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

Margarita M Ivanova, Brandie N Radde, Jieun Son1, Fabiola F Mehta1, Sang-Hyuk Chung1 and Carolyn M Klinge

Department of Biochemistry and Molecular Biology, Center for Genetics and Molecular Medicine, University of Louisville School of Medicine, Louisville, Kentucky 40292, USA
1Department of Biology and Biochemistry, Center for Nuclear Receptors and Cell Signaling, University of Houston, 3605 Cullen Blvd, Houston, Texas 77204, USA

Abstract

Nuclear respiratory factor-1 (NRF-1) stimulates the transcription of nuclear-encoded genes that regulate mitochondrial (mt) genome transcription and biogenesis. We reported that estradiol (E2) and 4-hydroxytamoxifen (4-OHT) stimulate NRF-1 transcription in an estrogen receptor α (ERα)- and ERβ-dependent manner in human breast cancer cells. The aim of this study was to determine whether E2 and 4-OHT increase NRF-1 in vivo. Here, we report that E2 and 4-OHT increase NRF-1 expression in mammary gland (MG) and uterus of ovariectomized C57BL/6 mice in a time-dependent manner. E2 increased NRF-1 protein in the uterus and MG; however, in MG, 4-OHT increased Nrf1 mRNA but not protein. Chromatin immunoprecipitation assays revealed increased in vivo recruitment of ERα to the Nrf1 promoter and intron 3 in MG and uterus 6 h after E2 and 4-OHT treatment, commensurate with increased NRF-1 expression. E2- and 4-OHT-induced increases in NRF-1 and its target genes Tfam, Tfb1m, and Tfb2m were coordinated in MG but not in uterus due to uterine-selective inhibition of the expression of the NRF-1 coactivators Ppargc1a and Ppargc1b by E2 and 4-OHT. E2 transiently increased NRF-1 and PGC-1α nuclear staining while reducing PGC-1α expression in uterus. E2, not 4-OHT, activates mt biogenesis in MG and uterus in a time-dependent manner. E2 increased mt outer membrane Tomm40 protein levels in MG and uterus whereas 4-OHT increased Tomm40 only in uterus. These data support the hypothesis of tissue-selective regulation of NRF-1 and its downstream targets by E2 and 4-OHT in vivo.

Key Words

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

Introduction

Uterus and mammary gland (MG) are classical estrogen target tissues in which estrogens, e.g. 17β-estradiol (E2), bind estrogen receptors α (ERα) and ERβ to regulate gene expression and physiological functions (Faulds et al. 2012). Studies in female ERα knockout (αERKO) and βERKO mice demonstrated that ERα is critical for MG formation and sexual maturation of the reproductive tract whereas ERβ is essential for ovarian function, but not for MG or uterus (Couse & Korach 1999). Although many tissue-specific phenotypes for αERKO, βERKO, and other αERKO mice have been reported (Pendaries et al. 2002, Korach et al. 2003, Billon-Galés et al. 2009), how estrogens...
regulate mitochondrial (mt) biogenesis and energy production by oxidative phosphorylation (OXPHOS) in uterus and MG 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 & 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 mt biogenesis and function in normal and cancer cells through nuclear-, mt-, plasma membrane-initiated events that have been reviewed (Yager & Chen 2007, Klinge 2008, Simpkins et al. 2008, 2010). 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 Rodrigo & 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 Rodrigo & Gustafsson 2011), and involve complex cross talk with multiple signaling pathways including other nuclear receptors, e.g. peroxisome proliferator-activated receptor α (PPARα) and PPARγ (Papi et al. 2013) and estrogen-related receptors (ERRs; Deblois & Gigueré 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 E2 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 NRF-1 in MCF-7 breast cancer cells (Mattingly et al. 2008, Ivanova et al. 2011). NRF-1 regulates the transcription of nuclear-encoded, mt genes, e.g. mt transcription factors Tfam, Tfβ1m, and Tfβ2m, and nuclear-encoded components of OXPHOS, e.g. cytochrome c (Cycs; Cam et al. 2004, Scarpulla 2006, 2008a, Richard 2011). In MCF-7 cells, E2-induced NRF-1 resulted in an increase in expression of TFAM and increased mt biogenesis (Mattingly et al. 2008). NRF-1’s activity depends on its interaction with coactivators PGC-1α, PGC-1β, and PRC (Scarpulla 2008a, 2012, Gleyzer & Scarpulla 2011, Richard 2011). Little is known about how estrogens or antiestrogens regulate the expression or activity of these coactivators in uterus or MG. One study reported that E2 reduced PGC-1α specifically in the uteri of immature and adult mice (Macari et al. 2010). Concordantly, ovariectomy increased PGC-1α in mouse brain endothelial cells (Kemper et al. 2013).

Most studies of the regulation of mt 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 & Kelly 2006, 2007, Scarpulla 2008b). There are no specific studies of how estrogens regulate mt biogenesis in MG and only one study in mouse uterus (Macari et al. 2010). Although microarray analysis of gene expression in mouse uterus identified many genes regulated by E2 and TAM (Moggs et al. 2004, Fong et al. 2007, 2010), 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 E2 treatment (Hewitt et al. 2012). Another study of uterine gene expression revealed lower Nrf1 in Src2−/− compared with WT adult mice (Jeong et al. 2007). NCOA2 (SRC-2) is a well-established coactivator for ERα and upregulated by E2 in adult uterus (Klinge 2000, Jeong et al. 2007, O’Malley & Kumar 2009). Microarray profiling of E2-regulated genes in aortas of WT, 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). E2–ERα increased whereas E2–ERβ reduced expression of Nrf1 and MRC genes in mouse aorta (O’Lone et al. 2007). Nrf1 was not included in a microarray profiling E2-regulated genes in mouse MG (Derou et al. 2009). Hence, how E2 and TAM regulate NRF-1, PGC-1 coactivators, NRF-1 regulated genes, and mt biogenesis in uterus and MG in vivo is unknown. Similarly, regulation of mt 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-1B while increasing PGC-1α in isolated brain endothelial cells from C57BL/6 mice (Kemper et al. 2013). Ovariectomy reduced the mt biogenesis ~20% and decreased the expression of MnSOD and ATP synthase subunit α, complex 5, suggesting that ovarian hormones normally stimulate mt biogenesis but suppress reactive oxygen species (ROS) in brain endothelium by inducing antioxidant 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 E2 and 4-OHT stimulate Nrf1 gene expression and
E2 and 4-OHT regulate NRF-1

M. M. IVANOVA and others

E2 and 4-OHT regulate NRF-1, its coregulators, and its downstream gene targets in mouse MG and uterus.

Materials and methods

Chemicals

E2, 4-OHT, and sesame oil were purchased from Sigma–Aldrich.

Antibodies

Antibodies were purchased as follows: ERα (HC-20) and Tomm40 (H-300), Santa Cruz Biotechnology; NRF-1, Rockland Immunocchemicals, 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), and 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 the 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-day quarantine, mice were randomized into three groups of five per group. Mice were given a single s.c. injection (between the shoulders) of vehicle control (sesame oil, 100 μl), E2 (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 0830 h followed by E2 at 0900 h, and 4-OHT at 1000 h 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 darkness) 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, 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 eight generations; i.e. 99.61% of the genome was contributed by C57BL/6). Mice were ovariectomized at 4–6 weeks of age and rested for 2 weeks. They were then i.p. injected with ethanol vehicle or 1 μg E2 (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 IHC

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 min as described previously (Balsitis et al. 2003). Non-specific antibody binding was blocked by incubating sections in 5% donkey serum (Santa Cruz Biotechnology) in PBS for 1 h. 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, Littleton, CO, USA) 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

RNA was extracted using the RNeasy Mini Kit from Qiagen. The High Capacity cDNA archive kit (PE Applied Biosystems) was used to reverse transcribe RNA using random hexamers. Supplementary Table 1, see section on supplementary data given at the end of this article lists gene and protein names and abbreviations. Murine mRNA transcript levels of Nrf1, Tfam, CycS, and Cox4 were analyzed using
SYBR Green dye (RT<sup>2</sup>SYBR Green ROX qPCR, Qiagen). Tfb1m and Tfb2m expression levels were analyzed by one-step quantitative real-time PCR (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. 2010). 18S rRNA primers and probes were purchased from Assays-on-Demand Gene Expression Products (PE Applied Biosystems). Murine Tfb1m and Tfb2m primers were from RealTimePrimers (Elkins Park, PA, USA). The mouse Ppargc1a, Tfam, cytochrome c, and 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<sub>2</sub>- 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 \( \Delta \Delta Ct \) 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 radioimmuno-precipitation 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).

**Western blot**

Western blotting followed standard procedures (Mattingly et al. 2008, Ivanova et al. 2011). In brief, 30–40 μg 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 normalized to the loading control, e.g.

![Figure 1](http://jme.endocrinology-journals.org/C209/2013/Society_for_Endocrinology/DOI:10.1530/JME-13-0051_PRINTED_IN_GREAT_BRITAIN_PUBLISHED_BY_BIOSCIENTIFICA_LTD/.)

**Figure 1**

E<sub>2</sub> and 4-OHT increase Nrf1 mRNA and NRF-1 protein expression in mouse mammary gland (MG) and uterus. Ovariectomized C57BL/6 mice were given a single s.c. injection of sesame oil (vehicle control), 100 ng E<sub>2</sub>, or 50 μg 4-OHT and were killed 6, 24, or 72 h post injection. (A and B) Q-RT-PCR analysis of Nrf1 mRNA level in 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 ± S.E.M. 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 vs vehicle control (Student’s t-test). Representative western blots are shown in Supplementary Figure 1, see section on supplementary data given at the end of this article.

Published by Bioscientifica Ltd.
β-actin or Ponceau S staining, and the normalized value of the control was set to 1 for comparison between separate experiments.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) was performed with the MAGnify Chromatin Immunoprecipitation System (Invitrogen) using the manufacturer’s protocol. Half of the uterus or the left inguinal MG 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. Lysed extracts (300 μg) 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 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 containing the mouse Nrf1 promoter (−962 bp) region with estrogen response element (ERE) were sense, 5'-GATCTCTGGGTTGGAGGTC-3' A; antisense, 5'-ATAATGCCCCACATGGCTG-3' (see diagram in Fig. 2A) and ‘close to ERE’ primers were sense, 5'-CTCTTAACCGCTGAGGCATC-3'; antisense, 5'-AGGGAGGGAGGAGATTTA-3'. The primers containing the Nrf1 intron 3 region with AP-1 and 1/2 ERE binding sites were sense, 5'-TTGTGACTGATACATGGTAATG-3' and antisense, 5'-CTCTTGGAAATAACTGACAGAAAT-3'. Real-time PCR was performed using 2 μl 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'-CAGGGCAAGGGAGCTAAC-3' and 5'-CAGGGGCCCCATGCAAATA-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.

**mt:nuclear DNA ratios**

The relative mt content of MG and uterus was determined using SYBR green qPCR measuring the ratio of mt encoded nicotinamide adenine dinucleotide dehydrogenase-5.
(mt-Nd5): nuclear-encoded cystic fibrosis (Cftr) (Bauerly et al. 2006). For nuclear DNA and mtDNA quantification, 10 and 0.1 ng DNA were used as template respectively (Bauerly et al. 2006). Each sample was analyzed in triplicate. Relative mt copy number to nuclear copy number was assessed by a comparative Ct method, using the following equation: 
\[ \Delta Ct_{\text{mitochondria/nuclear}} = Ct_{\text{mitochondria}} - Ct_{\text{nuclear}} \]. The fold-change relative to control animals was calculated using the following equation: 
\[ 2^{(-\Delta Ct_{\text{mitochondria/nuclear}})} \]. Values represent mean fold change ± S.E.M.

**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 (San Diego, CA, USA).

**Results**

**E2 and 4-OHT increase Nrf1 expression in both MG and uterus**

We previously reported that E2 and 4-OHT increase NRF-1 transcription in MCF-7 and T47D breast cancer cells and human umbilical vein endothelial cells (Mattingly et al. 2008, Ivanova et al. 2011, Mattingly & Klinge 2012), but the effect of E2 and 4-OHT on NRF-1 transcription in estrogen target tissues in vivo is unknown. The expression of Nrf1 was measured in the MG and uterus of ovariectomized C57BL/6 mice after treatment with vehicle (sesame oil), E2, or 4-OHT for 6, 24, or 72 h (Fig. 1A and B). Nrf1 transcript expression was increased by E2 in MG 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 MG and uterus 6 h after treatment but reduced Nrf1 in the MG, but not in the uterus, after 24 h. Nrf1 expression returned to basal in both MG and uterus 72 h after 4-OHT treatment. Basal Nrf1 transcript expressed was unchanged during the time course of treatment (Supplementary Figure 1).

**Tissue-specific regulation of NRF-1 protein expression by E2 and 4-OHT**

Commensurate with E2- 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. 1D) and Supplementary Figure 1). E2 increased NRF-1 protein in MG only 72 h after E2 treatment while 4-OHT was without effect (Fig. 1C and Supplementary Figure 1).

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

ChIP assays were used to investigate ERα binding to the mouse Nrf1 promoter. The location of the primers for ChIP are shown in Fig. 2A. These regions were chosen based on known or predicted EREs and other elements regulated

Figure 3

E2 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 ovariectomized mice treated with vehicle control, E2, or 4-OHT for the indicated time. (C and D) TFAM protein expression was examined in MG (C) and uterus (D) 72 h after E2 or 4-OHT treatment. The data shown are the mean ± S.E.M. of 5–10 mice/treatment group. \(* P < 0.05\) vs vehicle control (Student's t-test). Representative western blots are shown in Supplementary Figure 3, see section on supplementary data given at the end of this article.
by ER-tethering mechanisms (Supplementary Figure 2, see section on supplementary data given at the end of this article). E2 increased ERα recruitment to the ERE-containing region within the 5' promoter in MG and uterus 6 h after treatment (Fig. 2B and C). However, E2–ERα was not recruited to the 1/2 ERE/AP-1 site located in intron 3 in either MG or uterus (Fig. 2B and C). 4-OHT increased ERα recruitment to the ERE in both tissues and to the 1/2 ERE/AP-1 region (ChIP primer 1) in the MG. ERα recruitment corresponded to increased Nrf1 expression detected 6 h after treatment with E2 in both the MG and the uterus (Fig. 1A and B). 4-OHT–ERα was recruited to the 1/2 ERE/AP-1 binding site in the intron 3 region in the MG, but not in the uterus.

**E2 and 4-OHT increase selected NRF-1 target genes in MG**

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 MG 6 h after treatment with E2 and 4-OHT (Fig. 3A). The pattern of Tfam, Tfb1m, and Tfb2m gene expression was different in uterus. E2 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. 3B). While unchanged at 24 h, E2 increased Tfam and Tfb1m expression after 72 h in both MG and uterus (Fig. 3A and B). No increase in Tfb2m was detected in response to either E2 or 4-OHT in either MG or uterus (Fig. 3A and B). E2 and 4-OHT reduced Tfb2m in the uterus 24 h after treatment and 4-OHT reduced Tfbm2 in the MG 72 h after treatment (Fig. 3A and B).

**E2 and 4-OHT increase TFAM protein in MG and 4-OHT reduces TFAM protein in uterus**

E2 and 4-OHT increased TFAM protein in MG 72 h after treatment (Fig. 3C and Supplementary Figure 3). Reflecting the lack of induction of Tfam mRNA expression by E2 or 4-OHT in uterus (Fig. 3B), no increase in TFAM protein was detected in uterus 6 or 24 h after treatment or with E2 72 h after treatment (Fig. 3D and Supplementary Figure 3). Uterine TFAM protein levels were lower than control 72 h after 4-OHT-treatment. Concordantly, expression of mt encoded cytochrome c oxidase I (mt-Cox1) was significantly reduced by 4-OHT-treatment in uterus 24 and 72 h after treatment while no changes were detected in MG (Supplementary Figure 4).

**E2 activated and 4-OHT inhibited mt biogenesis**

TFAM is crucial for the initiation of mt transcription and DNA replication (Campbell et al. 2012). As E2 and 4-OHT increased TFAM protein in the MG, we examined the mt/nuclear DNA as an index of mt biogenesis. As recently reviewed (Kemper et al. 2013), mt:nuclear DNA ratios are a better marker of mt biogenesis than mt staining techniques. The mt:nuclear DNA ratio was determined by qPCR using primers for mt-Nd5 as a mitochondria gene and Cfr (cystic fibrosis) as a nuclear gene (Bauerly et al. 2006). Although E2 increased the mt:nuclear DNA ratio in MG at 6 h, this was not statistically significant until 24 h (Fig. 4A). 4-OHT decreased mt biogenesis in the MG after 72 h. In the uterus, E2 increased mt biogenesis 24 and 72 h after E2 treatment whereas 4-OHT had no effect (Fig. 4B). We conclude that E2 increased mt biogenesis in both MG and uterus, with a higher increase in uterus, which may
**E2 and 4-OHT regulate nuclear-encoded NRF-1 target genes Cox4 and CycS**

Cox4 and 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 MG with E2 treatment at 6 and 24 h whereas uterine expression was unaffected by E2 and inhibited by 4-OHT at all time points (Fig. 5A and B). Cox4 protein was increased in MG and uterus 72 h after E2 and 4-OHT treatment (Fig. 5C and D and Supplementary Figure 6A, see section on supplementary data given at the end of this article). 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 an 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 E2 in MG and decreased by 4-OHT in the uterus 72 h after

---

**Tissue-specific regulation of outer mt Tomm40 protein expression**

Because E2 increased the mt:nuclear DNA ratio at 24 h in MG and uterus, with more of an increase in uterus vs MG, while 4-OHT increased the mt:nuclear DNA ratio only in uterus (Fig. 4A), we examined the protein level of one component of the translocase of the outer mitochondrial membrane (TOM) complex that controls the transport of nuclear-encoded proteins into mitochondria as a representative outer mt membrane protein (Hoogenraad & Ryan 2001). Tomm40 is the pore-forming unit of the TOM complex (Hill et al. 1998). E2 increased Tomm40 protein levels in MG and uterus 72 h after treatment (Fig. 4C and D and Supplementary Figure 5), a result that correlates with E2-induced mt biogenesis at 24 h in these tissues, which would then be detected at the protein level, e.g. 72 h as measured here. 4-OHT increased mt biogenesis only in the uterus and not in MG after 24 h and likewise Tomm40 protein was increased only in the uterus after 72 h.

---

**Figure 5**

E2 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), E2, or 4-OHT for the indicated time. Values are the mean ± s.e.m. of 10–15 mice/treatment group. *P<0.05 vs vehicle control (Student’s t-test).

(C) COX4 and (D) cytochrome C protein expression was examined in MG and uterus 72 h after the indicated treatment. Values are the mean ± s.e.m. of 5–12 mice/treatment group. *P<0.05 vs vehicle control (Student’s t-test). Representative western blots are shown in Supplementary Figure 5, see section on supplementary data given at the end of this article.

---

**Figure 6**

E2 and 4-OHT increase Ppargc1b expression in mammary gland (MG) and reduce Ppargc1a and Ppargc1b expression in the uterus. Q-RT-PCR analysis of Ppargc1a and Ppargc1b expression in MG (A) and uterus (B) from ovex C57BL/6 mice treated with vehicle (control), E2, or 4-OHT for the indicated time. Values are the mean ± s.e.m. of 5–10 mice/treatment group. *P<0.05 vs vehicle control (Student’s t-test).
treatment (Fig. 5A and B). Cytochrome c protein levels did not change in MG of mice treated with either E2 or 4-OHT (Fig. 5D and Supplementary Figure 6B). However, E2 increased cytochrome c protein in uterus (Fig. 5C and Supplementary Figure 6B), a result in contrast to the lack of change in CycS mRNA (Fig. 5B).

**Expression of NRF-1 coactivators PGC-1α and PGC-1β in mouse MG and uterus**

PGC1-α and PGC1-β are essential for NRF-1 regulation of gene expression (Scarpulla 2006, 2008a). Ppargc1b was increased in MG 6 h after E2 and 4-OHT treatment (Fig. 6A). In uterus, Ppargc1a was reduced 6 h after 4-OHT treatment, and 24 and 72 h after E2 and 4-OHT treatment (Fig. 6B). Ppargc1b expression was reduced in uterus 24 h after 4-OHT treatment and 72 h after E2 and 4-OHT treatment (Fig. 6B). In conclusion, Ppargc1a expression was not regulated by E2 or 4-OHT in MG and decreased in uterus (Fig. 6A), consistent with the reports that E2 repressed PGC-1α in mouse uterus (Macari et al. 2010). GeoProfile GDS1058 showed Ppargc1c in uterus of ovex control, but not E2-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 E2 and 4-OHT in both MG 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 E2 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 E2 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 E2 for 24 and 48 h compared with vehicle. These results indicate that E2 enhances transient nuclear translocation of NRF-1 and PGC-1α in adult uterus. Our results also indicate that E2 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 mt biogenesis in normal mouse MG or uterus by E2 and 4-OHT. This is because Nrf1 was not included in microarrays previously used to identify E2- and TAM-regulated genes in mouse uterus (Moggs et al. 2004, Fong et al. 2007, 2010) and MG (Deroo et al. 2009). Earlier, we reported that E2 and 4-OHT increase NRF-1 transcription in breast cancer cells and HUVECs (Mattingly et al. 2008, Ivanova et al. 2011, Mattingly & Klinge 2012) and that E2 activates NRF-1 transcriptional activity and mt biogenesis in human breast cancer cells (Mattingly et al. 2008, Ivanova et al. 2011). Because NRF-1 is a master transcriptional regulator of genes regulating mt function (Scarpulla 2008a), we proposed a model that nuclear ER upregulation of NRF-1 which, in turn, stimulates the transcription of nuclear-encoded mt genes, thus coordinately regulates nuclear–mt transcription and function (Klinge 2008, Mattingly et al. 2008, Ivanova et al. 2011). In normal tissue, E2 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 mt biogenesis has been largely defined by studies in skeletal and cardiac muscle, liver, and brown fat (Finck & Kelly 2006, 2007, Scarpulla 2008b). This study demonstrates that E2 and 4-OHT increase Nrf1 mRNA and protein expression in a time- and tissue-specific manner in MG and uterus. Ppargc1a, Ppargc1b, and NRF-1-regulated nuclear-encoded genes are also regulated by E2 and 4-OHT in a tissue-specific manner as summarized Fig. 8. Although Nrf1 expression was increased by E2 and 4-OHT 6 h after treatment in both MG and uterus, NRF-1 protein was higher in uterus and E2 transiently increased nuclear NRF-1, and PGC-1α staining 4-OHT increased Nrf1 mRNA but not NRF-1 protein in MG. The discrepancy between Nrf1 mRNA and protein may result from differences in mRNA stability but will require further investigation. Our results for E2 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 MG appears to be more responsive than uterus to upregulation of NRF-1 target genes Tfb1m, Tfb2m, and Cox4 after E2 or 4-OHT treatment. Serial analysis of gene expression (SAGE) identified four OXPHOS genes Cox6a2, Mt-Co1, Mt-Co3, and Mt-Nd3, upregulated in MG 3 h after a single, s.c. injection of E2 (50 ng) in zERKO mice, allowing the authors to conclude that E2 regulates the expression of Cox6a2 and the mt encoded genes mainly through ERβ (Aboghe et al. 2009). Importantly, and commensurate
with our results that E2 increases TFAM in MG, all three mt encoded genes upregulated by E2 (MtCo1, MtCo3, and MtNd3 (Aboghe et al. 2009)) are regulated by TFAM (Scarpulla 2008b). Based on the results summarized in Fig. 8 and modeled in Fig. 9, we suggest that NRF-1 upregulates its target genes selectively in the MG at least in part because of the expression of Ppargc1b in MG. In contrast, uterine Ppargc1a and Ppargc1b expression are decreased with E2 and 4-OHT treatment. Our results agree with other reports showing that E2 repressed Ppargc1a transcription and reduced PGC-1α protein in mouse uterus (Macari et al. 2010) 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 that revealed increased ERα occupation of the ERE in the Nrf1 promoter in MG and uterus after E2 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 0.25 μg E2 injection of ovex C57BL/6J mice in this ChIP-seq analysis; however, the treatment time, route, and dose of E2 are different between our study and that from the Korach Laboratory.

Figure 9
Model of E2–ERα and 4-OHT–ERα regulation of Nrf1 transcription, NRF-1 regulation of its target genes, PGC-1 coactivator family expression, Tomm40, and mitochondrial biogenesis in mouse mammary gland (MG) and uterus. The E2–ERα-stimulated changes are shown in the top two cell models with MG on the left side and uterus on the right side. The 4-OHT–ERα-stimulated changes are shown in the bottom two cell models with MG on the left side and uterus on the right side. Of course, as 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.
Interestingly, ERz was recruited to the Tfam gene with E2 treatment in mouse uterus (Hewitt et al. 2012), commensurate with the increase in Tfam mRNA expression with E2 in mouse uterus seen 72 h after treatment (Fig. 3B). The ERz occupation of AP-1/2 ERE site in Nrf1 intron 3 in MG 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 ERs with AP-1 (Paech et al. 1997, Cerillo et al. 1998, Heldring et al. 2011), but further experiments will be required to dissect the molecular mechanisms involved. ChIP-seq demonstrated ERz bound to the first intron of the Wnt4 and Cdkn1 genes in E2-treated mouse uterus and upregulated expression (Hewitt et al. 2012), establishing the ability of intron-bound ERz to regulate gene transcription.

Although TFAM, Tfb1m, and Tfb2m are essential for mt gene transcription and DNA replication (Scarpulla 2008a), their regulation by E2 and 4-OHT did not completely match effects of E2 and 4-OHT on mt biogenesis. E2 increased mt biogenesis in the MG and uterus after 24 h, in agreement with increases in Tfam and Tfb1m only in the MG. Although 4-OHT increased Tfam and Ppargc1b in MG, the mt biogenesis was not increased. In fact, after 72 h treatment, 4-OHT reduced MG mt 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 pro-apoptotic activity of TAM in MG (Kotoula et al. 1993).

A summary of our findings is diagramed in Fig. 8 and modeled in Fig. 9. E2 and 4-OHT increase in vivo ERz recruitment to the Nrf1 gene and regulate NRF-1 signaling in time- and tissue-dependent manner in mouse MG and uterus. NRF-1 activated more of its target genes in MG than in uterus after E2 or 4-OHT treatment, whereas more genes were downregulated by E2 and 4-OHT in the uterus. These data are congruent with a report where only ten genes were regulated by E2 in mouse MG (Aboghe et al. 2009) whereas in mouse uterus, E2 altered the expression of 3538 genes (Mogggs et al. 2004). The increase in NRF-1 target gene Tfam in MG and uterus 6 h after E2 and 4-OHT treatment was different from the time-delayed increase in TFAM expression detected after E2, not 4-OHT, treatment of ERz-expressing MCF-7 breast cancer cells (Ivanova et al. 2011), reflecting a report that in vivo uterine gene regulation by E2-ERz is different from MCF-7 cells and may reflect direct recruitment of liganded ERz to the mouse Tfam promoter (Hewitt et al. 2012). In conclusion, our data demonstrate that E2 and 4-OHT differentially regulate NRF-1 and its downstream gene targets in MG and uterus with a stronger impact of E2 on stimulating mt biogenesis in uterus than MG.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-13-0051.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported by NIH R01 DK053220 to C M K.

Acknowledgements
The authors thank Dr Numan Al-Rayyan and Kathleen Gajewski for their help in collecting mouse tissues and confocal microscopy respectively. They also 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. They thank Dr Barbara J Clark for her thoughtful suggestions for this manuscript.

References


Couse JF & Korach KS 1999 Estrogen receptor null mice: what have we learned and where will they lead us? Endocrine Reviews 20 338–417. (doi:10.1210/er.20.3.338)


Received in final form 17 July 2013
Accepted 26 July 2013
Accepted Preprint published online 26 July 2013