Published in final edited form as:
J Mol Endocrinol. 2013 October ; 51(2): 233–246. doi:10.1530/JME-13-0051.

Estradiol and tamoxifen regulate NRF-1 and mitochondrial function in mouse mammary gland and uterus

Margarita M. Ivanova[1], **Brandie N. Radde**[1], **Jieun Son**[2], **Fabiola F. Mehta**[2], **Sang-Hyuk Chung**[2], and **Carolyn M. Klinge**[1]

[1]Department of Biochemistry & Molecular Biology; Center for Genetics and Molecular Medicine, University of Louisville School of Medicine, Louisville, KY 40292

[2] Center for Nuclear Receptors and Cell Signaling, Department of Biology and Biochemistry, University of Houston, 3605 Cullen Blvd., Houston, TX 77204

Abstract

Nuclear respiratory factor-1 (NRF-1) stimulates the transcription of nuclear-encoded genes that regulate mitochondrial genome transcription and biogenesis. We reported that estradiol (E_2) and 4-hydroxytamoxifen (4-OHT) stimulate NRF-1 transcription in an estrogen receptor α- and β- (ERα, ERβ) dependent manner in human breast cancer cells. The aim of this study was to determine if E_2 and 4-OHT increase NRF-1 *in vivo*. Here we report that E_2 and 4-OHT increase NRF-1 expression in mammary gland and uterus of ovariectomized C57BL/6 mice in a time-dependent manner. E_2 increased NRF-1 protein in the uterus and mammary gland; however, in mammary gland, 4-OHT increased *Nrf1* mRNA but not protein. Chromatin immunoprecipitation (ChIP) assays revealed increased *in vivo* recruitment of ERα to the *Nrf1* promoter and intron 3 in mammary gland and uterus 6 h after E_2 and 4-OHT treatment, commensurate with increased NRF-1 expression. E_2 and 4-OHT- induced increases in NRF-1 and its target genes *Tfam*, *Tfb1m*, and *Tfb2m* were coordinated in mammary gland but not uterus, due to uterine-selective inhibition of the expression of the NRF-1 coactivators *Ppargc1a* and *Ppargc1b* by E_2 and 4-OHT. E_2 transiently increased NRF-1 and PGC-1α nuclear staining while reducing PGC-1α in uterus. E_2, not 4-OHT, activates mitochondrial biogenesis in mammary gland and uterus in a time-dependent manner. E_2 increased mitochondrial outer membrane Tom40 protein levels in mammary gland and uterus whereas 4-OHT increased Tom40 only in uterus. These data support the hypothesis of tissue-selective regulation of NRF-1 and its downstream targets by E_2 and 4-OHT *in vivo*.

Keywords

nuclear respiratory factor-1; estrogen receptor; mitochondria; mouse

Introduction

Uterus and mammary gland are classical estrogen target tissues in which estrogens, *e.g.*, 17β-estradiol (E_2), bind estrogen receptors α and β (ERα and ERβ) to regulate gene expression and physiological functions (Faulds, et al. 2012). Studies in female ERα and ERβ knockout mice (αERKO and βERKO) demonstrated that ERα is critical for mammary gland formation and sexual maturation of the reproductive tract whereas ERβ is essential for

* author to whom correspondence should be addressed: Carolyn M. Klinge, Department of Biochemistry & Molecular Biology; Center for Genetics and Molecular Medicine, University of Louisville School of Medicine, Louisville, KY 40292 USA; carolyn.klinge@louisville.edu Phone: 502-852-3668; Fax: 502-852-3659.

DISCLOSURE STATEMENT: The authors have nothing to disclose

ovarian function, but not mammary gland or uterus (Couse and Korach 1999). Although many tissue-specific phenotypes for αERKO, βERKO, and other ERα knockout mice have been reported (Billon-Galés, et al. 2009; Korach, et al. 2003; Pendaries, et al. 2002), how estrogens regulate mitochondrial biogenesis and energy production by oxidative phosphorylation (OXPHOS) in uterus and mammary gland is largely undefined.

Weight gain by postmenopausal women and ovariectomized rodents and the preservation of muscle mass and inhibition of adipogenesis by hormone replacement therapy (Cooke and Naaz 2004) provide clear evidence for the stimulatory effects of endogenous estrogens on metabolism, but does not address the effects of endogenous estrogens on specific tissues. Estrogens regulate mitochondrial biogenesis and function in normal and cancer cells through nuclear, mitochondrial, plasma membrane - initiated events that have been reviewed (Klinge 2008; Simpkins, et al. 2008; Simpkins, et al. 2010; Yager and Chen 2007). The specific roles for ERα and ERβ in regulating estrogens' tissue-specific effects on energy, glucose homeostasis, and insulin sensitivity were recently reviewed (Faulds et al. 2012). In addition to central brain actions to regulate appetite and satiety (Barros and Gustafsson 2011), ERα maintains metabolic control in skeletal muscle, white adipose tissue, liver, and pancreas by stimulating insulin signaling, while activated ERβ results in a diabetogenic/adipogenic phenotype, although questions remain (Faulds et al. 2012). The mechanisms and gene targets by which ERα and ERβ regulate metabolic pathways are largely unknown, certainly tissue-specific (Barros and Gustafsson 2011), and involve complex crosstalk with multiple signaling pathways including other nuclear receptors (NRs), *e.g.*, peroxisome proliferator-activated receptors α and γ (PPARα, PPARγ) (Papi, et al. 2013) and estrogen related receptors (ERRs) (Deblois and Giguere 2013); coregulators, *e.g.*, PPARγ coactivator (PGC-1) family proteins (Scarpulla, et al. 2012); and plasma membrane receptors (Renoir, et al. 2013).

We reported that E_2 and 4-hydroxytamoxifen (4-OHT), an active tamoxifen (TAM) metabolite, stimulate transcription of nuclear-encoded nuclear respiratory factor 1 (NRF-1) by binding ERα and ERβ, respectively, and increasing ERα, ERβ, and RNA polymerase II recruitment to the promoter of *NRF1* in MCF-7 breast cancer cells (Ivanova, et al. 2011; Mattingly, et al. 2008). NRF-1 regulates the transcription of nuclear-encoded, mitochondrial genes, *e.g.*, mitochondrial transcription factors *TFAM*, *TFB1M*, and *TFB2M*, and nuclear encoded components of OXPHOS, *e.g.*, cytochrome *c* (*CYCS*) (Cam, et al. 2004; Richard C 2011; Scarpulla 2006; Scarpulla 2008a). In MCF-7 cells, E_2-induced NRF-1 resulted in an increase in expression of Tfam and increased mitochondrial biogenesis (Mattingly et al. 2008). NRF-1's activity depends on its interaction with coactivators PGC-1α, PGC-1β, and PRC (Gleyzer and Scarpulla 2011; Richard C 2011; Scarpulla 2008a, 2012). Little is known about how estrogens or antiestrogens regulate the expression or activity of these coactivators in uterus or mammary gland. One study reported that E_2 reduced PGC-1α specifically in the uteri of immature and adult mice (Macari, et al. 2010a). Concordantly, ovariectomy increased PGC-1α in mouse brain endothelial cells (Kemper, et al. 2013).

Most studies of the regulation of mitochondrial biogenesis by NRF-1 and the PGC-1 family have been performed in tissues with high oxidative activity: skeletal muscle, liver, brown fat, and heart (Finck and Kelly 2006, 2007; Scarpulla 2008b). There are no specific studies of how estrogens regulate mitochondrial biogenesis in mammary gland and only one study in mouse uterus (Macari et al. 2010a). Although microarray analysis of gene expression in mouse uterus identified many genes regulated by E_2 and TAM (Fong, et al. 2007; Fong, et al. 2010; Moggs, et al. 2004), *Nrf1* was not included in the microarray platforms. GEO profile # GDS2208 / 9902 revealed that *Nrf1* was increased in C57BL/6 mouse uterus by 6 h of E_2 treatment (Hewitt, et al. 2012). Another study of uterine gene expression revealed lower *Nrf1* in *SRC-2*$^{-/-}$ compared to wild type adult mice (Jeong, et al. 2007). SRC-2 is a

well-established coactivator for ERα and upregulated by E_2 in adult uteri (Klinge 2000; O'Malley and Kumar 2009) (Jeong et al. 2007). Microarray profiling of E_2-regulated genes in aortas of wild type, ERα, and ERβ knockout mice revealed opposite regulation of *Nrf1* and many NRF-1-regulated mitochondrial respiratory chain (MRC) genes by ERα and ERβ (O'Lone, et al. 2007). E_2-ERα increased whereas E_2-ERβ reduced expression of *Nrf1* and MRC genes in mouse aorta (O'Lone et al. 2007). *Nrf1* was not included in a microarray profiling E_2-regulated genes in mouse mammary gland (Deroo, et al. 2009). Hence, how E_2 and TAM regulate of NRF-1, PGC-1 coactivators, NRF-1 regulated genes, and mitochondrial biogenesis in uterus and mammary gland i*n vivo* is unknown. Similarly, regulation of mitochondrial biogenesis, by antiestrogens, *e.g.*, TAM, in uterus where TAM is an agonist (Sourla, et al. 1997) is largely undefined (Klinge 2008).

Ovariectomy was recently shown to decrease the expression of NRF-1, TFAM, and PGC-1β while increasing PGC-1α in isolated brain endothelial cells from C57BL/6 mice (Kemper et al. 2013). Ovariectomy reduced the mitochondrial biogenesis ~ 20% and decreased the expression of manganese superoxide dismutase (MnSOD) and ATP synthase subunit α, Complex 5 suggesting that ovarian hormones normally stimulate mitochondrial biogenesis but suppress ROS in brain endothelium by inducing anti-oxidant proteins (Kemper et al. 2013). This agrees with many reports on the protective effect of estrogens against brain injury (Simpkins et al. 2010).

The goal of this study was to test the hypothesis that E_2 and 4-OHT stimulate NRF-1 gene expression and coordinately activate downstream nuclear and mitochondrial gene expression and mitochondrial biogenesis *in vivo* in a tissue-selective manner. To address this hypothesis, ovariectomized (ovex) mice were administered a single dose of E_2 (100 ng/mouse) or 4-OHT (50 μg/mouse). NRF-1 and its target genes, both nuclear and mitochondrial (mt), mt biogenesis, and mt/nuclear DNA ratio were examined 6, 24, and 72 h post injection based on a previous study (O'Brien, et al. 2006). Our results support our hypothesis and reveal novel tissue-selective actions of E_2 and 4-OHT in regulating NRF-1, its coregulators, and its downstream gene targets in mouse mammary gland and uterus.

Materials and Methods

Chemicals

17β-estradiol (E_2), 4-hydroxytamoxifen (4-OHT), and sesame oil were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Antibodies

Antibodies were purchased as follows: ERα (HC-20) and Tom40 (H-300), Santa Cruz Biotechnology (Santa Cruz, CA, USA); NRF-1, Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA); Tfam (DO1P), Abnova, (Taipei, Taiwan); nuclear-encoded cytochrome *c* oxidase subunit IV (*COX4-1*, nuclear-encoded, MitoSciences, Eugene, OR, USA); Cytochrome C Ab-2, Thermo Fisher Scientific (Fremont, CA, USA), β-actin, Sigma-Aldrich.

Animals

C57BL/6 mice, ovariectomized on postnatal day 20, were purchased from Charles River Laboratories (Wilmington, MA, USA) on postnatal day 25. All animal studies were conducted in accordance with procedures outlined in the NIH Guide to the Care and Use of Experimental Animals as approved by the AALAC-accredited University of Louisville Institutional Animal Care and Use Committee. After 14 d quarantine, mice were randomized into three groups of five/group. Mice were given a single subcutaneous (sc) injection

(between the shoulders) of vehicle control (sesame oil, 100 μl), E_2 (100 ng/mouse), or 4-OHT (50 μg/mouse) and tissues were harvested at 6, 24 and 72 h after injection. A total of 15 mice were included in each treatment group and time point within each experiment and three separate experiments were performed. All mice were injected with vehicle control at 8:30 am, followed by E_2 at 9 am, and 4-OHT at 10 am with tissues harvested 6, 24, and 72 h after injection. There is no variation in *Nrf1* transcript expression in mouse prefrontal cortex during a 24 h (12 h light:12 h dark) cycle (GDS3080), similar to the conditions used in our experiment. There is no evidence that *Nrf1* gene expression varies with circadian rhythm (Bozek, et al. 2009; Bozek, et al. 2010).

Mice and treatments for IHC studies

Female mice were obtained by mating near congenic C57BL/6 animals (129/C57BL/6 mice were backcrossed to C57BL/6 for 8 generations; *i.e.*, 99.61% of the genome was contributed by C57BL/6). Mice were ovariectomized at four to six weeks of age and rested for 2 weeks. They were then i.p. injected with ethanol vehicle or 1 μg of 17β-estradiol (Sigma-Aldrich) for 24 or 48 h. All procedures were carried out according to animal protocols approved by the University of Houston Institutional Animal Care and Use Committee.

Tissue processing and immunohistochemistry

Uteri were harvested, fixed in 4% paraformaldehyde, and embedded in paraffin. Serial sections were made longitudinally at 5-μm thickness. Sections were deparaffinized, rehydrated, and microwaved in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes as previously described (Balsitis, et al. 2003). Non-specific antibody binding was blocked by incubating sections in 5% donkey serum (Santa Cruz Biotechnology) in PBS for 1hr. Sections were serially incubated with rabbit anti-NRF-1 antibody (1:200 in 5% donkey serum, Sigma-Aldrich) and then goat anti-PGC-1α (1:50 in 5% donkey serum, Novus Biologicals) at 4°C overnight; normal rabbit IgG and normal goat IgG (Santa Cruz Biotechnology) were used as negative controls. Anti-rabbit IgG-Alexa Fluor 488 (Invitrogen) and anti-goat IgG-CFL 594 (Santa Cruz Biotechnology) were used as secondary antibodies. Nuclei were stained with Hoechst 33258 (Sigma-Aldrich). Tissues were visualized with an Olympus FV1000 laser scanning confocal microscope.

RNA Isolation and Quantitative Real-Time-PCR (qPCR)

RNA was extracted using the RNeasy Mini Kit from Qiagen (Valencia, CA, USA). The High Capacity cDNA archive kit (PE Applied Biosystems, Foster City, CA, USA) was used to reverse-transcribe RNA using random hexamers. Supplementary Table 1 lists gene and protein names and abbreviations. Murine mRNA transcript levels of *Nrf1, Tfam, CycS, Cox4* were analyzed using SYBR Green dye (RT^2SYBR®Green ROX qPCR, QIAGEN). *Tfb1m* and *Tfb2m* expression levels were analyzed by one-step qPCR (Power SYBR® Green RNA-to-Ct 1-step kit, Applied Biosystems). Relative transcription levels were normalized to 18S rRNA or 36b4 mRNA (Macari, et al. 2010b). 18S rRNA primers and probe were purchased as Assays-on-Demand™ Gene Expression Products (PE Applied Biosystems). Murine *Tfb1m* and *Tfb2m* primers were from RealTimePrimers (Elkins Park, PA). The mouse *Ppargc1a, Tfam, cytochrome c, Cox4* primer sequences were reported in (Safdar, et al. 2011) and were purchased from IDT (Coralville, IA, USA). The mouse *Nrf1* primers were also purchased from IDT: forward: 5'-GCACCTTTGGAGAATGTGGT-3'; reverse: 5'-CTGAGCCTGGGTCATTTTGT-3'.

Transcript expression analysis was determined between the control and E_2 or 4-OHT treated groups and individual samples were run in triplicate. qPCR was performed in the ABI PRISM 7900 SDS 2.1 or ViiA7 real-time PCR systems (PE Applied Biosystems) using relative quantification. Analyses and fold differences were determined using the

comparative CT method. Fold change was calculated from the $\Delta\Delta C_T$ values with the formula $2^{-\Delta\Delta CT}$ and data are presented as relative to expression in control (sesame oil-injected) mice.

Protein Isolation

Whole tissue extracts were prepared in radioimmunoprecipitation (RIPA) buffer and protease inhibitor (Roche) (Mattingly et al. 2008) with a mechanical homogenizer (Omni International, Kennesaw, GA, USA). Protein concentrations were determined using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA).

Western Blot

Western blotting followed standard procedures (Ivanova et al. 2011; Mattingly et al. 2008). In brief, 30-40 μg of protein lysates (see individual Figures) were separated on 10 or 14 % SDS PAGE gels and electroblotted to PVDF membranes. Data were captured and analyzed by Carestream Image Station 4000 R Pro with Carestream Molecular Imaging Software, version 5.0, (Carestream Health, Inc., New Haven, CT, USA). The values from regions of interest (ROI) normalized to the loading control, *e.g*., β-actin or Ponceau S staining, and the normalized value of the control was set to 1 for comparison between separate experiments.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP was performed with the MAGnify™ Chromatin Immunoprecipitation System (Invitrogen) using the manufacturer's protocol. Half of the uterus or the left inguinal mammary gland from one mouse was fixed in 1% formaldehyde at room temperature 20 min. Then the reaction was terminated by addition of 0.125 M glycine for 5 min at room temperature followed by sedimentation and a wash with ice cold PBS. After aspiration of the PBS, the tissues were frozen at −80°C. The tissues were mechanical homogenized in ChIP buffer (Ivanova et al. 2011) and genomic DNA was shared by sonication on ice. 300 μg of lysed extracts were incubated with anti-ERα (HC-20) antibody or normal rabbit IgG (both from Santa Cruz). DNA was eluted from the beads by incubating with 100 μl of 10% Chelex (Bio-Rad) at 95°C for 10 min, followed by Proteinase K treatment and heat inactivation at 95°C for 10 min. Following centrifugation, the supernatant was used for PCR amplification. The primers used for qPCR contained the mouse *Nrf1* promoter (−962 bp) region with estrogen response element (ERE) were sense 5'- GATCTCTGGGTTGGAGGTC-3' A and antisense 5'-ATAAATGCCCCACATGGTGT-3' (see diagram in Fig. 2*A*); and 'close to ERE' primers were sense: 5' CTCTTAACCGCTGAGCCATC-3' and antisense: 5'-AGGGAAAGGGAGGAGATTCA-3'. The primers containing the *Nrf1* intron 3 region with AP-1 and ½ERE binding sites were sense: 5'-TTTGACTTGATACATGTGAATGC-3', and antisense: 5'-TCTCTGTGAAATAACTGACAGAAAT-3'. Real time-PCR was performed using 2 μl of purified DNA and SYBR Green Master Mix (SuperArray Bioscience Corp., Frederick, MD, USA). Relative promoter enrichment was compared with IgG for each treatment. ChIP using primers to an intron 6 region of *Nrf1*: 5'-CAGGCAGAGGTGGAGCTAAC-3' and 5'-CACTGGGCCCTAGCAAATAC–3' were used as a negative control. The Ct values for ChIP with IgG to any of the primers or ChIP for ERα with primers for intron 6 were "undetectable" > 35 ± 0.5.

Mitochondrial/Nuclear DNA Ratios

The relative mitochondrial content of mammary gland and uterus was determined using SYBR green qPCR measuring the ratio of mitochondrial- encoded nicotinamide adenine dinucleotide dehydrogenase-5 (mt-Nd5)/ nuclear-encoded cystic fibrosis (Cftr) (Bauerly, et al. 2006). For nuclear and mitochondrial DNA quantification, 10 ng and 0.1 ng DNA were used as template, respectively (Bauerly et al. 2006). Each sample was analyzed in triplicate.

Relative mitochondrial copy number to nuclear copy number was assessed by a comparative Ct method, using the following equation: $\Delta Ct^{mitochondria/nuclear} = Ct^{mitochondria} - Ct^{nuclear}$. The fold-change relative to control animals was calculated using the following equation: $2(-\Delta\Delta Ct)^{mitochondria/nuclear}$, where $\Delta\Delta Ct^{mitochondria/nuclear} = \Delta Ct^{control\ mitochondria/nuclear} - \Delta C^{tmitochondria/nuclear}$ of each animal from different treatment and time groups (Bauerly et al. 2006). Values represent mean fold change ± SEM.

Statistical Analysis

Statistical evaluation of the data was performed using Student's t-test in Excel and one-way ANOVA followed by Student-Newman-Keuls or Dunn's post-hoc tests using GraphPad Prism.

Results

E_2 and 4-OHT increase *Nrf1* expression in both mammary gland and uterus

We previously reported that E_2 and 4-OHT increase NRF1 transcription in MCF-7 and T47D breast cancer cells and human umbilical vein endothelial cells (Ivanova et al. 2011; Mattingly et al. 2008; Mattingly and Klinge 2012), but the effect of E_2 and 4-OHT on NRF1 transcription in estrogen target tissues *in vivo* is unknown. The expression of *Nrf1* was measured in the mammary gland and uterus of ovariectomized C57BL/6 mice after treatment with vehicle (sesame oil), E_2, or 4-OHT for 6, 24, or 72 h (Fig. 1*A* and 1*B*). *Nrf1* transcript expression was increased by E_2 in mammary gland after 6 and 72 h and in the uterus after 6 and 24 h, but not at 72 h. 4-OHT increased *Nrf1* expression in both mammary gland and uterus 6 h after treatment, but reduced *Nrf1* in the mammary gland, but not in the uterus, after 24 h. *Nrf1* expression returned to basal in both mammary gland and uterus 72 h after 4-OHT treatment. Basal Nrf1 transcript expressed was unchanged during the time course of treatment (Supplementary Fig. 1)

Tissue-specific regulation of NRF-1 protein expression by E_2 and 4-OHT

Commensurate with E_2-and 4-OHT-induced *Nrf1* mRNA, NRF-1 protein increased in uterus 6 and 24 h after treatment with a return to basal levels after 72 h (Fig. 1*D* and Supplementary Fig. 1). E_2 increased NRF-1 protein in mammary gland only 72 h after E_2 treatment while 4-OHT was without effect (Fig. 1*C* and Supplementary Fig. 1).

ERα binding to mouse *Nrf1* promoter *in vivo*

Chromatin immunoprecipitation (ChIP) assays were used to investigate ERα binding to the mouse *Nrf1* promoter. The location of the primers for ChIP are shown in Figure 2A. These regions were chosen based on known or predicted EREs and other elements regulated by ER tethering mechanisms (Supplementary Fig. 2). E_2 increased ERα recruitment to the ERE-containing region within the 5' promoter in mammary gland and uterus 6 h after treatment (Fig. 2*B, C*). However, E_2-ERα was not recruited to the ½ ERE/AP-1 site located in intron 3 in either mammary gland or uterus (Fig. 2B, *C*). 4-OHT increased ERα recruitment to the ERE in both tissues and to the ½ ERE/AP-1 region (ChIP primer 1) in the mammary gland. ERα recruitment corresponds to increased *Nrf1* expression detected 6 h after treatment with E_2 in both the mammary gland and uterus (Fig. 1*A, B*). 4-OHT-ERα was recruited to the ½ ERE /AP-1 binding site in the intron 3 region in the mammary gland, but not uterus.

E_2 and 4-OHT increase selected NRF-1 target genes in mammary gland

An increase in NRF-1 protein is expected to increase the transcription of its target genes, *e.g., Tfam, Tfb1m*, and *Tfb2m*, involved in regulation of mtDNA transcription and replication. Correspondingly, we detected an increase in *Tfam* and *Tfb1m* in mammary gland

6 h after treatment with E_2 and 4-OHT (Fig. 3*A*). The pattern of *Tfam, Tfb1m*, and *Tfb2m* gene expression was different in uterus. E_2 and 4-OHT repressed *Tfb1m* in the uterus 6 h after treatment and in the 4-OHT-treated mice, *Tfb1m* remained repressed after 24 h. 4-OHT reduced uterine *Tfam* below control levels after 24 h, but expression returned to control levels by 72 h (Fig. 3*B*). While unchanged at 24 h, E_2 increased *Tfam* and *Tfb1m* expression after 72 h in both mammary gland and uterus (Fig. 3 *A*, *B*). No increase in *Tfb2m* was detected in response to either E_2 or 4-OHT in either mammary gland or uterus (Fig. 3*A*, *B*). E_2 and 4-OHT reduced *Tfb2m* in the uterus 24 h after treatment and 4-OHT reduced *Tfbm2* in the mammary gland 72 h after treatment (Fig. 3*A*, *B*).

E_2 and 4-OHT increase Tfam protein in mammary gland and 4-OHT reduces Tfam protein in uterus

E_2 and 4-OHT increased Tfam protein in mammary gland 72 h after treatment (Fig. 3*C* and Supplementary Fig. 3). Reflecting the lack of induction of *Tfam* mRNA expression by E_2 or 4-OHT in uterus (Fig. 3*B*), no increase in Tfam protein was detected in uterus 6 or 24 h after treatment or with E_2 72 h after treatment (Fig. 3*D* and Supplementary Fig. 3). Uterine Tfam protein levels were lower than control 72 h after 4-OHT treatment. Concordantly, expression of mitochondrial-encoded cytochrome c oxidase I (*mt-Co1*) was significantly reduced by 4-OHT-treatment in uterus 24 and 72 h after treatment while no changes were detected in mammary gland (Supplementary Fig. 4).

E_2 activated and 4-OHT inhibited mitochondrial biogenesis

Tfam is crucial for the initiation of mitochondrial transcription and DNA replication (Campbell, et al. 2012). Since E_2 and 4-OHT increased Tfam protein in the mammary gland, we examined the mitochondrial/nuclear DNA as an index of mitochondrial biogenesis. As recently reviewed (Kemper et al. 2013), mitochondrial /nuclear DNA ratios are a better marker of mitochondrial biogenesis than mitochondrial staining techniques. The mitochondrial/nuclear DNA ratio was determined by qPCR using primers for mt-Nd5 as a mitochondria gene and *Cftr* (cystic fibrosis) as a nuclear gene (Bauerly et al. 2006). Although E_2 increased the mitochondrial /nuclear DNA ratio in mammary gland at 6 h, this was not statistically significant until 24 h (Fig. 4*A*). 4-OHT decreased mitochondrial biogenesis in the mammary gland after 72 h. In the uterus, E_2 increased mitochondrial biogenesis 24 and 72 h after E_2 treatment whereas 4-OHT had no effect (Fig. 4*B*). We conclude that E_2 increased mitochondrial biogenesis in both mammary gland and uterus, with a higher increase in uterus, which may reflect E_2-induced increases in transcription, protein synthesis, and cell proliferation, all metabolically demanding, in the ovex mouse uterus at these time points (Moggs et al. 2004).

Tissue-specific regulation of outer mitochondrial Tom40 protein expression

Because E_2 increased the mt/nuclear DNA ratio at 24 h in mammary gland and uterus, with more of an increase in uterus versus mammary gland, while 4-OHT increased the mt/nuclear DNA ratio only in uterus (Fig. 4*A*), we examined the protein level of one component of the translocase of the outer mitochondrial membrane (TOM) complex which controls the transport of nuclear-encoded proteins into mitochondria as a representative outer mt membrane protein (Hoogenraad and Ryan 2001). Tom40 is the pore-forming unit of the TOM complex (Hill, et al. 1998). E_2 increased Tom40 protein levels in mammary gland and uterus 72 h after treatment (Fig. 4*C, D* and Supplementary Fig. 5), a result that correlates with E_2-induced mitochondrial biogenesis at 24 h in these tissues which would then detected at the protein level later, *e.g*., 72 h as measured here. 4-OHT increased mitochondrial biogenesis only in the uterus and not in mammary gland after 24 h and likewise Tom40 protein was increased only in the uterus after 72 h.

E_2 and 4-OHT regulate nuclear-encoded NRF-1 target genes *Cox4* and *CycS*

Cytochrome oxidase subunit IV (*Cox4*) and cytochrome C (*CycS*) are nuclear-encoded, NRF-1 target genes for OXPHOS. We tested the hypothesis that an increase in NRF-1 protein should result in an increase in the expression of its target genes. *Cox4* mRNA expression was increased in mammary gland with E_2 treatment at 6 and 24 h whereas uterine expression was unaffected by E_2 and inhibited by 4-OHT at all time points (Fig. 5*A* and *B*). Cox4 protein was increased in mammary gland and uterus 72 h after E_2 and 4-OHT treatment (Fig. 5*C, D* and Supplementary Fig. 6*A*). The reason for the discrepancy between the absence of an effect of 4-OHT on *Cox4* transcript expression and the increase in Cox4 protein detected in MG and the inhibition of *Cox4* transcript expression but increase in Cox4 protein in uterus at the 72 h time point is unknown. Among the possible explanations are increased protein stability, and altered mRNA stability.

CycS expression was significantly increased by E_2 in mammary gland and decreased by 4-OHT in the uterus 72 h after treatment (Fig. 5*A, B*). Cytochrome C protein levels did not change in mammary gland of mice treated with either E_2 or 4-OHT (Fig. 5*D* and Supplementary Fig. 6*B*). However, E_2 increased cytochrome *c* protein in uterus (Fig. 5*C* and Supplementary Fig. 6*B*), a result in contrast to the lack of change in *CycS* mRNA (Fig. 5*B*).

Expression of NRF-1 coactivators PGC-1α and PGC-1β in mouse mammary gland and uterus

PGC1-α and PGC-1β are essential for NRF-1 regulation of gene expression (Scarpulla 2006; Scarpulla 2008a). *Ppargc1b* was increased in mammary gland 6 h after E_2 and 4-OHT treatment (Fig. 6*A*). In uterus, *Ppargc1a* was reduced 6 h after 4-OHT treatment, and 24 and 72 h after E_2 and 4-OHT treatment (Fig. 6*B*). *Ppargc1b* expression was reduced in uterus 24 h after 4-OHT treatment and72 h after E_2 and 4-OHT treatment (Fig. 6*B*). In conclusion, *Ppargc1a* expression was not regulated by E_2 or 4-OHT in mammary gland and decreased in uterus (Fig. 6*A*), consistent with reports that: E_2 repressed PGC-1α in mouse uterus (Macari et al. 2010a), GeoProfile GDS1058 showed *Pparg1c* in uterus of ovex control, but not E_2-treated ovex CD1 mice 4, 8 or 24 h after treatment. Ovariectomy increased PGC-1α in mouse brain endothelial cells (Kemper et al. 2013). Our data suggest that *Ppargc1a* and *Ppargc1b* are differentially regulated by E_2 and 4-OHT in both mammary gland and uterus.

Colocalization of NRF-1 and coactivator PGC-1α in mouse uterus

To address whether NRF-1 and PGC-1α are expressed in the same cell types in mouse uterus, IHC staining was performed on tissues from ovex mice treated with E_2 for 24 or 48 h (Fig. 7). NRF-1 staining was more prominent in the cytoplasm in vehicle-treated uterine tissues. However, both stromal and epithelial cells showed enhanced nuclear staining upon E_2 treatment for 24 h but not for 48 h. PGC-1α staining was observed in both cytoplasm and nucleus of stromal and epithelial cells in vehicle-treated tissues; however, PGC-1α staining was primarily in the nucleus in 24 h-treated tissues but not in 48 h-treated tissues. Consistent with Q-RT-PCR results (*see* Fig. 6B), staining was reduced in uteri treated with E_2 for 24 h and 48 h compared to vehicle. These results indicate that E_2 enhances transient nuclear translocation of NRF-1 and PGC-1α in adult uteri. Our results also indicate that E_2 regulates NRF-1 and PGC-1α in both immature and mature uteri. Most importantly, it was evident that NRF-1 and PGC-1α were expressed in virtually all cells in stroma and epithelium and that they colocalized in the nucleus regardless of treatment.

Discussion

The data presented here are the first study of the regulation of endogenous *Nrf1* expression, NRF-1 target gene expression, and mitochondrial biogenesis in normal mouse mammary

gland or uterus by E_2 and 4-OHT. This is because *Nrf1* was not included in microarrays previously used to identify E_2 and TAM regulated genes mouse uterus (Fong et al. 2007; Fong et al. 2010; Moggs et al. 2004) and mammary gland (Deroo et al. 2009). Earlier, we reported that E_2 and 4-OHT increase NRF-1 transcription in breast cancer cells and HUVECs (Ivanova et al. 2011; Mattingly et al. 2008; Mattingly and Klinge 2012) and that E_2 activates NRF-1 transcriptional activity and mitochondrial biogenesis in human breast cancer cells (Ivanova et al. 2011; Mattingly et al. 2008). Because NRF-1 is a master transcriptional regulator of genes regulating mitochondrial function (Scarpulla 2008a), we proposed a model that nuclear ER upregulation of NRF-1 which, in turn, stimulates the transcription of nuclear-encoded mitochondrial genes, thus coordinately regulates nuclear-mitochondrial transcription and function (Ivanova et al. 2011; Klinge 2008; Mattingly et al. 2008). In normal tissue, E_2 increased NRF-1 protein in rat brain (Stirone, et al. 2005) and reduced *Nrf1* mRNA expression in mouse brown adipocytes (Rodriguez-Cuenca, et al. 2007).

Regulation of mitochondrial biogenesis has been largely defined by studies in skeletal and cardiac muscle, liver, and brown fat (Finck and Kelly 2006, 2007; Scarpulla 2008b). This study demonstrates that E_2 and 4-OHT increase *Nrf1* mRNA and protein expression in a time and tissue-specific manner in mammary gland and uterus. *Ppargc1a, Ppargc1b*, and NRF-1-regulated nuclear-encoded genes are also regulated by E_2 and 4-OHT in a tissue-specific manner as summarized Fig. 8. Although *Nrf1* expression was increased by E_2 and 4-OHT 6 h after treatment in both mammary gland and uterus, NRF-1 protein was higher in uterus and E_2 transiently increased nuclear NRF-1 and PGC-1α staining 4-OHT increased *Nrf1* mRNA but not NRF-1 protein in mammary gland. The discrepancy between *Nrf1* mRNA and protein may result from differences in mRNA stability, but will require further investigation. Our results for E_2 regulation show an expected inverse correlation with recent data showing that ovariectomy decreased NRF-1, Tfam, and *Ppargc1b* while increasing *Ppargc1a* at both mRNA and protein levels in isolated mouse brain endothelial cells (Kemper et al. 2013).

Interestingly, our study suggests that the mammary gland appears to be more responsive than uterus to upregulation of NRF-1 target genes *Tfam*, *Tfb1m* and *Cox4* after E_2 or 4-OHT treatment. Serial analysis of gene expression (SAGE) identified four OXPHOS genes: *Cox6a2, MtCo1, MtCo3*, and *MtNd3*, upregulated in mammary gland 3 h after a single, subcutaneous injection of E_2 (50 ng) in αERKO mice, allowing the authors to conclude that E_2 regulates the expression of *Cox6a2* and the mitochondrial-encoded genes mainly through ERβ (Aboghe, et al. 2009). Importantly, and commensurate with our results that E_2 increases Tfam in mammary gland, all three mitochondrial-encoded genes upregulated by E_2 (*MtCo1, MtCo3*, and *MtNd3*(Aboghe et al. 2009)) are regulated by Tfam (Scarpulla 2008b). Based on the results summarized in Figure 8 and modeled in Figure 9, we suggest that NRF-1 upregulates its target genes selectively in the mammary gland at least in part because of the expression of *Ppargc1b* in mammary gland. In contrast, uterine *Ppargc1a* and P*pargc1b* expression are decreased with E_2 and 4-OHT treatment. Our results agree with other reports showing that E_2 repressed *Ppargc1a* transcription and reduced PGC-1α protein in mouse uterus (Macari et al. 2010a) and ovariectomy increased PGC-1α and reduced PGC-1β in mouse brain endothelial cells (Kemper et al. 2013).

To explain the mechanisms involved in regulation of mouse *Nrf1* transcription, we performed ChIP which revealed increased ERα occupation of the ERE in the *Nrf1* promoter in mammary gland and uterus after E_2 and 4-OHT treatment. Similar to ERα ChIP-seq identification of ERα binding sites in a whole genome study of mouse uterus (Hewitt et al. 2012), we also observed ERα bound directly to the ERE-containing region of the *Nrf1* promoter in the uterus of the ovex, control-treated mice. At the same time, our results differ

from the apparent lack of ERα recruitment to the *Nrf1* gene in uterus with 1 h of 0.25 μg E_2 injection of ovex C57BL/6J mice in this ChIP-seq analysis; however, the treatment time, route, and dose of E_2 are different between our study and that from the Korach lab (Hewitt et al. 2012). Interestingly, ERα was recruited to the *Tfam* gene with E_2 treatment in mouse uterus (Hewitt et al. 2012), commensurate with the increase in *Tfam* mRNA expression with E_2 in mouse uterus seen 72 h after treatment (Fig. 3*B*). The ERα occupation of AP-1/ ½ERE site in *Nrf1* intron 3 in mammary gland from 4-OHT treated mice is intriguing because it corresponds to inhibition of NRF-1 expression in our study. Mechanistically, this observation may be related to the tethering interaction of ERα with AP-1 (Cerillo, et al. 1998; Heldring, et al. 2011; Paech, et al. 1997), but further experiments will be required to dissect the molecular mechanisms involved. ChIP-seq demonstrated ERα bound to the first intron of the *Wnt4* and *Cdkn1* genes in E_2-treated mouse uterus and upregulated expression (Hewitt et al. 2012), establishing the ability of intron-bound ERα to regulate gene transcription.

Although Tfam, Tfb1m, and Tfb2m are essential for mitochondrial gene transcription and DNA replication (Scarpulla 2008a), their regulation by E_2 and 4-OHT did not completely match effects of E_2 and 4-OHT on mitochondrial biogenesis. E_2 increased mitochondrial biogenesis in the mammary gland and uterus after 24 h, in agreement with increases in *Tfam* and *Tfb1m* only in the mammary gland. Although 4-OHT increased *Tfam* and *Pgpargc1b* in mammary gland, the mitochondrial biogenesis was not increased. In fact, after 72 h treatment, 4-OHT reduced mammary gland mitochondrial biogenesis, in agreement with reduced *Tfb2m* and the return of *Tfb1m* to basal 24 h after treatment. This may relate to the fact that Tfam and Tfb1m alone are insufficient to initiate mtDNA replication and Tfb2m promotes mtDNA replication more efficiently than Tfb1m (Cotney, et al. 2007). It may also relate to the proapoptotic activity of tamoxifen in mammary gland (Kotoula, et al. 1993).

A summary of our findings is diagramed in Figure 8 and modeled in Figure 9. E_2 and 4-OHT increase *in vivo* ERα recruitment to the *Nrf1* gene and regulate NRF-1 signaling in time- and tissue-dependent manner in mouse mammary gland and uterus. NRF-1 activated more of its target genes in mammary gland than in uterus after E_2 or 4-OHT treatment, whereas more genes were downregulated by E_2 and 4-OHT in the uterus. These data are congruent with a report where only 10 genes were regulated by E_2 in mouse mammary gland (Aboghe et al. 2009) whereas in mouse uterus, E_2 altered the expression of 3,538 genes (Moggs et al. 2004). The increase in NRF-1 target gene *Tfam* in mammary gland and uterus 6 h after E_2 and 4-OHT treatment was different from the time-delayed increase in TFAM expression detected after E_2, not 4-OHT, treatment of ERα-expressing MCF-7 breast cancer cells (Ivanova et al. 2011), reflecting a report that *in vivo* uterine gene regulation by E_2-ERα is different from MCF-7 cells and may reflect direct recruitment of liganded ERα to the mouse *Tfam* promoter (Hewitt et al. 2012). In conclusion, our data demonstrate that E_2 and 4-OHT differentially regulate NRF-1 and its downstream gene targets in mammary gland and uterus with a stronger impact of E_2 on stimulating mitochondrial biogenesis in uterus than mammary gland.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Numan Al-Rayyan and Kathleen Gajewski for their help in collecting mouse tissues and confocal microscopy, respectively. We thank high school student Meghan Hyman whose summer research was supported by a grant from the James Graham Brown Cancer Center and Maria Lohr, a summer student from Centre College,

Danville, KY for their assistance with some of the experiments. We thank Dr. Barbara J. Clark for her thoughtful suggestions for this manuscript.

Research Support: This work was supported by NIH R01 DK053220 to C.M.K.

References

Aboghe DH, Yoshioka M, Phaneuf D, St-Amand J. Regulation of gene expression by estrogen in mammary gland of wild type and estrogen receptor alpha knockout mice. The Journal of Steroid Biochemistry and Molecular Biology. 2009; 113:116–126. [PubMed: 19126430]

Balsitis SJ, Sage J, Duensing S, Münger K, Jacks T, Lambert PF. Recapitulation of the Effects of the Human Papillomavirus Type 16 E7 Oncogene on Mouse Epithelium by Somatic Rb Deletion and Detection of pRb-Independent Effects of E7 In Vivo. Molecular and Cellular Biology. 2003; 23:9094–9103. [PubMed: 14645521]

Barros Rodrigo PA, Gustafsson J-Å. Estrogen Receptors and the Metabolic Network. Cell Metabolism. 2011; 14:289–299. [PubMed: 21907136]

Bauerly KA, Storms DH, Harris CB, Hajizadeh S, Sun MY, Cheung CP, Satre MA, Fascetti AJ, Tchaparian E, Rucker RB. Pyrroloquinoline quinone nutritional status alters lysine metabolism and modulates mitochondrial DNA content in the mouse and rat. Biochimica et Biophysica Acta (BBA) - General Subjects. 2006; 1760:1741–1748.

Billon-Galés A, Fontaine C, Filipe C, Douin-Echinard V, Fouque M-J, Flouriot G, Gourdy P, Lenfant F, Laurell H, Krust A, et al. The transactivating function 1 of estrogen receptor alpha is dispensable for the vasculoprotective actions of 17beta-estradiol. Proceedings of the National Academy of Sciences. 2009; 106:2053–2058.

Bozek K, Relogio A, Kielbasa SM, Heine M, Dame C, Kramer A, Herzel H. Regulation of clock-controlled genes in mammals. PLoS ONE. 2009; 4:e4882. [PubMed: 19287494]

Bozek K, Rosahl AL, Gaub S, Lorenzen S, Herzel H. Circadian transcription in liver. Biosystems. 2010; 102:61–69. [PubMed: 20655353]

Cam H, Balciunaite E, Blais A, Spektor A, Scarpulla RC, Young R, Kluger Y, Dynlacht BD. A Common Set of Gene Regulatory Networks Links Metabolism and Growth Inhibition. Molecular Cell. 2004; 16:399–411. [PubMed: 15525513]

Campbell CT, Kolesar JE, Kaufman BA. Mitochondrial transcription factor A regulates mitochondrial transcription initiation, DNA packaging, and genome copy number. Biochimica et biophysica acta. 2012

Cerillo G, Rees A, Manchanda N, Reilly C, Brogan I, White A, Needham M. The oestrogen receptor regulates NFkappaB and AP-1 activity in a cell- specific manner. Journal of Steroid Biochemistry and Molecular Biology. 1998; 67:79–88. [PubMed: 9877207]

Cooke PS, Naaz A. Role of Estrogens in Adipocyte Development and Function. Experimental Biology and Medicine. 2004; 229:1127–1135. [PubMed: 15564439]

Cotney J, Wang Z, Shadel GS. Relative abundance of the human mitochondrial transcription system and distinct roles for h-mtTFB1 and h-mtTFB2 in mitochondrial biogenesis and gene expression. Nucleic Acids Res. 2007; 35:4042–4054. [PubMed: 17557812]

Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocrine Reviews. 1999; 20:358–417. [PubMed: 10368776]

Deblois G, Giguere V. Oestrogen-related receptors in breast cancer: control of cellular metabolism and beyond. Nat Rev Cancer. 2013; 13:27–36. [PubMed: 23192231]

Deroo BJ, Hewitt SC, Collins JB, Grissom SF, Hamilton KJ, Korach KS. Profile of estrogen-responsive genes in an estrogen-specific mammary gland outgrowth model. Molecular Reproduction and Development. 2009; 76:733–750. [PubMed: 19484750]

Faulds MH, Zhao C, Dahlman-Wright K, Gustafsson J-Å. The diversity of sex steroid action: regulation of metabolism by estrogen signaling. Journal of Endocrinology. 2012; 212:3–12. [PubMed: 21511884]

Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. Journal of Clinical Investigation. 2006; 116:615–622. [PubMed: 16511594]

Finck BN, Kelly DP. Peroxisome Proliferator-Activated Receptor {gamma} Coactivator-1 (PGC-1) Regulatory Cascade in Cardiac Physiology and Disease. Circulation. 2007; 115:2540–2548. [PubMed: 17502589]

Fong CJ, Burgoon LD, Williams KJ, Forgacs AL, Zacharewski TR. Comparative temporal and dose-dependent morphological and transcriptional uterine effects elicited by tamoxifen and ethynylestradiol in immature, ovariectomized mice. BMC Genomics. 2007; 8:151. [PubMed: 17555576]

Fong CJ, Burgoon LD, Williams KJ, Jones AD, Forgacs AL, Zacharewski TR. Effects of tamoxifen and ethynylestradiol cotreatment on uterine gene expression in immature, ovariectomized mice. Journal of Molecular Endocrinology. 2010; 45:161–173. [PubMed: 20628019]

Gleyzer N, Scarpulla RC. PGC-1-related Coactivator (PRC), a Sensor of Metabolic Stress, Orchestrates a Redox-sensitive Program of Inflammatory Gene Expression. Journal of Biological Chemistry. 2011; 286:39715–39725. [PubMed: 21937425]

Heldring N, Isaacs GD, Diehl AG, Sun M, Cheung E, Ranish JA, Kraus WL. Multiple Sequence-Specific DNA-Binding Proteins Mediate Estrogen Receptor Signaling through a Tethering Pathway. Molecular Endocrinology. 2011; 25:564–574. [PubMed: 21330404]

Hewitt SC, Li L, Grimm SA, Chen Y, Liu L, Li Y, Bushel PR, Fargo D, Korach KS. Research Resource: Whole-Genome Estrogen Receptor α Binding in Mouse Uterine Tissue Revealed by ChIP-Seq. Molecular Endocrinology. 2012; 26:887–898. [PubMed: 22446102]

Hill K, Model K, Ryan MT, Dietmeier K, Martin F, Wagner R, Pfanner N. Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins. Nature. 1998; 395:516–521. [PubMed: 9774109]

Hoogenraad NJ, Ryan MT. Translocation of proteins into mitochondria. IUBMB Life. 2001; 51:345–350. [PubMed: 11758801]

Ivanova MM, Luken KH, Zimmer AS, Lenzo FL, Smith RJ, Arteel MW, Kollenberg TJ, Mattingly KA, Klinge CM. Tamoxifen increases nuclear respiratory factor 1 transcription by activating estrogen receptor β and AP-1 recruitment to adjacent promoter binding sites. The FASEB Journal. 2011; 25:1402–1416.

Jeong J-W, Lee KY, Han SJ, Aronow BJ, Lydon JP, O'Malley BW, DeMayo FJ. The p160 Steroid Receptor Coactivator 2, SRC-2, Regulates Murine Endometrial Function and Regulates Progesterone-Independent and -Dependent Gene Expression. Endocrinology. 2007; 148:4238–4250. [PubMed: 17556502]

Kemper MF, Zhao Y, Duckles SP, Krause DN. Endogenous ovarian hormones affect mitochondrial efficiency in cerebral endothelium via distinct regulation of PGC-1 isoforms. Journal of Cerebral Blood Flow and Metabolism. 2013; 33:122–128. [PubMed: 23093066]

Klinge CM. Estrogen receptor interaction with co-activators and co-repressors. Steroids. 2000; 65:227–251. [PubMed: 10751636]

Klinge CM. Estrogenic control of mitochondrial function and biogenesis. Journal of Cellular Biochemistry. 2008; 105:1342–1351. [PubMed: 18846505]

Korach KS, Emmen JMA, Walker VR, Hewitt SC, Yates M, Hall JM, Swope DL, Harrell JC, Couse JF. Update on animal models developed for analyses of estrogen receptor biological activity. The Journal of Steroid Biochemistry and Molecular Biology. 2003; 86:387–391. [PubMed: 14623535]

Kotoula V, Karkavelas G, Economou L, Sionga A, Boutis L, Kerameos-Foroglou C. Effects of tamoxifen and CV 205502 on the morphology and the evolution of the noncancerous mouse mammary gland. Histology and Histopathology. 1993; 8:627–636. [PubMed: 7905759]

Macari C, Teyssier C, Tribollet V, Mouzat K, Forcet C, Horard B, Lobaccaro J-M, Vanacker JM. Estrogens repress PGC1-α expression in the uterus. Molecular and Cellular Endocrinology. 2010a; 330:33–40. [PubMed: 20816721]

Macari C, Teyssier C, Tribollet V, Mouzat K, Forcet C, Horard B, Lobaccaro JM, Vanacker JM. Estrogens repress PGC1-alpha expression in the uterus. Molecular and cellular endocrinology. 2010b; 330:33–40. [PubMed: 20816721]

Mattingly KA, Ivanova MM, Riggs KA, Wickramasinghe NS, Barch MJ, Klinge CM. Estradiol stimulates transcription of Nuclear Respiratory Factor-1 and increases mitochondrial biogenesis. Molecular Endocrinology. 2008; 22:609–622. [PubMed: 18048642]

Mattingly KA, Klinge CM. Diesel exhaust particulate extracts inhibit transcription of nuclear respiratory factor-1 and cell viability in human umbilical vein endothelial cells. Archives of Toxicology. 2012; 86:633–642. [PubMed: 22105178]

Moggs JG, Tinwell H, Spurway T, Chang HS, Pate I, Lim FL, Moore DJ, Soames A, Stuckey R, Currie R, et al. Phenotypic anchoring of gene expression changes during estrogen-induced uterine growth. Environmental Health Perspectives. 2004; 112:1589–1606. [PubMed: 15598610]

O'Brien JE, Peterson TJ, Tong MH, Lee E-J, Pfaff LE, Hewitt SC, Korach KS, Weiss J, Jameson JL. Estrogen-induced Proliferation of Uterine Epithelial Cells Is Independent of Estrogen Receptor {alpha} Binding to Classical Estrogen Response Elements. Journal of Biological Chemistry. 2006; 281:26683–26692. [PubMed: 16847062]

O'Lone R, Knorr K, Jaffe IZ, Schaffer ME, Martini PGV, Karas RH, Bienkowska J, Mendelsohn ME, Hansen U. Estrogen Receptors {alpha} and {beta} Mediate Distinct Pathways of Vascular Gene Expression, Including Genes Involved in Mitochondrial Electron Transport and Generation of Reactive Oxygen Species. Molecular Endocrinology. 2007; 21:1281–1296. [PubMed: 17374850]

O'Malley BW, Kumar R. Nuclear Receptor Coregulators in Cancer Biology. Cancer Research. 2009; 69:8217–8222. [PubMed: 19843848]

Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science. 1997; 277:1508–1510. [PubMed: 9278514]

Papi A, Storci G, Guarnieri T, De Carolis S, Bertoni S, Avenia N, Sanguinetti A, Sidoni A, Santini D, Ceccarelli C, et al. Peroxisome Proliferator Activated Receptor-α/Hypoxia Inducible Factor-1α Interplay Sustains Carbonic Anhydrase IX and Apoliprotein E Expression in Breast Cancer Stem Cells. PLoS ONE. 2013; 8:e54968. [PubMed: 23372804]

Pendaries C, Darblade B, Rochaix P, Krust A, Chambon P, Korach KS, Bayard F, Arnal JF. The AF-1 activation-function of ERalpha may be dispensable to mediate the effect of estradiol on endothelial NO production in mice. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99:2205–2210. [PubMed: 11854517]

Renoir J-M, Marsaud V, Lazennec G. Estrogen receptor signaling as a target for novel breast cancer therapeutics. Biochemical Pharmacology. 2013; 85:449–465. [PubMed: 23103568]

Richard CS. Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2011; 1813:1269–1278.

Rodriguez-Cuenca S, Monjo M, Gianotti M, Proenza AM, Roca P. Expression of mitochondrial biogenesis-signaling factors in brown adipocytes is influenced specifically by 17beta-estradiol, testosterone, and progesterone. Am J Physiol Endocrinol Metab. 2007; 292:E340–346. [PubMed: 16954335]

Safdar A, Little JP, Stokl AJ, Hettinga BP, Akhtar M, Tarnopolsky MA. Exercise increases mitochondrial PGC-1alpha content and promotes nuclear-mitochondrial cross-talk to coordinate mitochondrial biogenesis. The Journal of biological chemistry. 2011; 286:10605–10617. [PubMed: 21245132]

Scarpulla RC. Nuclear control of respiratory gene expression in mammalian cells. Journal of Cellular Biochemistry. 2006; 97:673–683. [PubMed: 16329141]

Scarpulla RC. Nuclear Control of Respiratory Chain Expression by Nuclear Respiratory Factors and PGC-1-Related Coactivator. Annals of the New York Academy of Sciences. 2008a; 1147:321–334. [PubMed: 19076454]

Scarpulla RC. Transcriptional Paradigms in Mammalian Mitochondrial Biogenesis and Function. Physiological Reviews. 2008b; 88:611–638. [PubMed: 18391175]

Scarpulla RC. Nucleus-encoded regulators of mitochondrial function: Integration of respiratory chain expression, nutrient sensing and metabolic stress. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms. 2012; 1819:1088–1097.

Scarpulla RC, Vega RB, Kelly DP. Transcriptional integration of mitochondrial biogenesis. Trends in Endocrinology & Metabolism. 2012; 23:459–466. [PubMed: 22817841]

Simpkins JW, Yang S-H, Sarkar SN, Pearce V. Estrogen actions on mitochondria--Physiological and pathological implications. Molecular and Cellular Endocrinology. 2008; 290:51–59. [PubMed: 18571833]

Simpkins JW, Yi KD, Yang S-H, Dykens JA. Mitochondrial mechanisms of estrogen neuroprotection. Biochimica et Biophysica Acta (BBA) - General Subjects. 2010; 1800:1113–1120.

Sourla A, Luo S, Labrie C, Bélanger A, Labrie F. Morphological Changes Induced by 6-Month Treatment of Intact and Ovariectomized Mice with Tamoxifen and the Pure Antiestrogen EM-800. Endocrinology. 1997; 138:5605–5617. [PubMed: 9389549]

Stirone C, Duckles SP, Krause DN, Procaccio V. Estrogen Increases Mitochondrial Efficiency and Reduces Oxidative Stress in Cerebral Blood Vessels. Molecular Pharmacology. 2005; 68:959–965. [PubMed: 15994367]

Yager JD, Chen JQ. Mitochondrial estrogen receptors – new insights into specific functions. Trends in Endocrinology & Metabolism. 2007; 18:89–91. [PubMed: 17324583]

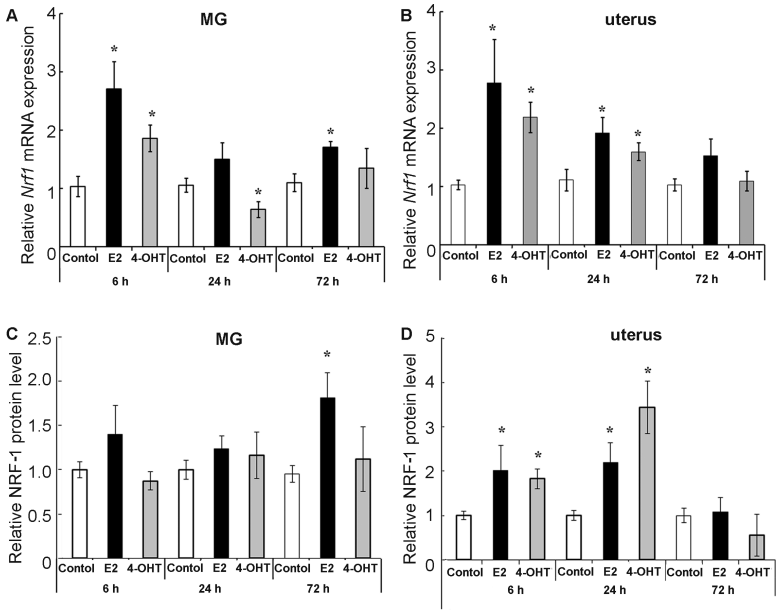

Figure 1. E_2 and 4-OHT increase *Nrf1* mRNA and NRF-1 protein expression in mouse mammary gland and uterus

Ovariectomized C57BL/6 mice were given a single sc injection of sesame oil (vehicle control), 100 ng E_2, or 50 μg 4-OHT and were euthanized 6, 24, or 72 h post injection. A and B. Q-RT-PCR analysis of *Nrf1* mRNA level in mammary gland (MG) (A) and uterus (B). C and D. NRF-1 protein expression was examined relative to β-actin in MG (C) and uterus (D) after the indicated time of treatment. Values are the mean ± SEM of 5-10 mice/ treatment group in which control was set to 1 for each treatment time within each blot for comparison. * $p < 0.05$ versus vehicle control (Student's t test). Representative western blots are shown in Supplementary Fig. 1.

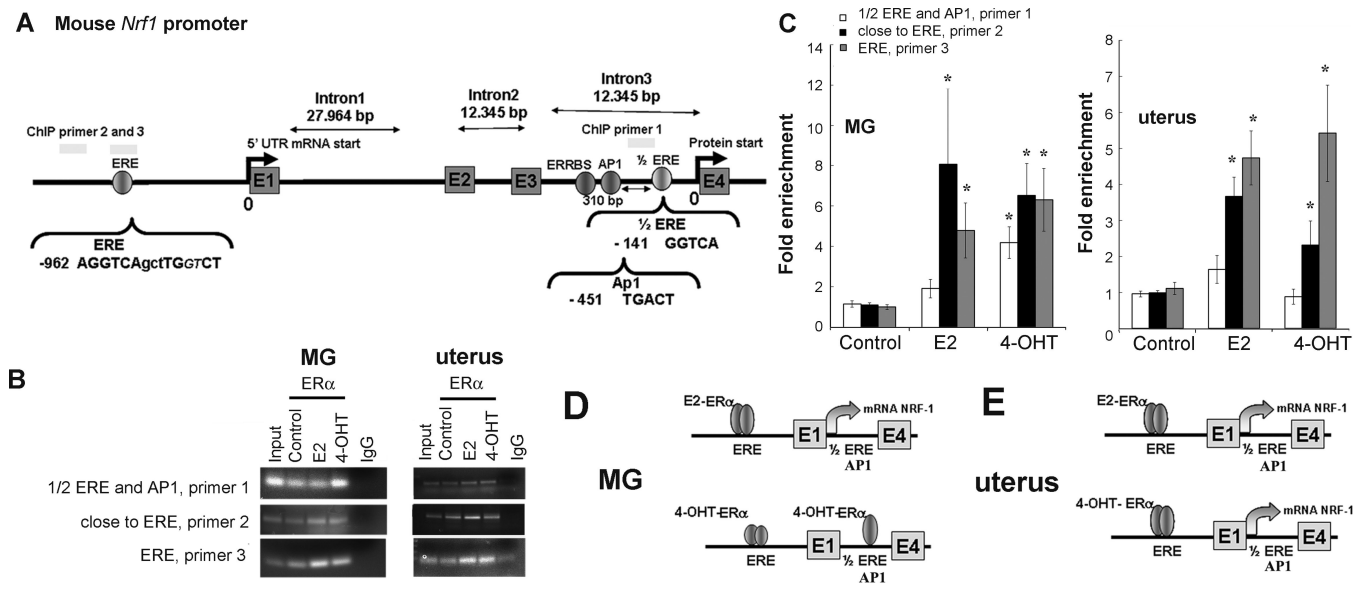

Figure 2. E_2 and 4-OHT increase ERα recruitment to the *Nrf1* promoter *in vivo* in a tissue-dependent manner

A, Diagram of the mouse *Nrf1* gene promoter showing locations of ERE, AP-1, ½ ERE, and ERR-binding sites (ERRBS), transcription and protein coding start sites, as indicated. Exons (E1, E2, E3, and E4) are indicated as grey boxes. The location of the 3 primers used for ChIP for ERα on *Nrf1* are indicated as light grey boxes above the *Nrf1* gene. B, Ovex C57BL/6 mice were treated with vehicle (control), E_2, or 4-OHT for 6 h. ChIP with ERα antibody or with rabbit pre-immune serum (IgG) was performed with the 3 pairs of ChIP primers (locations shown in A) for the mouse *Nrf1* gene promoter. C, Immunoprecipitated DNA was normalized to input and IgG control and Q-PCR data are expressed relative to IP for control-treated mammary gland (MG, left) and uterus (right). Values are the mean ± SEM of 5 mice/treatment group. * $p < 0.05$ from control for each primer. D and E, ChIP results are diagrammed for E_2-ERα and 4-OHT-ERα recruitment to the *Nrf1* promoter in MG (D) and uterus (E). The bent arrows indicate transcriptional upregulation of *Nrf1* mRNA as measured by Q-PCR (see Fig. 1*A*).

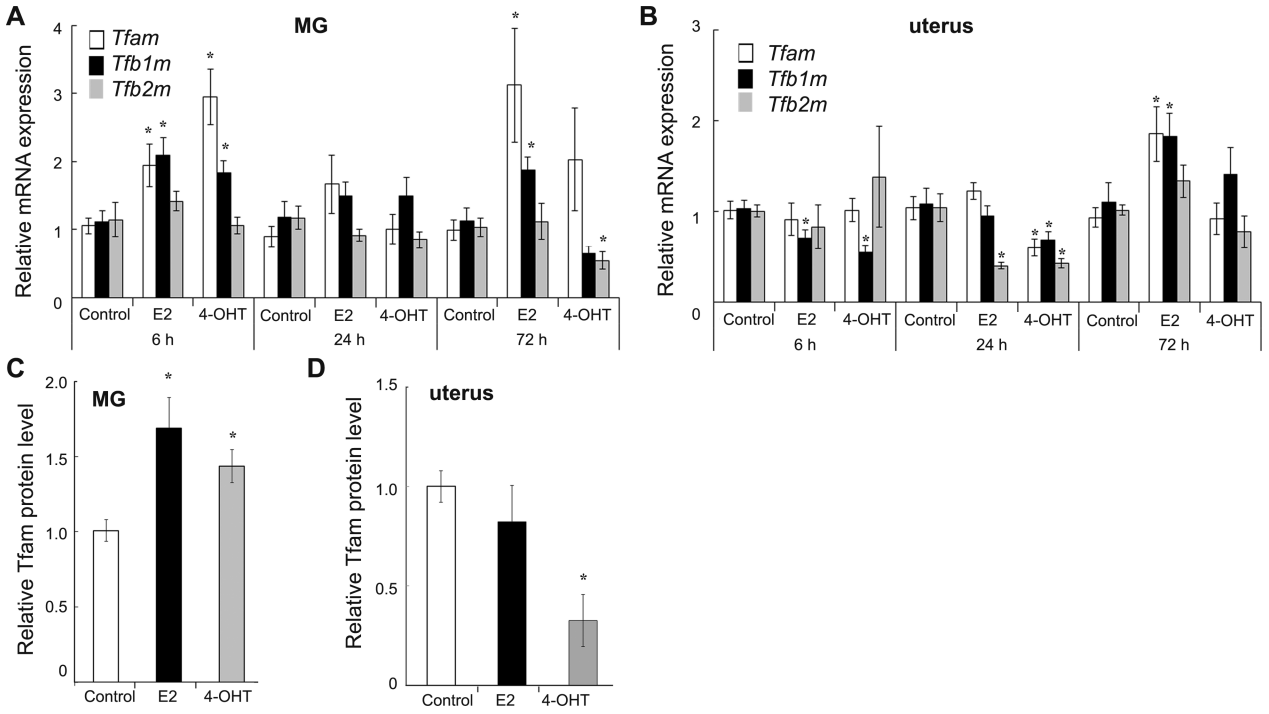

Figure 3. E_2 and 4-OHT selectively regulate the expression of NRF-1 target genes for mtDNA transcription and replication

A and B, Q-RT-PCR analysis of the mRNA expression of Nrf1-regulated *Tfam*, *Tfb1m*, and *Tfb2m* expression in mammary gland (MG) (A) and uterus (B) from ovex mice treated with vehicle control, E_2, or 4-OHT for the indicated time. C and D, Tfam protein expression was examined in mammary gland (MG in panel C) and uterus (D) 72 h after E_2 or 4-OHT treatment. The data shown are the mean ± SEM of 5-10 mice/treatment group. * $p < 0.05$ versus vehicle control (Student's t-test). Representative western blots are shown in Supplementary Fig. 3.

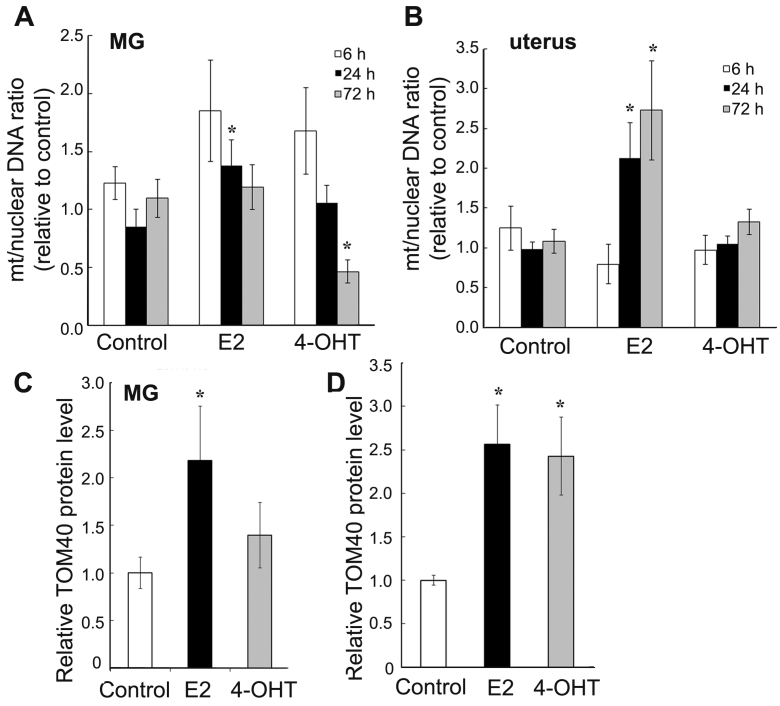

Figure 4. E_2 and 4-OHT differently regulate mitochondrial biogenesis in mouse mammary gland and uterus

A-B, Total DNA was purified from mammary gland (MG) and uterus of ovex mice treated with vehicle (control), E_2, or 4-OHT for 6, 24 and 72 h. The data are the ratio of the mitochondrial genome encoded gene mt-ND5 normalized to nuclear-encoded gene *Ctfr* as determined by qPCR. Values are the mean ± SEM of 6-15 mice/treatment group. * $p < 0.05$ versus vehicle control (Student's t-test). C-D, Tom40 protein was examined in MG (C) and uterus (D) after 72 h treatment. The values are the mean ± SEM of 4-7 mice/treatment groups. *$p < 0.05$ versus vehicle control (Student's t-test). Representative western blots are shown in Supplementary Fig.4.

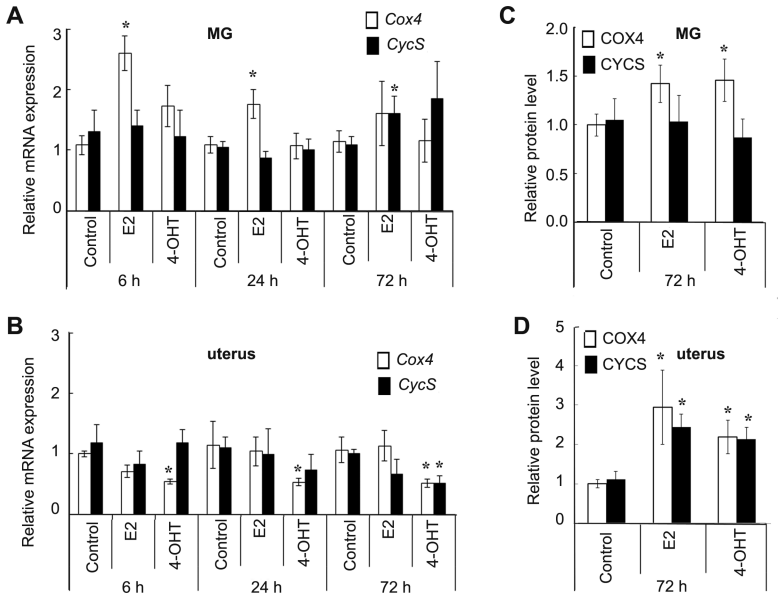

Figure 5. E_2 and 4-OHT differently regulate the level of NRF-1 target genes involved in mitochondrial respiration

A and B, Q-RT-PCR analysis of the mRNA expression of Nrf1-regulated genes *Cox4* and *CycS* in mammary gland (MG) (A) and uterus (B) from ovex mice treated with vehicle (control), E_2, or 4-OHT for the indicated time. Values are the mean ± SEM of 10-15 mice/treatment group. *p < 0.05 *versus* vehicle control (Student's t-test). C-D, Cox4 (C) and cytochrome *c* (D) protein expression was examined in MG and uterus 72 h after the indicated treatment. Values are the mean ± SEM of 5-12 mice/treatment group. *p < 0.05 versus vehicle control (Student's t-test). Representative Western blots are shown in Supplementary Fig. 5.

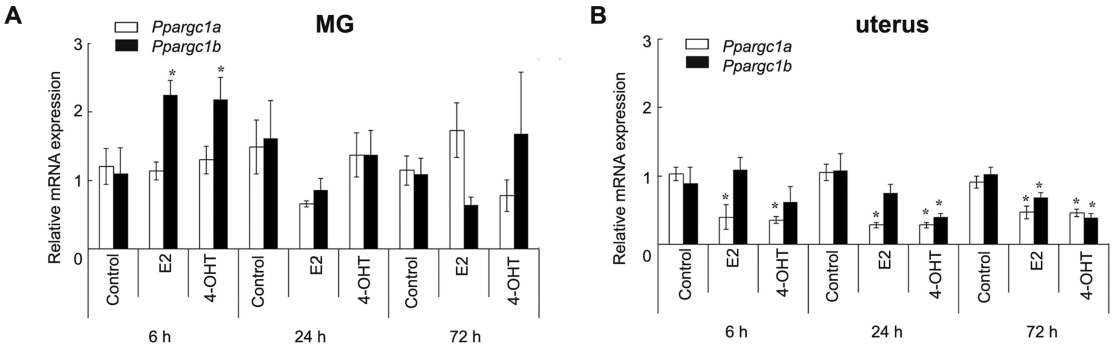

Figure 6. E_2 and 4-OHT increase *Ppargc1b* expression in mammary gland and reduce *Ppargc1a* and *Ppargc1b* expression in the uterus

Q-RT-PCR analysis of *Ppargc1a* and *Ppargc1b* expression in mammary gland (MG) (A) and uterus (B) from ovex C57BL/6 mice treated with vehicle (control), E_2, or 4-OHT for the indicated time. Values are the mean ± SEM of 5-10 mice/treatment group. *$p < 0.05$ versus vehicle control (Student's t-test).

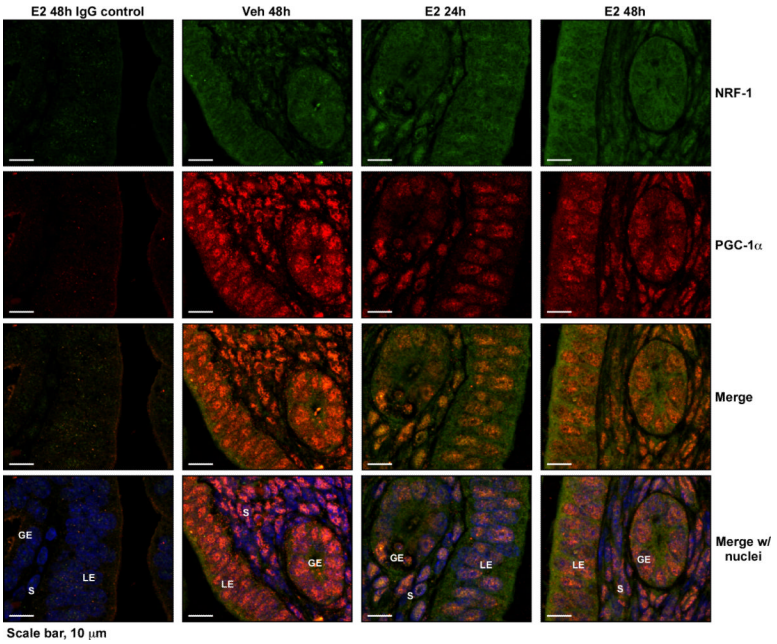

Figure 7. E_2 increases NRF-1 and PGC-1a nuclear colocalization in uterus and decreases PGC-1a protein levels

Ovex mice were treated with vehicle (veh, EtOH) or 1 μg E_2 for 24 or 48 h. Uterine tissue sections were stained for NRF-1 (green) and PGC-1α (red). Nuclei were stained with Hoechst 33258 (blue). LE, luminal epithelium; GE, glandular epithelium; S, stroma. White scale bar is 10 μm.

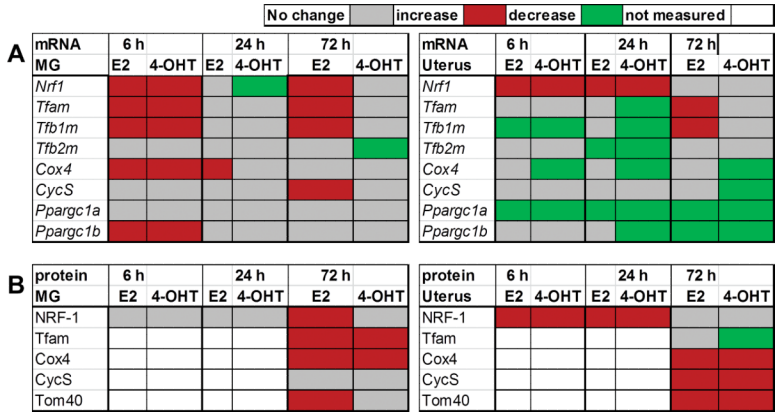

Figure 8. Summary of E_2 and 4-OHT regulation of *Nrf1*, *Ppargc1a*, *Ppargc1b*, and Nrf1 target gene expression in mouse mammary gland and uterus

Changes in the expression of the indicated genes (A) and proteins (B) in mammary gland (MG) and uterus are expressed relative to vehicle control after the indicated treatment time (6, 24, or 72 h).

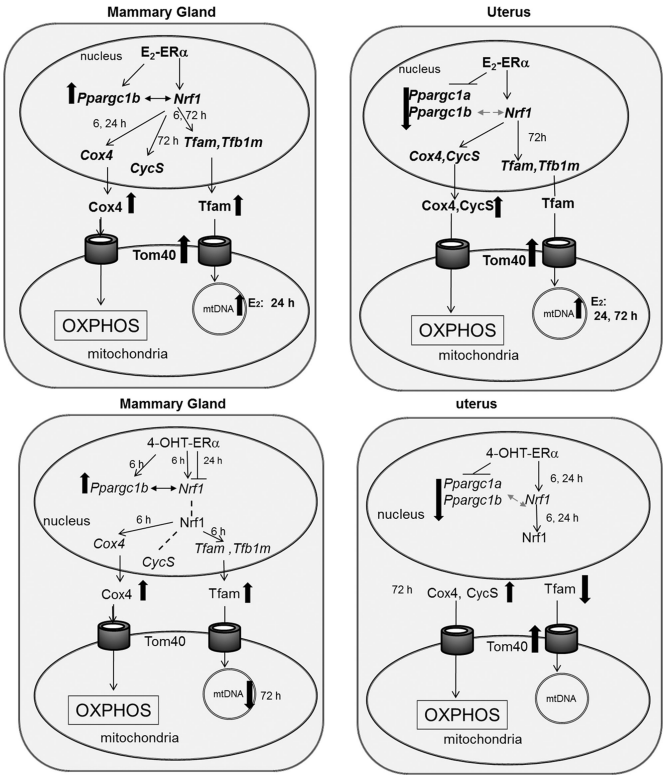

Figure 9. Model of E_2-ERα and 4-OHT-ERα regulation of *Nrf1* transcription, NRF-1 regulation of its target genes, PGC-1 coactivator family expression, Tom40, and mitochondrial biogenesis in mouse mammary gland and uterus

The E_2-ERα-stimulated changes are shown in the top two cell models with mammary gland on the left and uterus on the right. The 4-OHT-ERα-stimulated changes are shown in the bottom two cell models with mammary gland on the left and uterus on the right. Of course since we used whole tissue lysates for the experiments in this study, we cannot attribute gene/protein changes to a specific cell type. The time (h) at which changes in gene or protein expression were detected is indicated. Bold black arrows indicate the direction (up- or down-regulation) of the change of gene expression.