Published in final edited form as:
*Chem Res Toxicol*. 2010 August 16; 23(8): 1374–1383. doi:10.1021/tx100129h.

# Unexpected hormonal activity of a catechol equine estrogen metabolite reveals reversible glutathione conjugation

**Kuan-Wei Peng**[a,1], **Minsun Chang**[b,1], **Yue-Ting Wang**[a,1], **Zhican Wang**[a], **Zhihui Qin**[a], **Judy L. Bolton**[a], and **Gregory R. J. Thatcher**[a,*]

[a]Department of Medicinal Chemistry and Pharmacognosy (M/C 781), College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, Chicago, Illinois 60612-7231

[b]Division of Biological Sciences, College of Science, Sookmyung Women's University, Hyochanwon Road 52, Yongsan-gu, Seoul 140-742, South Korea

## Abstract

4-Hydroxyequilenin (4-OHEN) is a major phase I metabolite of the equine estrogens present in widely prescribed hormone replacement formulations. 4-OHEN is autoxidized to an electrophilic *o*-quinone that has been shown to redox cycle, generating ROS, and to covalently modify proteins and DNA and thus potentially to act as a chemical carcinogen. To establish the ability of 4-OHEN to act as a hormonal carcinogen at the estrogen receptor (ER), estrogen responsive gene expression and proliferation were studied in ER(+) breast cancer cells. Recruitment by 4-OHEN of ER to estrogen responsive elements (ERE) of DNA in MCF-7 cells was also studied and observed. 4-OHEN was a potent estrogen, with additional weak activity associated with binding to the arylhydrocarbon receptor (AhR). The potency of 4-OHEN towards classical ERα mediated activity was unexpected given the reported rapid autoxidation and trapping of the resultant quinone by GSH. Addition of thiols to cell cultures did not attenuate the estrogenic activity of 4-OHEN and pre-formed thiol conjugates added to cell incubations only marginally reduced ERE-luciferase induction. On reaction of the 4OHEN-GSH conjugate with NADPH, 4-OHEN was observed to be regenerated at a rate dependent upon NADPH concentration, indicating that intracellular non-enzymatic and enzymatic regeneration of 4-OHEN accounts for the observed estrogenic activity of 4-OHEN. 4-OHEN is therefore capable of inducing chemical and hormonal pathways that may contribute to estrogen-dependent carcinogenesis, and trapping by cellular thiols does not provide a mechanism of termination of these pathways.

### Keywords

Equine estrogen metabolite; estrogen receptor α; estrogen response element; estrogen carcinogenesis; glutathione conjugate

## Introduction

There is a clear association between prolonged exposure to estrogens and increased risk of breast and uterine cancer through early menarche, late menopause, and during hormone replacement therapy (HRT) (1-4). The Women's Health Initiative (WHI) study demonstrated increased breast cancer incidence due to long term use of HRT (5,6). Remarkably, the recent

*Corresponding authors. Department of Medicinal Chemistry and Pharmacognosy (M/C 781), College of Pharmacy, University of Illinois at Chicago, 833 South Wood Street, Chicago, Illinois 60612-7231. Fax: 312 996 7107, thatcher@uic.edu.
[1]Contributed equally to this work.

Supporting Information Available. This information is available free of charge via the Internet at http://pubs.acs.org/.

analysis from the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) registries suggested a correlation between discontinuation of HRT and a decreased incidence of breast cancer (7). In the absence of safer, efficacious alternatives to HRT, conjugated equine estrogens remain the most popular HRT agents in North America.

Both hormonal and chemical pathways are likely to contribute to estrogen induced carcinogenesis. The prevalent hormonal mechanism is thought to be classical estrogen receptor (ER) mediated signaling of cell proliferation (4,8), promoting carcinogenesis through tumor cell growth and mitosis (9). Chemical carcinogenesis results from oxidative metabolism of estrogens to catechols and *o*-quinones, which has been shown to cause both direct and indirect genotoxic effects (4,10). The catechol metabolite, 4-hydroxyestradiol (4-$OHE_2$), can be oxidized to an *o*-quinone that reacts with DNA, potentially yielding mutagenic apurinic sites (11). 4-$OHE_2$ has also been reported to possess hormonal estrogenic activity (12,13), for example, 4-$OHE_2$ (10 nM) induced estrogen sensitive genes and cell proliferation in MCF-7 cells (14,15). Therefore, 4-$OHE_2$ has the potential to mediate hormonal and chemical carcinogenesis.

4-Hydroxyequilenin (4-OHEN) is the major phase I metabolite of the equine estrogens that are present in the widely prescribed HRT agents Prempro® and Premarin® (16). In contrast to 4-$OHE_2$, 4-OHEN autoxidizes to an *o*-quinone that is reactive towards cellular proteins and DNA, both by alkylation/arylation and through generation of ROS leading to oxidation. This enhanced reactivity is argued to underlie the greater cytotoxicity and genotoxicity of 4-OHEN relative to 4-$OHE_2$. Moreover, DNA damage, generation of ROS, and cytotoxicity from 4-OHEN were observed to be enhanced in ERα(+) relative to ER(-) breast cancer cells (17,18). The rapid localization of ROS in the nucleus of ER(+), but not ER(-) cells, in response to 4-OHEN treatment, suggested that 4-OHEN is a ligand for ERα, since the receptor is estimated to be 85% localized in the nucleus (19). However, weak binding of 4-OHEN to isolated ERα was reported using radioligand displacement (binding affinity relative to $E_2$ = 0.02 and $IC_{50}$ = 1.5 μM (17). These observations are not entirely incompatible, since 4-OHEN autoxidation to the labile quinone electrophile should shift the binding curve to the right; whereas, 4-$OHE_2$ that does not autoxidize to a quinone is a high affinity estrogen.

The chemical and hormonal differences between human and equine estrogens have been relatively neglected in understanding the harmful side effects of HRT agents commonly taken by women. On the basis of the chemical differences between the oxidative catechol metabolites, 4-$OHE_2$ and 4-OHEN, it was predicted that in cell culture, 4-OHEN would autoxidize to the quinone, be trapped by GSH, and thereby act as a relatively weak estrogenic hormone. In contrast, 4-OHEN was observed to be a full classical estrogen towards ER-mediated gene transcription and cell proliferation. Importantly, trapping of the quinone as a GSH conjugate was observed to be reversible in the presence of NAD(P)H. Intriguingly, the normal role of GSH conjugates in detoxification, has been perverted by the chemistry of 4-OHEN to yield an NAD(P)H activated ERα ligand that acts as a full classical estrogen, in that it induces proliferation and estrogen-sensitive genes.

## Materials and methods

### Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Itasca, IL) unless stated otherwise. ICI 182,780 (ICI) was purchased from Tocris (Ellisville, MO). $E_2$ and ICI were dissolved in ethanol and stored at -20 °C. 4-OHEN was synthesized by treating equilin with Fremy's salt as described previously with minor modifications (20). 4-OHEN stock solutions were freshly prepared in DMSO or acetonitrile.

### Cell culture conditions

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless stated otherwise. MCF-7 human breast cancer cells were obtained from American Type Culture Collection and were maintained in RPMI 1640 containing 10% fetal bovine serum (Atlanta Laboratory, Atlanta, GA), 0.1 mM nonessential amino acids, and 6 μg/ml bovine insulin. Estrogen-free media were prepared by supplementing 3× dextran-coated charcoal-treated fetal bovine serum to phenol red-free RPMI 1640 media, whereas other components remained the same. S30 cells (a gift from Dr. V. C. Jordan) were derived from MDA-MB-231 human breast cancer cells by stable transfection of cDNA encoding the wild-type ERα (21) and maintained in phenol red-free MEM supplemented with 10% 3× dextran-coated charcoal-treated fetal bovine serum, 0.5 mg/ml geneticin, and concentrations of glutamine, non-essential amino acids, and insulin the same as those for MCF-7 cells. MCF-7 K1 cells were a kind gift from Dr. J. Frasor (University of Illinois at Chicago) and are shown to have higher level of ERα expression than the parent MCF-7 cells suitable for a chromatin immunoprecipitation (ChIP) assay. These cells were maintained in minimum essential medium (MEM) supplemented with 2% penicillin- streptomycin-fungizome, 2% glutamax, 5% calf serum (Hyclone), and 29.3 ml of 7.5% sodium bicarbonate solution. Cells were incubated in a humidified atmosphere with 5% $CO_2$ at 37 °C

### Transient transfection and luciferase assay

MCF-7 cells were cultured in estrogen-free media for four days before transfection. The cells were transfected with 2 μg of the pERE-luciferase plasmid, which contained three copies of the *Xenopus laevis* vitellogenin A2 ERE upstream of fire fly luciferase (22). To normalize transfection efficiency, pRL-TK plasmid (1 mg/plate, Promega, Madison, WI) which contained a cDNA encoding *Renilla* luciferase was co-transfected. In some experiments, pGudLuc1.1 (a gift from Dr. Michael S. Denison, UC Davis) containing four copies of the xenobiotic response element (XRE) upstream of fire fly luciferase was transfected. Cells ($5 \times 10^6$) in serum-free media were transfected by electroporation in a 0.4-cm cuvette (Bio-Rad Laboratories, Hercules, CA) at a voltage of 0.32 kV and a high capacitance of 950 mF in a GenePulser X-cell (Bio-Rad Laboratories). Cells were transfected with pERE-luciferase plasmid and treated with various compounds in different conditions as follows. In the inhibition of COMT experiment, cells were pre-treated with or without Ro 41-0960 (COMT inhibitor, 10 μM) for 24 h, and then treated with different concentrations of $E_2$, 4-OHEN, and 4-$OHE_2$ for 18 h. To study the effect of additional thiol agents on 4-OHEN mediated ERE-luciferase activity, two different experiments were performed. In one experiment MCF-7 cells were treated with different concentrations of thiols (*N*-acetyl cysteine (NAC), *N*-acetyl penicillamine (NAP)) used to trap 4-OHEN quinone for 30 min before a 18 h treatment of 10 nM 4-OHEN or 1 nM $E_2$. In the other experiment, thiols (NAC or glutathione (GSH)) were incubated with 4-OHEN (10 nM) first, the resulting mixture was then used to treat cells for 18 h. The luciferase activities in cell lysates were measured using Dual Luciferase assay system (Promega) with a FLUOstar OPTIMA (BMG LABTECH, Durham, NC). Data were reported as the relative percentage values compared to those obtained with 1 nM $E_2$ treatment set as 100% and initial luciferase activity values were calculated by dividing the firefly luciferase (ERE-luciferase) reading by the *Renilla* luciferase reading.

### Transient transfection of siRNAs

siRNA duplexes were prepared by Dharmacon Research (Lafayette, CO). Each siRNA was prepared with TT overhang to the end of 3′ position. The sense sequence (5′ to 3′) of siRNA duplexes for aryl hydrocarbon receptor (siAhR) was UAC UUC CAC CUC AGU UGG C (nucleotide position 774 to 792), CGU ACG CGG AAU ACU UCG A for firefly luciferase (siLuc; nucleotide positions 153 to 171), and CGCGCUUUGUAGGAUUCG for scrambled

inhibitory RNA (siSC; nucleotide positions 24750 to 24768). The sequence for siSc was derived from a message transcribed from the chloroplast genome of *Euglena gracilis*. Cells were in stripped medium for 4 days and were seeded at a density of $8 \times 10^5$ cells/well in 6-well plates. siRNA duplexes (100 nM/well), pERE-Luc plasmid (0.5 μg/well), and pRL-TK plasmid (0.5 μg/well) were transfected using Lipofectamin 2000 reagent (Invitrogen) and incubated for 24 h. Cells were treated with the appropriate compounds for another 24 h. Luciferase activity relative to *Renilla* luciferase activity was determined as described above.

### Chromatin immunoprecipitation assay (ChIP)

Cells were cultured in estrogen-free media for 4 days before treatment. Cells were treated with DMSO, $E_2$, 4-OHEN, or 4-$OHE_2$ for 45 min and then cells were washed and crosslinked by 1.5% formaldehyde for 15 min. ChIP assays were performed as previously reported (Wang *et al.*, 2009). Briefly, cells were lysed with SDS cell lysis buffer containing protease inhibitor (Roche, Indianapolis) followed by sonication. Samples were pre-cleared by protein A/G agarose slurry to prevent non-specific binding. Samples were incubated with either ERα antibody (1 μg) or IgG antibody (1 μg) overnight at 4 °C and then were added with protein A/G agarose slurry (40 μL) for 2 h at 4 °C to pull-down complexes. After centrifugation at 5,000 g for 1 min, the pellet containing the pull-down complexes was washed with 300 μL low salt buffer, 300 μL high salt buffer, and 300 μL TE buffer at least 3 times for 5 min at room temperature and then centrifuged at 5,000 g for 1 min. After bead pellets were eluted by 50 μL elution buffer, all samples including inputs were decrosslinked for 16 h at 65 °C and DNA was purified using QIAquick PCR purification kit. PCR experiments were performed to detect DNA fragments containing the estrogen sensitive gene (*pS2* ERE sequence). The *pS2*-ERE forward and reverse primers sequence (5′ to 3′) were GACGGAATGGGCTTCATGA and AGTGAGAGATGGCCGGAAAA, respectively. The *pS2*-upstream forward and reverse primers sequence (5′ to 3′) were GGGTCTCAGTGGTGGCAGTA and ACCGCTCATACCATCCAGTC, respectively.

### Total RNA isolation and quantitative PCR analysis

MCF-7 cells grown in estrogen-free media for 4 days were treated with the compounds as indicated. Total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed with Molomey murine leukemia virus reverse transcriptase using TaqMan® reverse transcription reagents kit (Applied Biosystems, Foster City, CA). Depending on the target gene, 1 or 2 μL of reaction mixture was used in real-time quantitative PCR. The PCR and subsequent analyses were performed using the GeneAmp 5700 Sequence Detection system (Applied Biosystems). Probes and primers were designed using Primer Express Software (Applied Biosystems) and synthesized by Applied Biosystems. *pS2* forward and reverse primers and probe sequences (5′ to 3′) were CGTGAAAGACAGAATTGTGGTTTT, CGTCGAAACAGCAGCCCTTA, and TGTCACGCCCTCCCAGTGTGCA, respectively. Progesterone receptor (*PR*) forward and reverse primers and probe sequences (5′ to 3′) were AGAGCACTGGATGCTGTTGCT, GCTTAGGGCTTGGCTTTCATT, and TCCCACAGCCATTGGGCGT, respectively. TGF-α forward and reverse primers and probe sequences (5′ to 3′) were GTTTTTGGTGCAGGAGGACAA, CACAGCGTGCACCAACGT, and CCAGCATGTGTCTGCCATTCTGGG, respectively. 18S rRNA Taqman® Pre-Developed Assay Reagent Control (Applied Biosystems) was used as an internal control. All probes were labeled with 6-carboxyfluorescein (FAM) and TAMRA was used as 3′-quencher in all cases. The PCR mixture contained TaqMan® Universal master mix (Applied Biosystems) and different concentrations of primers and probes depending on the genes subject to analysis. Real-time quantitative PCR consisted of 1 cycle of 50 °C for 2 min and 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The fluorescence signal was measured during the last 30 s of the annealing/

extension phase. The quantification of gene expression was related to a standard curve derived using RNA samples from vehicle control-treated cells. Data normalization was performed by dividing the expression level of a specific gene by that of 18S rRNA. Finally, results were expressed as fold-induction where the gene expression level in vehicle control-treated cells was set as 1.

### Cell proliferation

MCF-7 cells were seeded at $1.5 \times 10^4$ cells/well in 24-well plates after being cultured in estrogen-free media for 4 days. Cells were treated with DMSO, $E_2$, 4-OHEN, 4-$OHE_2$, ICI, and appropriate combination of two compounds every other day for 6 days in triplicate. The cell growth was determined by measuring the total DNA amount in each well (23). Briefly, cells were lysed with 100 μL/well 1× passive lysis buffer (Promega) and the lysates were resuspended in 400 μL/well 0.1 × PBS followed by sonication. DNA content was detected with a 96-well fluorescence reader (BMG Laboratories) using an excitation filter of 360 - 390 nm and an emission filter of 450 - 470 nm, after 30 μL of cell lysate was incubated with 200 μL of 2 μg/mL of Hoechst 33058 (Bio-Rad Laboratories) for 1 h at room temperature. The DNA amount in each sample was quantitated using calf thymus DNA as a standard.

### Preparation and purification of mono-GSH 4-OHEN-o-quinone Conjugate

A freshly made stock solution of 4-OHEN in acetonitrile (2 mM) and a fresh GSH stock solution (21 mM) in PBS were added into PBS (50 mM, pH 7.4) to achieve final concentrations of 0.1 mM and 10 mM respecitvely. The resulting solution was incubated at 37 °C for 30 min then immediately subjected to analysis using an Agilent 6310 ion trap spectrometer (Agilent Technologies, Santa Clara, CA) equipped with a model 1100 HPLC system and eletrospray ionization source. The compound of interest 4-OHEN mono-GSH conjugate was identified in positive ion detection mode as $[M + H]^+$ in both reduced form (m/z 588) and oxidized form (m/z 586). It was isolated from the reaction mixture by reverse phase HPLC (Shimadzu, Japan) using a Advantage LANCER C18 column (5 μm, 4.6 × 150 mm) with UV absorbance detection at 250 and 280 nm. The mobile phase was composed of a mixture of water with 0.1% (v/v) formic acid and 10% (v/v) MeOH as solvent (A), and acetonitrile with 0.1% formic acid as solvent (B). A constant flow rate was maintained at 1 mL/min. Elution was initiated with 5% B, held for 5 min, increased with a linear gradient to 60% B in 10 min and then to 90% B in 5 min, held for 5 min before decreasing to 5% B in 3 min and equilibrating at 5% B for another 2 min. The fraction of mono GSH conjugate was collected and stored at -20 °C as a stock solution for further use.

### NADPH reduction of mono-GSH 4-OHEN-o-quinone conjugate

Reaction between 4-OHEN mono-GSH conjugate and NADPH was studied by LC-MS. Solutions containing 4-OHEN mono-GSH conjugate (ca. $5 \times 10^{-3}$ mM in reduced form) and NAPDH (0.5 mM, 1 mM, 5 mM) in 50 mM PBS (pH 7.4) were combined to a total volume of 1 mL and immediately subjected to LC-MS analysis. The reaction was monitored by mass spectrometry and HPLC-UV at 4 wavelengths (210 nm, 230 nm, 250 nm, 280 nm) maintaining temperature at 28-29 °C in an autosampler. 4-OHEN generated during the course of the reaction was characterized by ESI-MS in positive ion mode as its oxidized form (m/z 281). Observed rate constants for the disappearance of mono-GSH conjugate (reduced form) and the formation of 4-OHEN were obtained from HPLC peak area vs. time data collected at 250 nm by fitting the data with a pseudo-first order rate equation. Self-decomposition of 4-OHEN mono-GSH conjugate in aqueous solution was also monitored under the same conditions but in the absence of NADPH. The LC conditions were described as above. The concentrations of 4-OHEN and 4-OHEN mono-GSH conjugate in reaction mixture were calculated or estimated based on the HPLC response factors of 4-OHEN. All

experiments were performed in duplicate within 3 days after the purification of mono-GSH conjugate.

### Quantification of equilin and equilenin in 4-OHEN

The contamination resulting from equilin (EQ) and/or equilenin (EN) were determined using positive mode electrospray ionization on API 3000 (Applied Biosystem, Forster City, CA) triple quadrupole mass spectrometer equipped with Agilent 1200 HPLC (Agilent Technologies) based on the calibration curves for dansyl-derivatized EQ and EN standards. The calibration standard compounds and 4-OHEN used for analysis were derivatized by dansyl chloride using Xu's procedure (24). Briefly, dansyl chloride solution (1 mg/1 mL in acetone) was mixed with analyte solution in pH 10 sodium bicarbonate buffer. After vortexing the resulting solution was incubated at 60 °C for 5 min to form the corresponding dansyl derivatives. Calibration curves for the range of dansylated EQ and EN concentrations from 50 pM to 500 nM were obtained (Figure S1 in Supporting Information). The detection limits for dansyl-EQ and dansyl-EN by LC-MS/MS were determined by seven replicate injections of calibration standards and calculated by the following equation: MDL = $t_{99\%} \times S_{(n = 7)}$, $t_{99\%}$ is Student's t at 99% confidence intervals ($t_{99\%, n = 7} = 3.143$) and S is the standard deviation of seven samples. The volume of each injection was 5 μL. The limits of detection for dansyl-EQ and dansyl-EN are, respectively, 251 pg and 625 pg. The MS and LC conditions were as follows. Multiple reaction monitoring (MRM) channels of 502/171 and 500/171 were set to detect EQ and EN. The ionspray voltage was 4.5 kV, the source temperature was 350 °C, the nebulizer gas was 12 instrument units, the curtain gas was 8 units, collision gas 5 units. The focusing potential for EQ and EN were 360 and 370 V, the declustering potential for EQ and EN were 86 and 81 V, the collision energy for EQ and EN were 49 and 53 V. HPLC was performed using a Xbridge™ C18 (3.5 μm, 3.0 × 100 mm) column (Waters Corporation, Milford, MA) at a flow rate of 0.3 mL/min. The elution solvent consisted of water with 10% MeOH and 0.1% formic acid (A) and MeOH with 0.1% formic acid (B). The mobile phase was initially composed of solvent A/solvent B (45/55), held for 5 min, then a linear gradient of B from 55 to 95% in 10 min, holding at 95% solvent B for 5 min then 5 min gradient of B from 95% to 55%. The column was re-equilibrated with a mobile phase composed of 45/55 A/B for 7 min before the next injection.

### Statistics

The data are reported as the mean ± s.d. or s.e.m. of triplicate or duplicate experiments. Statistical comparisons between control and treated groups were performed using either Student's t-test or one-way ANOVA. Differences in results among data sets were considered statistically significant when $p < 0.05$ unless stated otherwise.

## Results

### 4-OHEN is a full classical estrogen agonist in cell culture

MCF-7 cells were transiently transfected with an ERE-luciferase reporter plasmid and luciferase activity was determined after 18 h treatment, 4-OHEN being shown to act as a full classical estrogen with potency an order of magnitude less than the comparable 4-$OHE_2$ catechol (Fig. 1; Table 1). 4-OHEN is metabolized to 4-MeOEN by the actions of the phase II enzyme, COMT (25). Since 4-MeOEN was shown to be a full classical estrogen in cell culture (26), the influence of the COMT inhibitor, Ro 41-0960, was studied, and was shown to be without effect, demonstrating that the estrogenic activity of 4-OHEN was not due to formation of 4-MeOEN. Under these conditions, it was previously shown that Ro 41-0960 (10 μM) was not cytotoxic and reduced COMT activity by 87% and 90% at 6 h and 24 h, respectively (27). The pure antiestrogen, ICI, completely abolished the luciferase activity indued by 4-OHEN, 4-$OHE_2$, and $E_2$ (Fig. 1B), showing that the activity of these estrogens

was mediated by ER. These data suggest that 4-OHEN is an ER agonist towards classical ER/ERE-mediated transcription with nanomolar potency in MCF-7 cells.

Confirmation of the ability of 4-OHEN to induce endogenous estrogen-sensitive gene products was obtained by measuring mRNA for trefoil factor (*pS2*) and progesterone receptor (*PR*) in MCF-7 cells after 24 h exposure to test compounds (28-30): 4-OHEN (100 nM) was equiefficacious with $E_2$ (10 nM) (Fig. 2); and, $EC_{50}$ for 4-OHEN could be estimated at 30 nM for *pS2* expression with lower potency for PR expression. Once again, ICI treatment completely abolished gene expression induced by $E_2$ and 4-OHEN. To confirm the generality of these phenomena beyond MCF-7 cells, gene expression was measured in S30 cells, measuring mRNA for the estrogen-responsive transforming growth factor *a* (*TGF-a*) (28,29). 4-OHEN again acted as a full classical estrogen, antagonized by ICI.

Proliferation of MCF-7 cells is another well-characterized response to estrogen agonists (31,32). Cellular DNA was measured after treatment of cells for 6 days showing the expected picomolar potency of $E_2$ itself and again demonstrating that 4-OHEN is an estrogen with potency of $10^{-9}$ to $10^{-8}$ M (Fig. 3). In comparison, the cell proliferation data for 4-$OHE_2$ were similar to those reported in kidney tubule cells (13), with 4-$OHE_2$ marginally more potent than 4-OHEN. While both 4-$OHE_2$ and 4-OHEN induce cell proliferation, their potency for cell proliferation is about 1/300th that of $E_2$ whereas their potency for inducing reporter gene expression is about 1/100th that of $E_2$. It is now well accepted that classical estrogenic signaling leading to gene transcription is dependent on cellular context, uses a variety of co-regulators, and may be mediated by receptor elements in addition to ERE (33). In addition, extranuclear ER signaling may lead to genomic and non-genomic actions (34,35). Therefore, the potency of ER agonists may be variable for gene expression and functional effects (36,37).

In order to confirm that catechol estrogen activation of ERE-reporter was mediated by a ligand-activated ER-ERE complex, a ChIP assay was performed to pull down the ER-ERE complexes using an ERα antibody and primers specific for the ERE promoter region of the *pS2* gene. MCF-7.K1 ERα (+) cells were treated with DMSO, $E_2$ (10 nM), or different concentrations of 4-OHEN and 4-$OHE_2$. To detect the *pS2* ERE sequence, both *pS2* ERE and *pS2* upstream primers were designed; the latter 4,400 bp upstream of the *pS2* ERE sequence, and therefore suitable as a negative control. It was observed that the *pS2* gene could be selectively pulled down by the ERα antibody and not by IgG after treatment with $E_2$, 4-$OHE_2$, or 4-OHEN. This was confirmed by detecting the DNA fragments containing the *pS2* ERE sequence, which were the products of PCR performed with *pS2* ERE primers and template DNA eluted from the DNA-protein complexes (Fig. 4). When primers for the upstream sequence of the *pS2* gene were used in the PCR experiments, no PCR products were detected. These results revealed that 4-OHEN and 4-$OHE_2$ could activate ERα and specifically bind to the ERE promoter region of this estrogen-responsive gene.

## 4-OHEN is a ligand for the aryl hydrocarbon receptor (AhR) but does not activate ERE via AhR

Polyaromatic small molecules bind to AhR that is known to modulate estrogenic activity through AhR-ER crosstalk (38). Liganded AhR translocates to the nucleus, recruits AhR nuclear translocator (Arnt), and binds to DNA, activating XRE, however, it has also been proposed that liganded AhR can recruit unliganded ER to form an AhR-ER complex that binds to ERE and stimulates transcription of ER target genes (39,40). Potentially, polyaromatic 4-OHEN could act as a ligand for AhR, leading to expression of ERE-dependent gene products. This hypothesis was tested by measuring transcriptional activity elicited by 4-OHEN at the XRE-promoter, which would require 4-OHEN binding to AhR.

Induction of XRE-luciferase activity by the AhR agonist, 3-methylcholanthrene (3-MC; 10 nM), was observed to be equivalent to 4-OHEN (1 μM) (Fig. 5A), demonstrating that 4-OHEN is a novel ligand for AhR. Although a weaker AhR agonist than 3-MC, the observed potency of $10^{-8}$ M for 4-OHEN is in the range observed for estrogenic activity, therefore it was necessary to investigate AhR-ER crosstalk. Knockdown of AhR signaling was effected by use of siRNA for AhR, using as controls, siRNA for luciferase, and scrambled siRNA (*E. gracilis* as a control for RNA interference performance). The induction of ERE-reporter gene transcription activity by 3-MC was inhibited by AhR knockdown (Supplemental Information), however, induction by 4-OHEN was not affected by attenuating the expression of AhR with siRNA (Fig. 5B). These data do not provide support for induction of estrogenic activity by 4-OHEN through AhR binding and agonism.

### Thiol trapping of 4-OHEN quinone does not block the estrogenic activity of 4-OHEN

MCF-7 cells were pre-incubated with three different small molecule thiols at various concentrations: i) NAC as a cell permeable nucleophile and reductant; ii) NAP as a cell permeable reductant, but hindered nucleophile; and iii), GSH as the biologically relevant cellular nucleophile and reductant. Classical ER-mediated genomic activity was measured using the ERE-luciferase reporter after incubation with 4-OHEN, or as a control $E_2$, for 24 h (Fig. 6A). In short, no differences were observed between the thiols studied, because exogenous thiol did not attenuate the estrogenic activity of 4-OHEN. This observation was contrary to the expectation that trapping of the 4-OHEN quinone by cellular thiol would significantly shift the dose-response for ER-mediated activity to the right. Therefore, 4-OHEN was pre-incubated with thiol under aerobic conditions, prior to addition to cell culture. HPLC-UV and LC-MS/MS analysis demonstrated that the expected GSH conjugate was rapidly formed, in addition to other minor products, and that the signals from 4-OHEN and its quinone were lost after addition of thiol (Fig. 7A); similar observations were made with NAC. Remarkably, pre-incubation of 4-OHEN with GSH or NAC prior to incubation in cell culture led to a reduction in estrogenic activity of only 10 to 15%, even at millimolar thiol concentrations (Fig. 6 B,C). One possible explanation for these unexpected observations was that the potent estrogens, equilin (EQ) and/or equilenin (EN) (41,42), were carried through in synthesis of 4-OHEN. In order rigorously to exclude this possibility, an LC-MS/MS method was developed to quantify EQ and EN as their dansyl derivatives (24). Based upon calibration curves generated from authentic samples, EQ and EN were measured at 50 pM and 4 pM, respectively, in a 10 nM solution of 4-OHEN. With reference to the published activity of EQ and EN towards ERE-luciferase induction in MCF-7 cells, these trace amounts cannot explain observed estrogenic activity (43).

### Thiol conjugation of 4-OHEN is reversible in the presence of NADPH

Regeneration of 4-OHEN after thiol trapping of the quinone would represent a mechanism by which 4-OHEN incubation with thiol would not significantly attenuate ERE-luciferase induction in cell culture. The 4-OHEN GSH conjugate was synthesized from the reaction of 4-OHEN with a hundredfold excess of GSH in pH 7.4 PBS. The mono-GSH conjugate was identified by MS and gave a single peak in the HPLC-UV chromatogram in the presence of GSH; in simile with previous observations, this conjugate was assumed to be the 2-sulfanyl 4-OHEN conjugate (44). Conjugate purified from excess thiol was reinjected into the LC-MS showing two peaks for: (i) the quinone form of the conjugate (**4** m/z 586, $\lambda_{max}$ = 280 nm); and (ii) the catechol form of the conjugate (**3** m/z 588, $\lambda_{max}$ = 250 nm). Reaction of the conjugate with NADPH was monitored by LC-MS and quantified by HPLC with UV detection as a function of time (Fig. 7 A, B; Scheme 1). The rate of formation of 4-OHEN and depletion of GSH conjugate was dependent on the concentration of NADPH, as was the extent of conversion of conjugate to 4-OHEN (Fig. 7 C-F). The half life for regeneration of 4-OHEN in the presence of NADPH (500 μM) was 40 min. The mechanism of regeneration

likely involves the addition of hydride equivalents from NADPH to the GSH conjugate of 4-OHEN. In the absence of NADPH, the GSH conjugate decomposed within 24 h in pH 7.4 PBS giving products that were not further identified.

## Discussion

There is evidence to support both hormonal and chemical contributions to estrogen carcinogenesis (10). Catechol estrogen oxidative metabolites contribute to chemical carcinogenesis via formation of electrophilic quinones and concomitantly ROS that damage DNA by alkylation and oxidation, respectively. Catechol estrogens that are ERα agonists may therefore represent optimal carcinogens if the DNA damage incurred is poorly repaired and mutagenic. Of the endogenous estrogen metabolites, 4-$OHE_2$ is a potent estrogen agonist and carcinogenic, whereas 2-$OHE_2$ is both a weak agonist and either weakly or non-carcinogenic (12,45-47). It is argued that ERα agonism promotes carcinogenesis through cell proliferation and mitosis, however, in ER(+) cells and tissues, binding of a catechol estrogen to ERα will concentrate the catechol in the nucleus, since ERα itself is predominantly located in the nucleus; thus resulting in localized production of DNA damaging electrophiles and ROS. Evidence for localized nuclear production of ROS and DNA damage has recently been provided (19,48).

Detoxification of catechol estrogen metabolites is mediated by Phase 2 enzymes through glucuronidation, sulfation, GSH conjugation, and *O*-methylation. We reported that 4-MeOEN, the product of COMT action on 4-OHEN, was an estrogen with nanomolar potency in cell culture; in this case, the action of Phase 2 enzyme metabolism may block chemical, but not hormonal carcinogenesis (49,50). The observations herein have similarity to those reported for 4-MeOEN: in breast cancer cells, 4-OHEN was a full classical estrogen agonist, inducing ERE-reporter gene transcription, cell proliferation, and expression of endogenous estrogen-responsive genes, all with potency of 10-50 nM. Although 4-OHEN was observed to act as a novel micromolar ligand for AhR, activating XRE, 4-OHEN was much less potent at AhR than ER. Moreover, silencing of AhR did not perturb the classical ER mediated actions of 4-OHEN. Finally, the ability of 4-OHEN to induce the binding of ERα to ERE sequences of DNA was clearly demonstrated using the ChIP assay.

Given our previous report on the estrogenic activity of 4-MeOEN, perhaps these similar observations on 4-OHEN appear unremarkable (49). However, unlike 4-MeOEN, 4-OHEN is readily autoxidized to an electrophilic *o*-quinone that is rapidly trapped by GSH and protein thiols (44,51-53). Furthermore, unlike 4-$OHE_2$ the endogenous catechol estrogen, 4-OHEN is rapidly oxidized ($t_{1/2} \approx 0.5$ min) in aqueous solution, without the need for cytochrome P450 or other oxidative enzymatic catalysis (20). The observations of submicromolar estrogenic potency were therefore not entirely expected, and suggest that the intracellular reducing environment maintains 4-OHEN largely in the catechol form, in contrast to observations in aerobic aqueous buffer. Therefore, cells were pre-incubated with the thiols, GSH, NAC, and NAP. It was anticipated that NAC as a primary thiol nucleophile would trap the 4-OHEN quinone, substantially or completely reducing the potency of 4-OHEN; but that NAP, as a tertiary, hindered thiol nucleophile would not be effective in quinone trapping. However, neither thiol was observed substantially to attenuate the estrogenic activity of 4-OHEN. Reaction of thiol with 4-OHEN in aqueous, aerobic buffer solution is known to trap the quinone as the thiol conjugate. Therefore, pre-incubation of 4-OHEN with thiol was performed to yield the conjugate, identified by LC-MS, prior to addition to cell culture. Surprisingly, the estrogenic potency of both the GSH and NAC conjugates the 4-OHEN when applied to cells was only marginally reduced relative to 4-OHEN itself. A simple explanation for this observation would be provided by regeneration

of 4-OHEN from the thiol conjugate in the intracellular environment, as proposed in Scheme 1.

In aerobic aqueous solution, the GSH conjugate of 4-OHEN is readily formed and can be isolated by chromatography. The GSH conjugate is expected to be more readily oxidized than 4-OHEN itself, because of stabilization of the electron-deficient quinone by the sulfanyl ring substituent (54). The reversion of the conjugate to 4-OHEN requires addition of hydride to the quinone **4** (Scheme 1). Accordingly, the rate of 4-OHEN formation and conjugate loss was observed to be dependent on NADPH concentration. 4-OHEN (**1**) was observed to be non-enzymatically regenerated from the GSH conjugate (**3**) in the presence of the reducing agent NADPH. Mammary tumors are characterized by increased activity of enzymes involved in the generation of cytoplasmic NADPH (55), and subcellular fractions from MCF-7 cell lines have high cytochrome P450 reductase content (56), therefore intracellular regeneration of 4-OHEN is reasonable. In aerobic aqueous solution, regeneration of 4-OHEN from the GSH conjugate is not complete and other products are formed over a matter of hours leading to complete decomposition of 4-OHEN and conjugate to unknown final products, however at nanomolar concentrations in the intracellular milieu, enzymic or non-enzymic reduction clearly regenerates 4-OHEN at levels capable of induction of ER-mediated signaling. An alternative two step single electron pathway has been proposed for NADPH mediated conversion of a hydroquinone GSH conjugate to the hydroquinone (57), however, the hydride addition proposed in Scheme 1 generates an intermediate akin to a σ-complex in $S_NAr$ reactions. Further mechanistic studies are ongoing.

Cellular GSH provides a major antioxidant in the intracellular aqueous compartment and defense against electrophilic agents such as catechol estrogen quinones, although some toxicity has been associated with these GSH conjugates (54,58). The rapid formation of 4-OHEN GSH conjugates has been considered as a detoxification pathway for 4-OHEN. However, both 4-OHEN and its GSH conjugates can redox cycle generating ROS and thereby consuming GSH; it was previously shown that 4-OHEN rapidly autoxidizes and significantly depletes GSH within minutes (52,59). The novel observations herein, that 4-OHEN is a potent estrogen, regenerated from the 4-OHEN GSH conjugate, provide a new perspective to redox cycling, since 4-OHEN can also be expected to consume NADPH via formation of the GSH conjugate. 4-OHEN is a classical estrogen agonist that is known: to damage DNA by alkylation and oxidation; to generate ROS; and, to consume cellular reductants; furthermore conjugation by GSH does not block any of these potential contributions to carcinogenesis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported by NIH Grant CA73638 to J. L. B. and the SRC program of KOSEF (Research Center for Women's Diseases) to M. C.

## References

1. Liehr JG. Genotoxic effects of estrogens. Mutation Research. 1990; 238:269–276. [PubMed: 2160607]
2. Colditz GA. Relationship between estrogen levels, use of hormone replacement therapy, and breast cancer. J Natl Cancer Inst. 1998; 90:814–823. [PubMed: 9625169]

3. Feigelson HS, Henderson BE. Estrogens and breast cancer. Carcinogenesis. 1996; 17:2279–2284. [PubMed: 8968038]
4. Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. N Engl J Med. 2006; 354:270–282. [PubMed: 16421368]
5. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the women's health initiative randomized controlled trial. J Am Med Assoc. 2002; 288:321–333.
6. Beral V. Breast cancer and hormone-replacement therapy in the Million Women Study. Lancet. 2003; 362:419–427. [PubMed: 12927427]
7. Ravdin PM, Cronin KA, Howlader N, Berg CD, Chlebowski RT, Feuer EJ, Edwards BK, Berry DA. The decrease in breast-cancer incidence in 2003 in the United States. N Engl J Med. 2007; 356:1670–1674. [PubMed: 17442911]
8. Yager JD. Chapter 3: endogenous estrogens as carcinogens through metabolic activation. J Natl Cancer Inst Monographs. 2000; 2000:67–73. [PubMed: 10963620]
9. Santen RJ. To block estrogen's synthesis or action: that is the question. J Clin Endocrinol Metab. 2002; 87:3007–3012. [PubMed: 12107191]
10. Bolton JL, Thatcher GRJ. Potential mechanisms of estrogen quinone carcinogenesis. Chem Res Toxicol. 2008; 21:93–101. [PubMed: 18052105]
11. Cavalieri E, Chakravarti D, Guttenplan J, Hart E, Ingle J, Jankowiak R, Muti P, Rogan E, Russo J, Santen R, Sutter T. Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. Biochim Biophys Acta. 2006; 1766:63–78. [PubMed: 16675129]
12. Kim SH, Lee SU, Kim MH, Kim BT, Min YK. Mitogenic estrogen metabolites alter the expression of 17beta-estradiol-regulated proteins including heat shock proteins in human MCF-7 breast cancer cells. Mol Cells. 2005; 20:378–384. [PubMed: 16404153]
13. Li JJ, Li SA, Oberley TD, Parsons JA. Carcinogenic activities of various steroidal and nonsteroidal estrogens in the hamster kidney: relation to hormonal activity and cell proliferation. Cancer Res. 1995; 55:4347–4351. [PubMed: 7671246]
14. Schutze N, Vollmer G, Tiemann I, Geiger M, Knuppen R. Catecholestrogens are MCF-7 cell estrogen receptor agonists. J Steroid Biochem Mol Biol. 1993; 46:781–789. [PubMed: 8274412]
15. Schutze N, Vollmer G, Knuppen R. Catecholestrogens are agonists of estrogen receptor dependent gene expression in MCF-7 cells. J Steroid Biochem Mol Biol. 1994; 48:453–461. [PubMed: 8180106]
16. Bolton JL, Thatcher GR. Potential mechanisms of estrogen quinone carcinogenesis. Chem Res Toxicol. 2008; 21:93–101. [PubMed: 18052105]
17. Liu X, Yao J, Pisha E, Yang Y, Hua Y, van Breemen RB, Bolton JL. Oxidative DNA damage induced by equine estrogen metabolites: role of estrogen receptor alpha. Chem Res Toxicol. 2002; 15:512–519. [PubMed: 11952337]
18. Chen Y, Liu X, Pisha E, Constantinou AI, Hua Y, Shen L, van Breemen RB, Elguindi EC, Blond SY, Zhang F, Bolton JL. A metabolite of equine estrogens, 4-hydroxyequilenin, induces DNA damage and apoptosis in breast cancer cell lines. Chem Res Toxicol. 2000; 13:342–350. [PubMed: 10813650]
19. Wang Z, Wijewickrama GT, Peng KW, Dietz BM, Yuan L, van Breemen RB, Bolton JL, Thatcher GRJ. Estrogen Receptor {alpha} Enhances the Rate of Oxidative DNA Damage by Targeting an Equine Estrogen Catechol Metabolite to the Nucleus. J Biol Chem. 2009; 284:8633–8642. [PubMed: 19158089]
20. Shen L, Pisha E, Huang Z, Pezzuto JM, Krol E, Alam Z, van Breemen RB, Bolton JL. Bioreductive activation of catechol estrogen-ortho-quinones: aromatization of the B ring in 4-hydroxyequilenin markedly alters quinoid formation and reactivity. Carcinogenesis. 1997; 18:1093–1101. [PubMed: 9163701]
21. Jiang SY, Jordan VC. Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst. 1992; 84:580–591. [PubMed: 1556769]

22. Catherino WH, Jordan VC. Increasing the number of tandem estrogen response elements increases the estrogenic activity of a tamoxifen analogue. Cancer Lett. 1995; 92:39–47. [PubMed: 7757959]
23. Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure. Anal Biochem. 1980; 102:344–352. [PubMed: 6158890]
24. Xu L, Spink DC. Analysis of steroidal estrogens as pyridine-3-sulfonyl derivatives by liquid chromatography electrospray tandem mass spectrometry. Anal Biochem. 2008; 375:105–114. [PubMed: 18162162]
25. Spink DC, Zhang F, Hussain MM, Katz BH, Liu X, Hilker DR, Bolton JL. Metabolism of equilenin in MCF-7 and MDA-MB-231 human breast cancer cells. Chem Res Toxicol. 2001; 14:572–581. [PubMed: 11368557]
26. Chang M, Peng Kw, Kastrati I, Overk CR, Qin ZH, Yao P, Bolton JL, Thatcher GRJ. Activation of estrogen receptor-mediated gene transcription by the equine estrogen Metabolite, 4-methoxyequilenin, in human breast cancer cells. Endocrinology. 2007; 148:4793–4802. [PubMed: 17584965]
27. Lavigne JA, Goodman JE, Fonong T, Odwin S, He P, Roberts DW, Yager JD. The effects of catechol-*o*-methyltransferase inhibition on estrogen metabolite and oxidative DNA damage levels in estradiol-treated MCF-7 cells. Cancer Res. 2001; 61:7488–7494. [PubMed: 11606384]
28. Kraus WL, Montano MM, Katzenellenbogen BS. Identification of multiple, widely spaced estrogen-responsive regions in the rat progesterone receptor gene. Mol Endocrinol. 1994; 8:952–969. [PubMed: 7997237]
29. Giamarchi C, Solanas M, Chailleux C, Augereau P, Vignon F, Rochefort H, Richard-Foy H. Chromatin structure of the regulatory regions of pS2 and cathepsin D genes in hormone-dependent and -independent breast cancer cell lines. Oncogene. 1999; 18:533–541. [PubMed: 9927210]
30. Levenson AS, Catherino WH, Jordan VC. Estrogenic activity is increased for an antiestrogen by a natural mutation of the estrogen receptor. J Steroid Biochem Mol Biol. 1997; 60:261–268. [PubMed: 9219916]
31. Klotz DM, Beckman BS, Hill SM, McLachlan JA, Walters MR, Arnold SF. Identification of environmental chemicals with estrogenic activity using a combination of in vitro assays. Environ Health Perspect. 1996; 104:1084–1089. [PubMed: 8930550]
32. Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano FO. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ Health Perspect. 1995; 103:113–122. [PubMed: 8593856]
33. Katzenellenbogen BS, Choi I, Delage-Mourroux R, Ediger TR, Martini PG, Montano M, Sun J, Weis K, Katzenellenbogen JA. Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. J Steroid Biochem Mol Biol. 2000; 74:279–285. [PubMed: 11162936]
34. Levin ER. Plasma membrane estrogen receptors. Trends Endocrinol Metab. 2009
35. Prossnitz ER, Maggiolini M. Mechanisms of estrogen signaling and gene expression via GPR30. Mol Cell Endocrinol. 2009; 308:32–38. [PubMed: 19464786]
36. Levenson AS, Kliakhandler IL, Svoboda KM, Pease KM, Kaiser SA, Ward JE III, Jordan VC. Molecular classification of selective estrogen receptor modulators on the basis of gene expression profiles of breast cancer cells expressing estrogen receptor a. British Journal of Cancer. 2002; 87:449–456. [PubMed: 12177783]
37. Frasor J, Stossi F, Danes JM, Komm B, Lyttle CR, Katzenellenbogen BS. Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. Cancer Res. 2004; 64:1522–1533. [PubMed: 14973112]
38. Wormke M, Castro-Rivera E, Chen I, Safe S. Estrogen and aryl hydrocarbon receptor expression and crosstalk in human Ishikawa endometrial cancer cells. J Steroid Biochem Mol Biol. 2000; 72:197–207. [PubMed: 10822009]
39. Ohtake F, Takeyama K, Matsumoto T, Kitagawa H, Yamamoto Y, Nohara K, Tohyama C, Krust A, Mimura J, Chambon P, Yanagisawa J, Fujii-Kuriyama Y, Kato S. Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature. 2003; 423:545–550. [PubMed: 12774124]

40. Shipley JM, Waxman DJ. Aryl hydrocarbon receptor-independent activation of estrogen receptor-dependent transcription by 3-methylcholanthrene. Toxicol Appl Pharmacol. 2006; 213:87–97. [PubMed: 16257430]
41. Bhavnani BR, Woolever CA. Interaction of ring B unsaturated estrogens with estrogen receptors of human endometrium and rat uterus. Steroids. 1991; 56:201–210. [PubMed: 1871786]
42. Bhavnani BR, Tam SP, Lu X. Structure activity relationships and differential interactions and functional activity of various equine estrogens mediated via estrogen receptors (ERs) ERalpha and ERbeta. Endocrinology. 2008; 149:4857–4870. [PubMed: 18599548]
43. Oda T, Tsuchiya T, Sato Y, Yasukochi Y. Estrogen receptor-mediated transcriptional activation by 17 beta-estradiol and its analogs. Biol Pharm Bull. 1996; 19:1018–1022. [PubMed: 8874807]
44. Chang M, Shin YG, van Breemen RB, Blond SY, Bolton JL. Structural and functional consequences of inactivation of human glutathione S-transferase P1-1 mediated by the catechol metabolite of equine estrogens, 4-hydroxyequilenin. Biochemistry. 2001; 40:4811–4820. [PubMed: 11294649]
45. Liehr JG, Ricci MJ, Jefcoate CR, Hannigan EV, Hokanson JA, Zhu BT. 4-Hydroxylation of estradiol by human uterine myometrium and myoma microsomes: implications for the mechanism of uterine tumorigenesis. Proc Natl Acad Sci U S A. 1995; 92:9220–9224. [PubMed: 7568105]
46. Liehr JG, Avitts TA, Randerath E, Randerath K. Estrogen-induced endogenous DNA adduction: possible mechanism of hormonal cancer. Proc Natl Acad Sci U S A. 1986; 83:5301–5305. [PubMed: 3460092]
47. Newbold RR, Liehr JG. Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. Cancer Res. 2000; 60:235–237. [PubMed: 10667565]
48. Peng KW, Wang H, Qin Z, Wijewickrama GT, Lu M, Wang Z, Bolton JL, Thatcher GR. Selective estrogen receptor modulator delivery of quinone warheads to DNA triggering apoptosis in breast cancer cells. ACS Chem Biol. 2009; 4:1039–1049. [PubMed: 19839584]
49. Chang M, Peng KW, Kastrati I, Overk CR, Qin ZH, Yao P, Bolton JL, Thatcher GRJ. Activation of estrogen receptor-mediated gene transcription by the equine estrogen metabolite, 4-methoxyequilenin, in human breast cancer cells. Endocrinology. 2007; 148:4793–4802. [PubMed: 17584965]
50. Chang M, Overk CR, Kastrati I, Peng KW, Yao P, Qin ZH, Petukhov P, Bolton JL, Thatcher GRJ. Estrogenic activity of the equine estrogen metabolite, 4-methoxyequilenin. Adv Exp Med Biol. 2008; 617:601–607. [PubMed: 18497087]
51. Bolton JL, Yu L, Thatcher GRJ. Quinoids formed from estrogens and antiestrogens. Methods Enzymol. 2004; 378:110–123. [PubMed: 15038960]
52. Yao J, Chang M, Li Y, Pisha E, Liu X, Yao D, Elguindi EC, Blond SY, Bolton JL. Inhibition of cellular enzymes by equine catechol estrogens in human breast cancer cells: specificity for glutathione S-transferase P1-1. Chem Res Toxicol. 2002; 15:935–942. [PubMed: 12119004]
53. Yao J, Li Y, Chang M, Wu H, Yang X, Goodman JE, Liu X, Liu H, Mesecar AD, Van Breemen RB, Yager JD, Bolton JL. Catechol estrogen 4-hydroxyequilenin is a substrate and an inhibitor of catechol-O-methyltransferase. Chem Res Toxicol. 2003; 16:668–675. [PubMed: 12755597]
54. Butterworth M, Lau SS, Monks TJ. Formation of catechol estrogen glutathione conjugates and gamma-glutamyl transpeptidase-dependent nephrotoxicity of 17beta-estradiol in the golden Syrian hamster. Carcinogenesis. 1997; 18:561–567. [PubMed: 9067557]
55. Thomas M, Bader C, Monet JD. Sex steroid hormone modulation of NADPH pathways in MCF-7 cells. Cancer Res. 1990; 50:1195–1200. [PubMed: 2297768]
56. Sinha BK, Mimnaugh EG, Rajagopalan S, Myers CE. Adriamycin activation and oxygen free radical formation in human breast tumor cells: protective role of glutathione peroxidase in adriamycin resistance. Cancer Res. 1989; 49:3844–3848. [PubMed: 2544260]
57. Khalife KH, Lupidi G. Nonenzymatic reduction of thymoquinone in physiological conditions. Free Radic Res. 2007; 41:153–161. [PubMed: 17364941]
58. Slot AJ, Wise DD, Deeley RG, Monks TJ, Cole SP. Modulation of human multidrug resistance protein (MRP) 1 (ABCC1) and MRP2 (ABCC2) transport activities by endogenous and exogenous glutathione-conjugated catechol metabolites. Drug Metab Dispos. 2008; 36:552–560. [PubMed: 18079363]

59. Chang M, Zhang F, Shen L, Pauss N, Alam I, van Breemen RB, Blond SY, Bolton JL. Inhibition of glutathione S-transferase activity by the quinoid metabolites of equine estrogens. Chem Res Toxicol. 1998; 11:758–765. [PubMed: 9671538]

## Abbreviations

| | |
|---|---|
| **AhR** | aryl hydrocarbon receptor |
| **COMT** | catechol *O*-methyltransferase |
| **ChIP** | chromatin immunoprecipitation assay |
| **DMSO** | dimethylsufoxide |
| **DT-diaphorase** | NAD(P)H:quinine oxidoreductase |
| **EDTA** | ethylenediamine tetraacetic acid |
| **$E_2$** | 17β-estradiol |
| **EN** | equilenin, 1,3,5(10),6,8-estropentaen-3-ol-17-one |
| **ER** | estrogen receptor |
| **ERE** | estrogen response element |
| **HRT** | hormone replacement therapy |
| **3-MC** | 3-methylcholanthrene |
| **2-MeO$E_2$** | 2-methoxyestradiol |
| **4-MeO$E_2$** | 4-methoxyestradiol |
| **4-MeOEN** | 4-methoxyequilenin |
| **2-O$HE_1$** | 2-hydroxyestrone |
| **2-O$HE_2$** | 2-hydroxyestradiol |
| **4-O$HE_1$** | 4-hydroxyestrone |
| **4-O$HE_2$** | 4-hydroxyestradiol |
| **4-OHEN** | 4-hydroxyequilenin, 3,4-dihydroxy-5(10),6,8,-estrapentaen-17-one |
| **NAC** | N-acetyl cysteine, NAP, N-acetyl penicillamine |
| **PBS** | phosphate-buffered saline |
| **PCR** | polymerase chain reaction |
| **RBA** | relative binding affinity |
| **ROS** | reactive oxygen species |
| **siAhR** | AhR small interfering RNA |
| **siLuc** | luciferase small interfering RNA |
| **siSC** | scrambled small interfering RNA |
| **XRE** | xenobiotic response element |

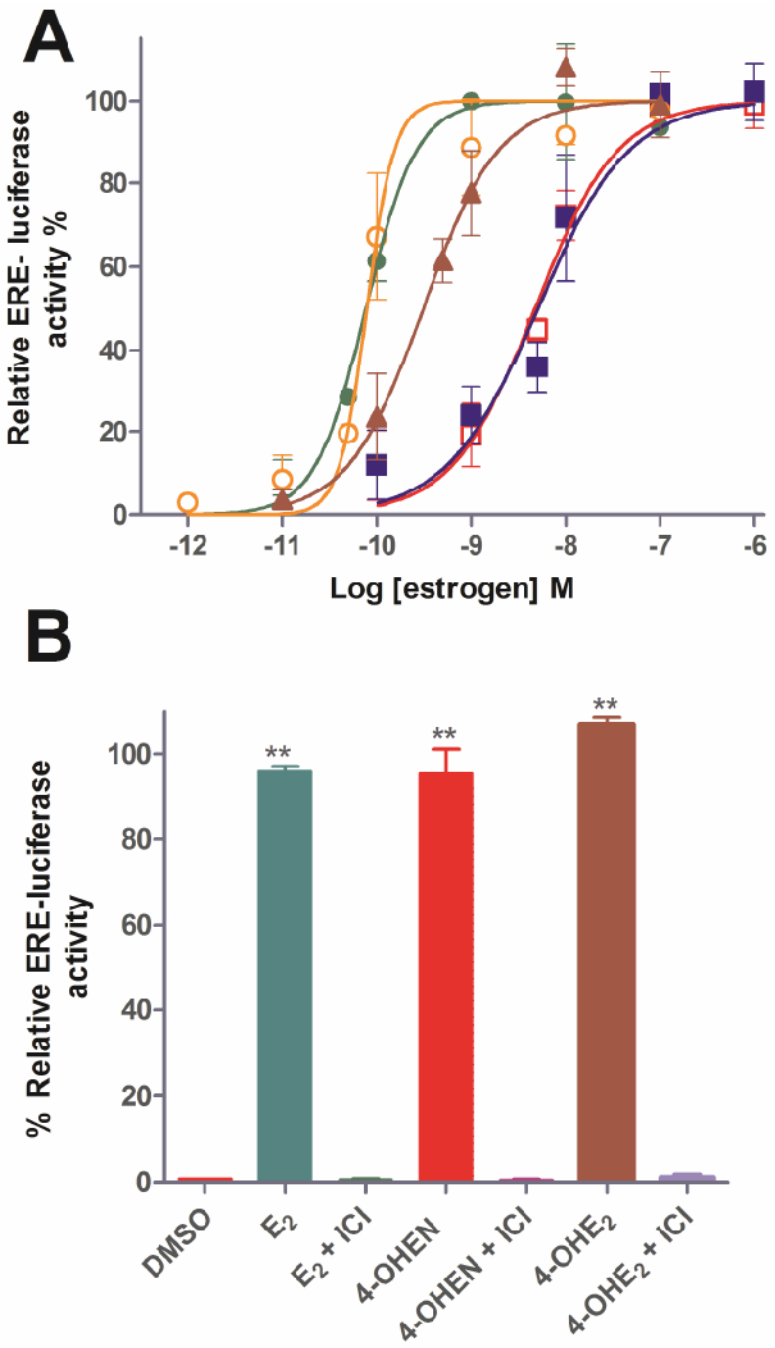



**Figure 1.**

Activation of ERE-luciferase reporter by $E_2$, 4-OHEN, and 4-$OHE_2$ in MCF-7 cells. (A) Cells were transfected with ERE-luciferase and pre-treated with or without 10 μM Ro41-0960 (COMT inhibitor) for 24 h, and then treated with different concentrations of $E_2$ (green circles), 4-OHEN (red open squares), 4-$OHE_2$ (burgundy triangles), $E_2$ + COMT inhibitor (orange open circles), 4-OHEN + COMT inhibitor (blue squares) for 18 h. (B) Antiestrogen, ICI 182780 (1 μM), abolished the activity of $E_2$ (1 nM green), 4-OHEN (100 nM red), and 4-$OHE_2$ (10 nM burgundy triangle). All data represent relative induction of ERE-luciferase activity compared to 1 nM $E_2$ set at 100%. Data showed mean and s.d. of

three independent experiments analyzed by ANOVA with Newman-Keuls post test (** $p<0.001$ vs. DMSO control).

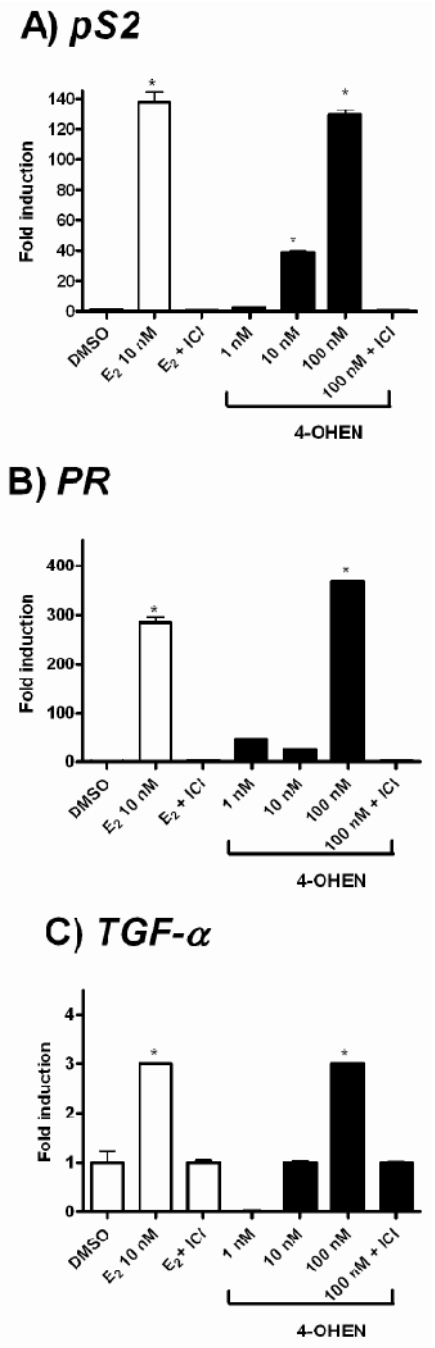



**Figure 2.**

Analysis of estrogen-responsive gene expression induced by 4-OHEN in breast cancer cells. Induction of mRNA for: A) trefoil factor 1 (*pS2*); and, B) progesterone receptor (*PR*) in MCF7 cells; in addition to C) *TGF-α* in S30 cells. Measurements from cells treated with $E_2$ (10 nM), ICI (1 μM), 4-OHEN (1 nM, 10 nM, 100 nM), or combinations as indicated, for 24 h. mRNA levels were quantified using real-time PCR. Assays were performed in three individual experiments and data represent the mean ± s.d; mRNA levels were significantly different ($p < 0.05$) compared to vehicle control.

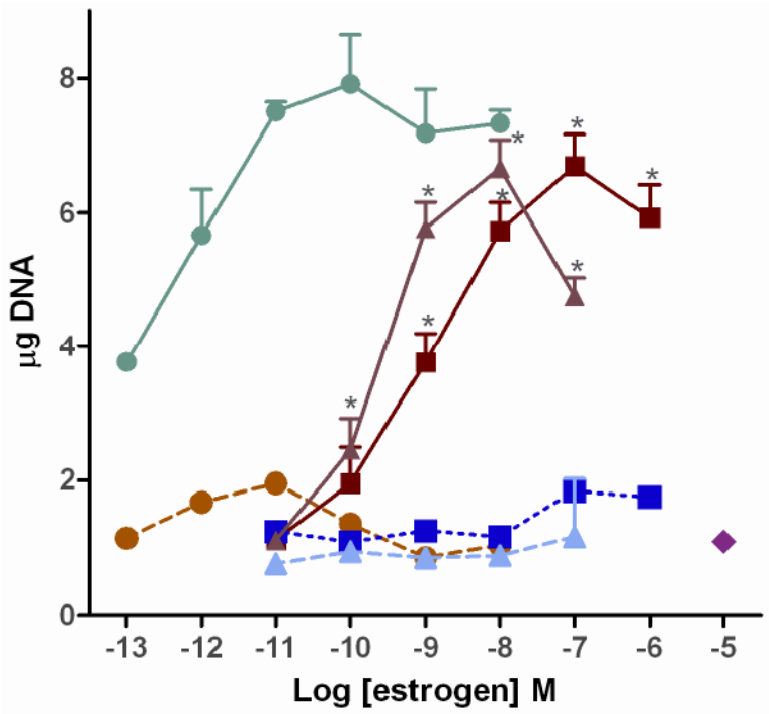



**Figure 3.**

Cell proliferation in MCF-7 breast cancer cells. Treatment for 6 days with vehicle (DMSO/ethanol, diamond), $E_2$ (green circle), 4-OHEN (red square), 4-$OHE_2$ (burgundy triangle), $E_2$ + 1 μM ICI (orange circle), 4-OHEN + 1 μM ICI (blue square), 4-$OHE_2$ + 1 μM ICI (lavender triangle). DNA content was quantified as a measure of cell growth. Assays were performed in three individual experiments and data represent the mean ± s.d.

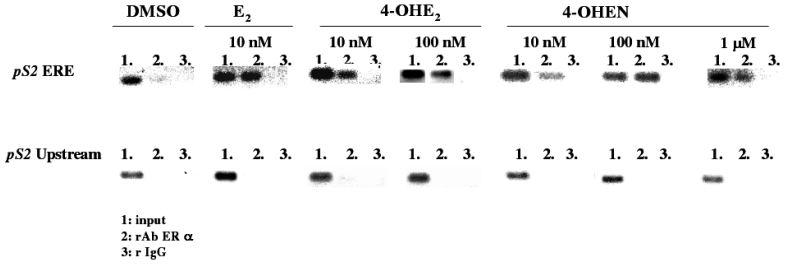



**Figure 4.**

Chromatin immunoprecipitation (ChIP) assay for liganded ER binding to ERE. PCR products for *pS2* ERE and a *pS2* upstream negative control from MCF-7 cells cultured in estrogen-free media for 4 days before treatment. Cells were treated with DMSO, 10 nM $E_2$, or different concentration of 4-$OHE_2$ or 4-OHEN for 45 min, and crosslinked by 1.5% formaldehyde for 15 min. All samples were pulled down by anti-ERα, IgG, and then incubated with a protein A/G agarose slurry. (1:Input, positive control, which was all DNA fragments without any antibody treatment; 2: rAb ERα, ERα Antibody; 3: rIgG, negative control (as a control for the specificity of antiERα). Images shown are representative of three individual experiments.

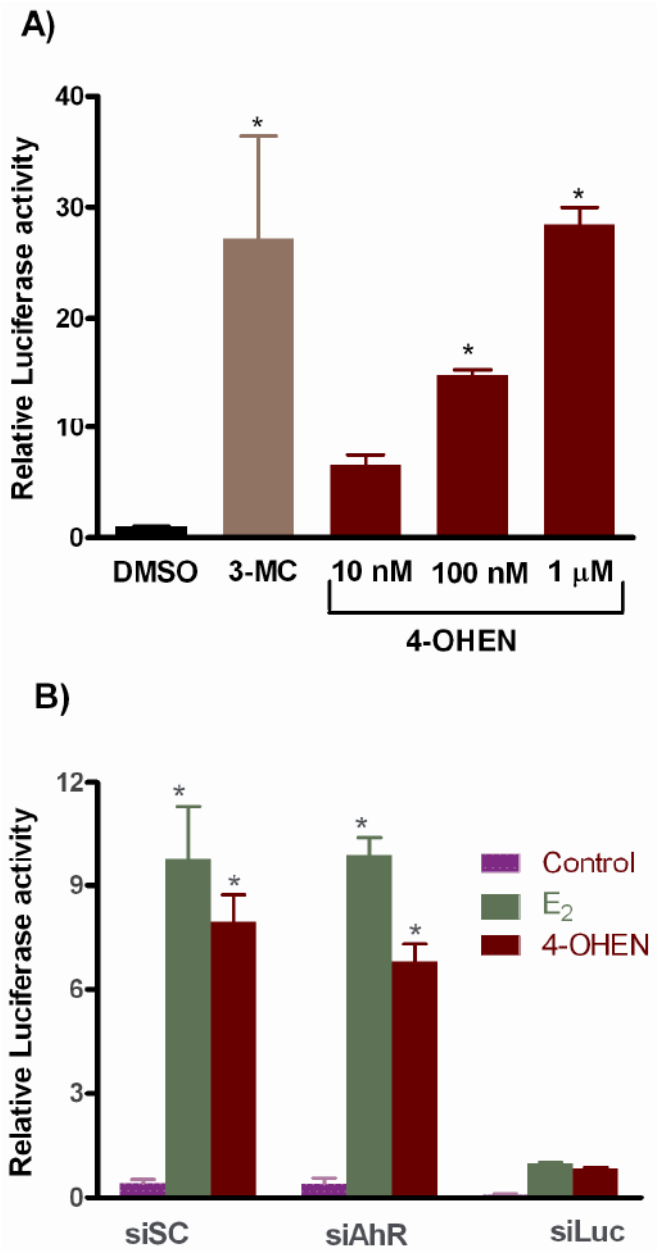



**Figure 5.**

Effect of AhR knockdown on XRE- and ERE-luciferase activities in MCF-7 cells. (A) Induction of XRE-luciferase activity by 4-OHEN compared to AhR agonist 3-MC. Cells were transiently transfected with 4 μg of an XRE-luciferase reporter plasmid. After transfection, cells were treated with DMSO, 3-MC (10 nM), 4-OHEN (10 nM, 100 nM, 1 μM) for 24 h. (B) siRNA for AhR does not affect ERE-luciferase activity induced by 4-OHEN. Cells were transfected with ERE-luciferase plasmid as well as with siSC (E. *gracilis*, as a control for RNA interference performance), siAhR, or siLuc (firefly luciferase, as a cross-check for luciferase induction). Cells were treated with 10 nM $E_2$ or 1 μM 4-OHEN for 24 h. Assays were performed in three individual experiments and data represent the mean ± s.d. (* $p<0.05$ vs. DMSO control).

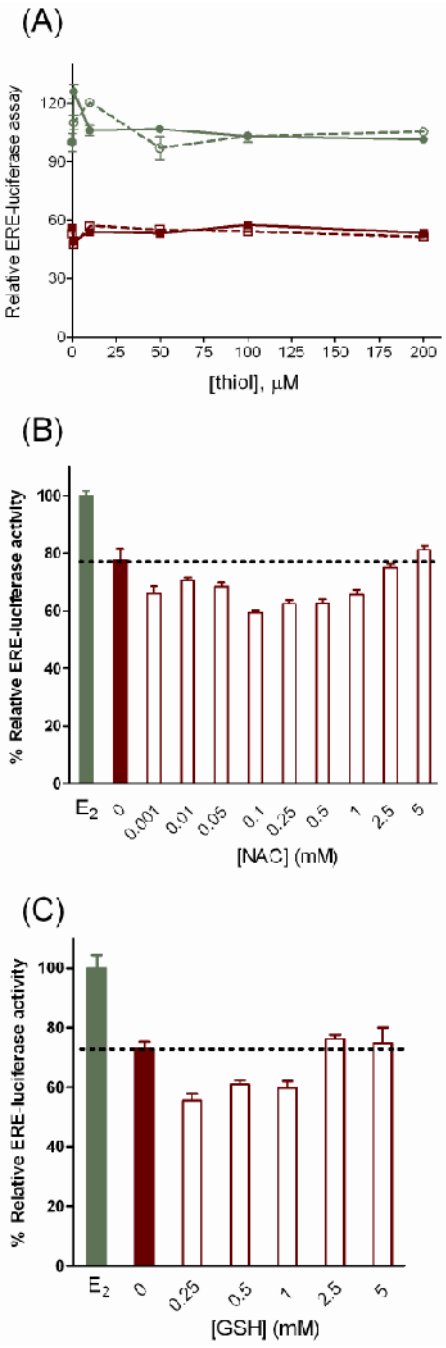



**Figure 6.**

Effect of added thiols on 4-OHEN mediated ERE-luciferase activity in MCF-7 cells. (A) Cells were pre-treated with NAC (solid lines and symbols) or NAP (open symbols and dash lines) for 30 min, followed by addition of 4-OHEN (10 nM, red) or $E_2$ (1 nM, green) for 18 h. (B and C) 4-OHEN (10 nM) was reacted with thiols at different concentrations for 30 min prior to addition to cells; cells were further incubated for 18 h. $E_2$ (1 nM) response is shown for comparison. Data show mean and s.d.

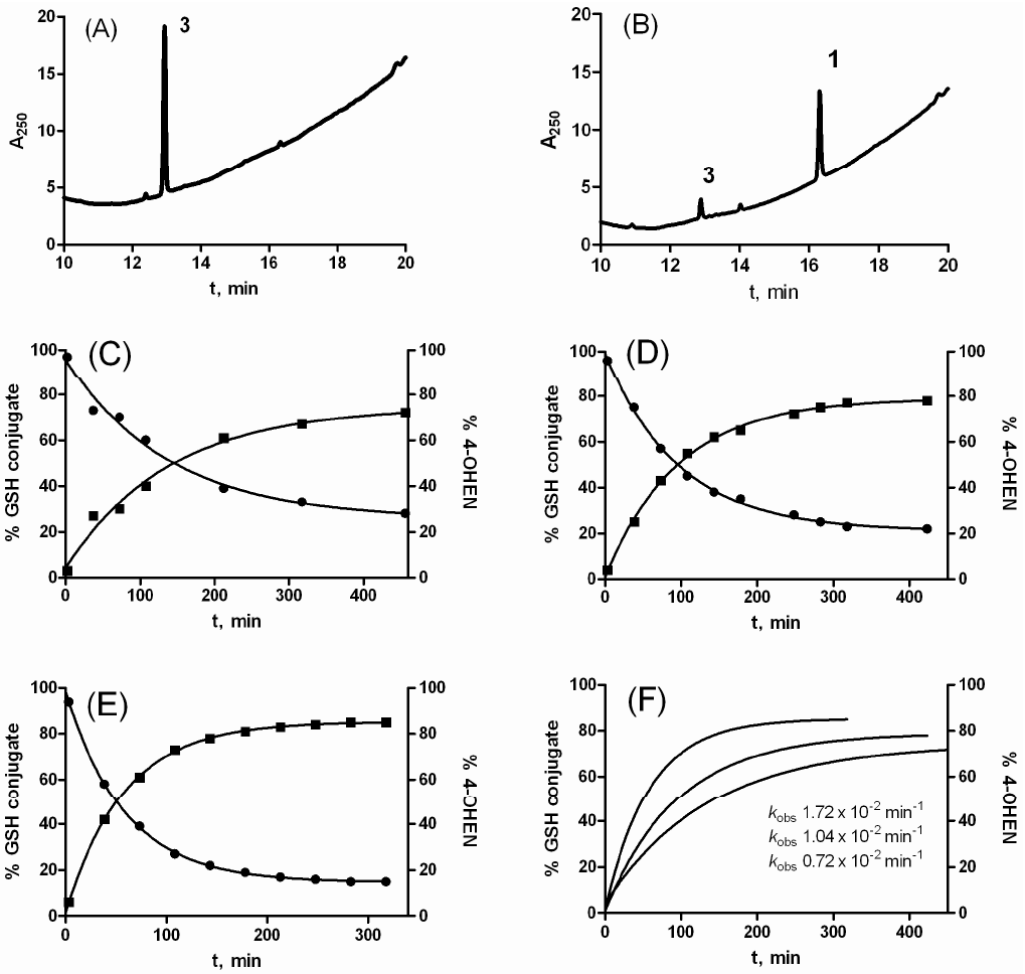
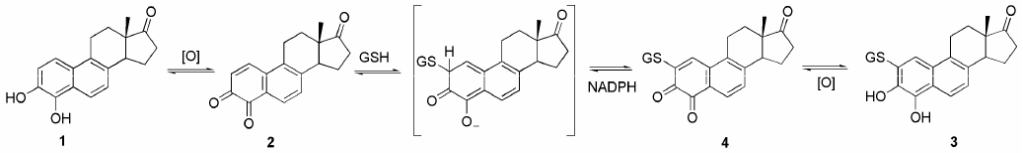



**Figure 7.**

(A & B) Non-enzymatic reduction of mono-GSH conjugate. (A) Chromatogram for conjugate (**3**) prior to addition of NADPH (5 mM). (B) Chromatogram after reaction with NADPH for 5 h at 28-29 °C showing formation of 4-OHEN (**1**). UV detection was at 250 nm. (C-F) Normalized HPLC peak area vs. time data for the formation of 4-OHEN **1** (squares) and the disappearance of 4-OHEN mono-GSH conjugate **3** (circles) at 250 nm: C, in the presence of 0.5 mM NADPH; D, in the presence of 1 mM NADPH; E, in the presence of 5 mM NADPH. Data were fitted to pseudo-first order rate equations to obtain the rate constant for the formation of 4-OHEN as, respectively, $0.72 \times 10^{-2}$ $min^{-1}$, $1.04 \times 10^{-2}$ $min^{-1}$, and $1.72 \times 10^{-2}$ $min^{-1}$, compiled in panel F.




**Scheme 1.**

## Table 1

Estrogenic and proliferative activities of estradiol compared with equilenin metabolites. [a]

| | IC $_{50}$ (nM) | EC $_{50}$ (nM) | EC$_{50}$ (nM) |
|---|---|---|---|
| | **ERα competitive binding affinity** | **ERE-reporter gene transcription activity** | **Cell proliferation** |
| $E_2$ | 6.5 ± 0.6[b] | 0.064 ± 0.05 | 0.004 ± 0.003 |
| 4-OH$E_2$ | 31 ± 2[b] | 0.4 ± 0.2 | 0.3 ± 0.2 |
| 4-OHEN | 1540 ± 200[b] | 5.7 ± 2.8 | 1.1 ± 0.2 |
| 4-MeOEN | > 50,000 | 6.5 ± 1.1[c] | 1.3 ± 1.2[c] |

[a] Mean and s.e.m.

[b] Data from reference (17)

[c] Data from reference (26)