females. Data were analyzed by repeated-measures, two-way ANOVA with post-hoc Newman-Keuls test. D. AUC analysis (mg/dL*min). Data were analyzed by a multifactorial ANOVA with post-hoc Newman-Keuls test. Sample sizes were 9-10 per group. For A-C: Letters denote an effect of steroid and underlined letters denote an effect of diet. For D: Capped lines denote comparisons within genotype between steroid (oil vs. EB) and diet (LFD vs. HFD).
combinations, and underlined letters denote comparisons between WT and either KIKO or KO within the
same treatment combination ($a = P < .05$; $b = P < .01$; $c = P < .001$; $d = P < .0001$).

Figure 4. Glucose clearance after insulin injection was not affected by diet or EB in WT and KIKO
females. ITT from A. WT, B. KIKO, and C. KO females. Data were analyzed by repeated-measures, two-
way ANOVA with post-hoc Newman-Keuls test. D. AUC analysis (mg/dL*min). Data were analyzed by
a multifactorial ANOVA with post-hoc Newman-Keuls test. Sample sizes were 9-10 per group. For A-C:
Letters denote an effect of steroid and underlined letters denote an effect of diet. For D: Capped lines
denote comparisons within genotype between steroid (oil vs. EB) and diet (LFD vs. HFD) combinations,
and underlined letters denote comparisons between WT and either KIKO or KO within the same
treatment combination ($a = P < .05$; $b = P < .01$; $c = P < .001$; $d = P < .0001$).

Figure 5. Regulation of peripheral peptide hormones and inflammatory cytokines by EB and HFD. A.
Plasma insulin levels (ng/ml). B. Plasma leptin levels (ng/ml). C. Plasma ghrelin levels (pg/ml). D.
Plasma MCP-1 levels (pg/ml). E. Plasma TNFα levels (pg/ml). F. Plasma IL-6 levels (pg/ml). Data were
analyzed by a multifactorial ANOVA with post-hoc Newman-Keuls test. Sample sizes were 9-10 per
group. Capped lines denote comparisons within genotype between steroid (oil vs. EB) and diet (LFD vs.
HFD) combinations, and underlined letters denote comparisons between WT and either KIKO or KO
within the same treatment combination ($a = P < .05$; $b = P < .01$; $c = P < .001$; $d = P < .0001$).

Figure 6. Regulation of arcuate neuropeptide and receptor genes by EB and HFD. A. Pomc, B. Npy, C.
Agrp, D. Ghsr, E. Lepr, F. Insr, G. 5ht2cr, and H. Pparγ expression calculated within each genotype.
Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls test within each genotype.
Sample size was 8 per group. Capped lines denote comparisons within genotype between steroid (oil vs.
EB) and diet (LFD vs. HFD) combinations ($a = P < .05$; $b = P < .01$; $c = P < .001$; $d = P < .0001$).
Figure 7. Regulation of receptor and signaling genes by EB and HFD in the liver. A. Insr, B. Lepr, C. Foxo1, and D. Ucp2 expression calculated within each genotype. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls test within each genotype. Sample size was 8 per group. Capped lines denote comparisons within genotype between steroid (oil vs. EB) and diet (LFD vs. HFD) combinations (a = P<.05; b = P<.01; c = P<.001; d = P<.0001).

Figure 8. Regulation of liver genes involved fatty acid metabolism by EB and HFD. A. Acc1, B. Acc2, C. Acly, D. Fatp2, E. Fatp5, and F. Fas expression calculated within each genotype. Data were analyzed by a two-way ANOVA with post-hoc Newman-Keuls test within each genotype. Sample size was 8 per group. Capped lines denote comparisons within genotype between steroid (oil vs. EB) and diet (LFD vs. HFD) combinations (a = P<.05; b = P<.01; c = P<.001; d = P<.0001).