Oxidative DNA Damage Induced by Equine Estrogen Metabolites: Role of Estrogen Receptor α

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Excessive exposure to synthetic and endogenous estrogens has been associated with the development of cancer in several tissues. 4-Hydroxyequilenin (4-OHEN), a major metabolite of equine estrogens present in estrogen replacement formulations, has been shown to induce cytotoxic/carcinogenic effects. In the present study, we have found that 4-OHEN caused DNA damage in breast cancer cells, and cells that contain estrogen receptor α (S30) are more sensitive to 4-OHEN-mediated DNA damage as compared to estrogen receptor negative cells (MDA-MB-231). For example, concentration-dependent increases in 8-oxo-deoxyguanosine (8-oxo-dG), as measured by LC-MS-MS or by the Fpg comet assay, were only detected in the S30 cells, and the amount of this lesion could be enhanced by agents, which catalyze redox cycling (NADH) or deplete GSH (diethyl maleate). The role of the estrogen receptor in modulating DNA damage was further established in incubations with the ER antagonist tamoxifen, where decreases in 8-oxo-deoxyguanosine were observed. Another equine estrogen metabolite, 4,17*β*-hydroxyequilenin (4,17*β*-OHEN), was found to have the same cytotoxicity and a similar ability to induce reactive oxygen species (ROS), and caused the same oxidative DNA damage in S30 cells as compared to 4-OHEN. However, 4,17*β*-OHEN induced twice as much single strand DNA breaks in S30 cells compared to 4-OHEN. Also 4,17*β*-OHEN was more estrogenic than 4-OHEN as demonstrated by a higher binding affinity for ERα and an enhanced induction in activity of estrogen-dependent alkaline phosphatase in Ishikawa cells. These data suggest that the mechanism of DNA damage induced by equine estrogen metabolites could involve oxidative stress and that the estrogen receptor may play a role in this process.

Introduction

Epidemiology studies have shown that the longer women are exposed to estrogens either through early menarche, late menopause, and/or through long-term estrogen replacement therapy, the higher the risk of developing breast or endometrial cancer (*1*−*8*). Premarin (Wyeth-Ayerst) is the most widely prescribed estrogen replacement formulation, which consists of the equine estrogens equilin and equilenin and their 17*β*-hydroxylated analogues, as well as the endogenous estrogens estrone and 17*β*-estradiol. Similar to studies in the hamster kidney tumor model with 17*β*-estradiol (*9*), it has been shown that treatment of hamsters for 9 months with the equine estrogens, equilin and equilenin, or sulfatase-treated Premarin resulted in 100% tumor incidence and abundant tumor foci (*10*). The mechanism of estrogen carcinogenesis is unknown; however, evidence suggests that metabolism of estrogens to catechols and further oxidation to highly reactive *o*-quinones could play a major role in induction of DNA damage leading to initiation and/or promotion of the carcinogenic process (*11*).

Previously we showed that the major catechol metabolite of both equilin and equilenin, 4-hydroxyequilenin (4-OHEN),[1] could autoxidize to potent cytotoxic quinoids and cause a variety of DNA lesions including formation of bulky stable adducts, apurinic sites, and oxidation of the phosphate sugar backbone and purine/pyrimidine bases in vitro (Scheme 1, *12*−*14*). The extent of DNA single strand breaks could be enhanced by agents which stimulate redox cycling in breast cancer cell lines and by the presence of estrogen receptor α but was inhibited by the estrogen receptor antagonist tamoxifen (*15*). In addition, our recent data suggest that 4-OHEN has the potential to be a much more effective tumor promoter and complete carcinogen in vitro in comparison with similar experiments with the endogenous catechol estrogen 4-hydroxyestrone (4-OHE) (*16*).

In the present investigation, we found that in the estrogen receptor positive cell line S30, both 4-OHEN and 4,17*β*-dihydroxyequilenin (4,17*β*-OHEN) could increase the levels of 8-oxo-deoxyguanosine (8-oxo-dG) formation,

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[1] Abbreviations: 4-OHEN, 4-hydroxyequilenin, 3,4-dihydroxy-1,3,5-(10),6,8-estrapenten-17-one; 4-OHE, 4-hydroxyestrone, 3,4-dihydroxy-1,3,5(10)-oestratrien-17-one; 4-OHE_2, 4-hydroxyestrodial; 4,17*β*-OHEN, 4,17*β*-dihydroxyequilenin; EN, equilenin, 1,3,5(10),6,8-estrapenten-3-ol-17-one; 17*β*EN, 17*β*-hydroxyequilenin; DMSO, dimethyl sulfoxide; ER, estrogen receptor; DEM, diethyl maleate; 8-oxo-dG, 8-oxo-2′-deoxyguanosine; GSH, glutathione; PBS, phosphate-buffered saline; PSF, penicillin−streptomycin−fungizome; LMAgarose, low-melting agarose; DCF, dichlorofluoroscein; DCDF-DA, 2′,7′-dichlorodiacylfluoroscein diacetate; ROS, reactive oxygen species; ED_{50}, dose of 50% cells killed; IC_{50}, dose of half-maximal induction; RBA, relative binding affinity.

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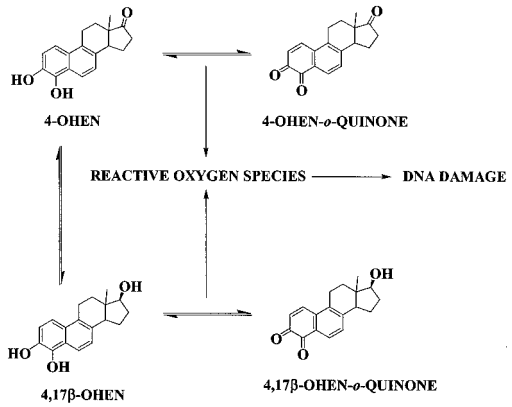
Scheme 1. Redox Cycling of 4-OHEN Inducing Oxidative Damage to DNA



a well-known marker for oxidative DNA damage (*17*). Our data also showed that 4,17β-OHEN had a higher binding affinity than 4-OHEN for estrogen receptor α and was more estrogenic than 4-OHEN as demonstrated by the enhanced induction of alkaline phosphatase activity in Ishikawa cells. In addition, 4,17β-OHEN significantly induced more DNA single strand breaks in S30 compared to 4-OHEN. Finally, both compounds showed an increased formation of ROS in both ER^+ and ER^- cells in a dose-dependent manner, indicating that ROS production was estrogen receptor independent. These data suggest that the mechanism of DNA damage induced by equine estrogen metabolites could involve oxidative stress and that the estrogen receptor may play a role in this process.

Materials and Methods

Materials. ***Caution:*** *The catechol estrogens were handled in accordance with NIH guidelines for the Laboratory Use of Chemical Carcinogens (18).* Chemicals were purchased from Fisher Scientific (Itasca, IL), Aldrich (Milwaukee, WI), or Sigma (St. Louis, MO) unless stated otherwise. 4-OHEN was synthesized by treating equilin with Fremy's salt as described previously (*19*, *20*) with minor modifications (*13*). 4,17β-OHEN was prepared by reduction of 4-OHEN with lithium tri-*tert*-butoxyaluminohydride (*21*). The Fpg FLARE comet assay kit for the detection of oxidative damage was purchased from Trevigen Inc. (Gaithersburg, MD). Isotopically labeled 8-oxo-dG (every carbon and nitrogen atom labeled with ^{13}C or ^{15}N) for use as an internal standard in LC-MS-MS studies was synthesized from isotopically labeled deoxyguanosine (Cambridge Isotope Laboratories, Andover, MA) using the published procedure (*22*). Human ERα was obtained from PanVera Corp. (Madison, WI).

Cell Culture Conditions. The MDA-MB-231 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in minimum essential medium (MEME) supplemented with 1% penicillin−streptomycin−fungizome (PSF), 6 μg/L insulin, 1% glutamax (Gibco-BRL, Grand Island, NY), 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), and 5% CO_2 at 37 °C. The S30 cell line, a stable ERα transfectant of MDA-MB-231 cells, was a generous gift from V. C. Jordan (Northwestern University, Evanston, IL). The S30 cells were maintained phenol-red-free MEME supplemented the same as MDA-MB-231 cells except for the inclusion of 5% charcoal−dextran-treated fetal bovine serum and 500 μg/mL G418. The cells were grown for 24−48 h to maintain logarithmic growth and then treated with various concentrations of catechol estrogens or DMSO in fresh medium. Compounds were freshly dissolved in DMSO, and the final DMSO concentration was 0.05%.

Analysis of Oxidized DNA Bases in Breast Cancer Cells by LC-MS-MS (*15*). S30 or MDA-MB-231 cells (5×10^6 cells) were treated with various concentrations of 4-OHEN for 90 min at 37 °C. In some cases, the cells were pretreated for 1 h with diethyl maleate (1 μM), NADH (15 μM), tamoxifen (1 nM), or vehicle (10 μL). Floating cells were collected by centrifugation at 3000 rpm for 5 min, and attached cells were first trypsinized and then harvested by centrifugation. The cells were combined and washed with PBS. The cell pellets were homogenized in 35 mL of lysis buffer (320 mM sucrose, 10 mM Tris, pH 7.4, 5 mM $MgCl_2$, 10 mM Triton X-100, and 50 mM mannitol) at 4 °C. After centrifugation, the nuclei pellet was treated for 30 min at 37 °C with RNase T1 (60 units) and RNase A (0.2 mg) in 1% SDS, 1 mM EDTA, 10 mM Tris, pH 7.4, and 0.45 M NaCl. The DNA concentration was measured at 260 nm, and the DNA was further incubated with Proteinase K (0.64 mg/mL) for 30 min at 37 °C. NaCl and Tris were added to achieve final concentrations of 0.62 M and 20 mM, respectively. An equal volume of 1-butanol was added, the sample was centrifuged, and the aqueous layer was isolated. After ethanolic precipitation, the DNA was washed twice with 70% ethanol at 4 °C. The DNA was dissolved in 100 μL of buffer (25 mM ammonium acetate, pH 5.3, 0.1 mM $ZnCl_2$) and hydrolyzed using nuclease P1 (3.9 units) and alkaline phosphatase (5 units) for 90 min at 37 °C. Labeled 8-oxo-dG was added as the internal standard, and the enzymes were removed by ultrafiltration in a centrifuge using a 10 000 molecular weight cutoff membrane. The ultrafiltrate was analyzed using LC-MS-MS on a Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with negative ion electrospray ionization according to the method of Hua et al. (*23*). The samples were separated using an ODS AQ C18 column (2.0 mm × 250 mm, S-5, YMC, Wilmington, NC) at a flow rate of 0.2 mL/min with a gradient mobile phase starting at 6% methanol/water and increasing to 13% methanol/water over 20 min and then increasing to 23% methanol/water in one step. 8-oxo-dG was detected using multiple-reaction monitoring with collision-induced dissociation for the fragmentation pathway of m/z 282 → 192. Under these conditions, the detection limit and retention time of the 8-oxo-dG were 7.4 fmol and 26 min, respectively.

Analysis of DNA Damage in Breast Cancer Cells Using the Alkaline Single Cell Gel Electrophoresis Assay (Comet Assay) (*24*, *25*). The comet assay was carried out as recommended by the manufacturer (Trevigen Inc., Gaithersburg, MD). Briefly, after incubation with the test compounds for 90 min, the attached cells were trypsinized and combined with the suspended cells in the medium by centrifugation. The cells were washed with PBS and resuspended in PBS at 2×10^5 cells/mL. Cells (30 μL) were combined with LMAgarose (300 μL, 42 °C), and 50 μL of the mixture was immediately placed onto a CometSlide. The slides were incubated at 4 °C in the dark for 10 min, followed by immersion in prechilled lysis solution at 4 °C for 30 min, and then incubated in freshly prepared alkali electrophoresis solution at room temperature in the dark for 45 min. Following electrophoresis in alkali solution at 320 mA for 30 min, the slides were immersed in ethanol for 5 min, and air-dried. The slides were then stained with SYBR Green and viewed under the fluorescence microscope. The DNA was scored from 0 (intact DNA) to 4 (completely damaged DNA with tail only). Scores were calculated using the following formula in which N_A, N_B, N_C, N_D, N_E were the number of cells demonstrating different comets tails, from intact nuclei (N_A) through completely damaged DNA (N_E):

$$\text{scores } (S) = \{(N_A \times 0 + N_B \times 1 + N_C \times 2 + N_D \times 3 + N_E \times 4)/(N_A + N_B + N_C + N_D + N_E)\} \times 100$$

Duplicate samples were prepared for each treatment, and at least 100 cells were scored per sample.

Oxidative DNA Damage Comet Assay. The instructions provided in the Trevigen Fpg FLARE (Fragment Length Analysis using Repair Enzymes) kit were followed. Briefly, following treatment, the cells were collected and washed as previously described and suspended in cold PBS (1×10^5 cells/mL). An

aliquot was combined with 1% low-melting agarose and pipetted onto Trevigen CometSlides. After fixing the cell/agar mixture to the slide, the cells were lysed at 4 °C in lysis buffer [100 mM Na_2EDTA, 2.5 M NaCl, 10 mM Tris (pH 10.0), 1% Triton X-100, 1% sodium lauroly sarcosinate] for 30 min. The slides were then equilibrated with the FLARE buffer [10 mM HEPES−KOH (pH 7.4), 0.1 M KCl] for 15 min. The slides were incubated for 60 min at 37 °C with the Fpg enzyme, and appropriate buffer-only controls were included. Following equilibration with alkali solution (500 mM EDTA adjusted with 5 M NaOH to pH 12.5), the slides underwent electrophoresis for 3 min at 1 V/cm. The slides were then fixed with 70% ethanol, stained with SYBR green, and scored as previously described. The difference between the score of the Fpg-treated samples and buffer controls was proportional to the amount of oxidative damage to the DNA bases.

Estrogen Receptor (ER) Competitive Binding Assays (*26*). The procedure of Obourn et al. (*27*) was used with minor modifications (*28*). Briefly, 24 h prior to the assay, a 50% v/v hydroxylapatite slurry was prepared using 10 g of hydroxylapatite in 60 mL of TE buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA) and stored at 4 °C. The ER binding buffer consisted of 10 mM Tris-HCl (pH 7.5), 10% glycerol, 2 mM dithiothrietol, and 1 mg/mL bovine serum albumin. The ERα wash buffer contained 100 mM KCl, 1 mM EDTA, and 40 mM Tris-HCl (pH 7.5). The reaction mixture consisted of 5 μL of test sample in DMSO, 5 μL of pure human recombinant diluted ERα (0.5 pmol) in ER binding buffer, 5 μL of 'Hot Mix' (400 nM, prepared fresh using 3.2 μL of 25 μM, 83 Ci/mmol [^{3}H]estradiol, 98.4 μL of ethanol, and 98.4 μL of ER binding buffer), and 85 μL of ER binding buffer. The incubations were carried out at room temperature for 2 h; then 100 μL of 50% hydroxylapatite slurry was added, and the tubes were incubated on ice for 15 min with vortexing every 5 min. The appropriate ER wash buffer was added (1 mL); the tubes were vortexed and then centrifuged at 2000*g* for 5 min. The supernatant was discarded, and this wash step was repeated 3 times. The hydroxylapatite pellet containing the ligand−receptor complex was resuspended in 200 μL of ethanol and transferred to scintillation vials. Cytoscint (4 mL/vial) was added, and the tubes were counted using a Beckman (Schaumburg, IL) LS 5801 liquid scintillation counter. The percent inhibition of [^{3}H]estradiol binding to each ER was determined as follows: $[1 - (dpm_{sample} - dpm_{blank})/(dpm_{DMSO} - dpm_{blank})] \times 100$. The binding capability (%) of the sample was calculated by comparison to estradiol (50 nM, 100%). The data represent the average ± SD of three determinations.

Induction of Alkaline Phosphatase Activity in Ishikawa Cells. The procedure of Pisha et al. (*29*) was used as described previously. Briefly, Ishikawa cells (5×10^4 cells/well) were incubated overnight with estrogen-free media in 96-well plates. Test samples in DMSO were added, and the cells in a total volume of 200 μL of media/well were incubated at 37 °C for 2 h followed by three successive washes with PBS. The cells were then incubated for 4 days in fresh medium. Enzyme activity was measured by reading the liberation of *p*-nitrophenol at 340 nm every 15 s for 16−20 readings with an ELISA reader (Power Wave 200 Microplate Scanning Spectrophotometer, Bio-Tek Instrument, Winooski, VT). The maximum slope of the lines generated by the kinetic readings were calculated using Table Curve software (SPSS, Chicago, IL) The percent induction of alkaline phosphatase activity was calculated as follows:

$$[(slope_{sample} - slope_{cells})/(slope_{estrogen} - slope_{cells})] \times 100$$

The data represent the average ± SD of triplicate determinations.

Evaluation of the Cytotoxic Potential of Catechol Estrogens in Breast Cancer Cell Lines. Cell viability was determined by trypan blue exclusion (*30*, *31*). Briefly, the cells (10^5 cells/mL) were incubated with various concentrations of catechol estrogens or DMSO for 18 h. After treatment, floating cells were collected by centrifugation at 3000 rpm for 5 min, and attached cells were first trypsinized and then harvested by centrifugation. Floating cells and attached cells were combined, washed with PBS, and stained with 0.4% trypan blue. A drop of cell suspension was placed on a hemocytometer, and the cell number was determined using a light microscope. The LC_{50} values were obtained by linear regression analysis, and the data represent the average ± SD of four determinations.

Table 1. LC-MS-MS Analysis of 8-Oxo-dG Formed in S30 and MDA-MB-231 Cells Treated with 4-OHEN and 4,17β-OHEN[a]

	8-oxo-dG/dG × 10^5			
	4-OHEN		4,17β-OHEN	
[catechol estrogen]	S30	MDA-MB-231	S30	MDA-MB-231
DMSO	0.26 ± 0.03	0.29 ± 0.04	0.32 ± 0.03	0.32 ± 0.01
2.5 μM	0.33 ± 0.03	0.21 ± 0.04	0.36 ± 0.04	0.23 ± 0.01
5.0 μM	0.38 ± 0.03	0.26 ± 0.05	0.44 ± 0.05	0.22 ± 0.01
7.5 μM	0.41 ± 0.03	0.22 ± 0.03	0.51 ± 0.03	0.22 ± 0.02
10 μM	0.44 ± 0.08	0.21 ± 0.03	0.55 ± 0.06	0.22 ± 0.02
15 μM	0.51 ± 0.05	0.27 ± 0.05	0.59 ± 0.02	0.29 ± 0.03

[a] Cells (5×10^4 cells/well) were treated with the compounds for 90 min. Experimental details are described under Materials and Methods. Data represent the average ± SD of duplicate determinations.

Evaluation of Intracellular ROS (*32*). Cells were collected and washed twice with PBS. The cell suspension (50 μL, 5×10^5 cells/mL) was added to a 96-well plate. Test compounds (1 μL) and 40 μg/mL DCDF-DA (25 μL) were added to the cells. The plates were read (excitation at 485 nm, emission at 530 nm) on a Packard FluorCount (Meriden, CT) at 3 h. A standard curve was generated using the fluorescent product DCF, which was read as described above.

Instrumentation. LC-MS-MS experiments were carried out using a Micromass Quattro II eletrospray triple-quadrupole mass spectrometer equipped with a Waters 2690 HPLC system consisting of a binary pump, UV absorbance detector, and autosampler. Parameters for MS-MS included a cone voltage of 20−25 V, an argon collision gas pressure of 2.5×10^{-3} mBar, and a source temperature of 110 °C. LC-MS-MS analysis was carried out using multiple-reaction monitoring, and the peak areas were calculated using Micromass MassLynx software. The fluorescence microscope used for the comet assays was the Axioplan universal microscope with 10 × 10 Plan-Neofluar objectives (Zeiss). The filter for visualizing DNA was the BP546 (Excitation), FT580 (Dichroic), and LP590 (Barrier) filter from Zeiss.

Results

Oxidation of DNA Bases in Breast Cancer Cells by 4-OHEN and 4,17β-OHEN. Previously we showed that 4-OHEN induced DNA damage and apoptosis in breast cancer cell lines (*15*). 8-Oxo-dG has been used as a biomarker for oxidative stress, and it has been extensively studied as an indication of DNA oxidation (*33*, *34*). In the present study, using an LC-MS-MS assay, we examined the relative ability of 4-OHEN and 4,17β-OHEN to increase the levels of 8-oxo-dG in S30 cells and in MDA-MB-231 cells. The S30 cell line is the MDA-MB-231 cell line stably transfected with ERα, allowing us to directly compare the role of the estrogen receptor in induction of oxidized DNA bases. Similar to what was observed previously in the ER^+ S30 cells (*15*), the data showed (Table 1) that both 4-OHEN and 4,17β-OHEN induced a concentration-dependent increase in the levels of 8-oxo-dG in this cell line. However, the level of 8-oxo-dG did not change in the ER^- cells (MDA-MB-231) even at the highest doses (15 μM) of the two compounds.

We have previously shown that agents, which stimulate redox cycling (NADH) or deplete GSH (DEM),

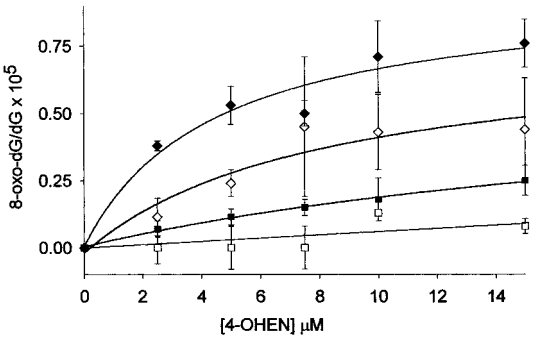


Figure 1. LC-MS-MS analysis of 8-oxo-dG formed in S30 cells treated with 4-OHEN, 4-OHEN + NADH (15 μM), 4-OHEN + DEM (1 μM), and 4-OHEN + tamoxifen (1 nM). Closed squares, 4-OHEN; open diamonds, 4-OHEN + NADH; closed diamonds, 4-OHEN + DEM; open squares, 4-OHEN + tamoxifen.

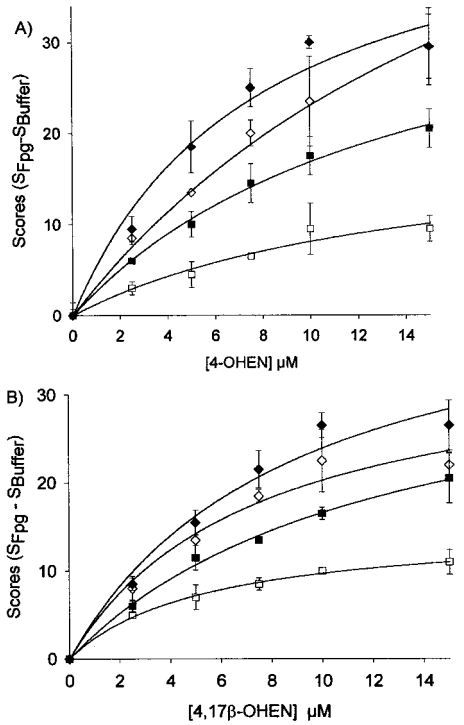


Figure 2. Fpg FLARE comet assay measuring oxidized DNA bases after incubation with 4-OHEN, 4,17β-OHEN for 90 min in S30 cells. (A) Closed squares, 4-OHEN; closed diamonds, 4-OHEN + DEM; open diamonds, 4-OHEN + NADH; open squares, 4-OHEN + tamoxifen. (B) Closed squares, 4,17β-OHEN; closed diamonds, 4,17β-OHEN + DEM; open diamonds, 4,17β-OHEN + NADH; open squares, 4,17β-OHEN + tamoxifen.

enhanced 4-OHEN-mediated induction of DNA single strand cleavage in S30 cells by using the comet assay (*15*). In this study, both 4-OHEN and 4,17β-OHEN showed a similar ability to induce the formation of oxidized DNA bases as measured by the Fpg comet assay (Figure 2). Increasing redox cycling with NADH or depleting GSH with DEM also increased the level of oxidized bases in S30 cells. In addition, nanomolar amounts of tamoxifen protected S30 cellular DNA from oxidative damage. We have also found that similar treatments with 4-OHEN increased the levels of 8-oxo-dG (Figure 1) in S30 cells using the LC-MS-MS assay. The LC-MS-MS assay gives precise measurements of 8-oxo-dG levels; however, the modified comet assay is much less labor-intensive and does not require expensive machinery, and samples can be processed more rapidly.

Table 2. Evaluation of ERα Binding Affinity of Estrogens Using the Estrogen Receptor (hER) Competitive Binding Assay[a]

compounds	IC_{50} (nM)	RBA[b]
4-OHE_2	31 ± 2	21
4-OHE	81 ± 3	8
4-OHEN	1540 ± 200	0.4
4,17β-OHEN	403 ± 30	1.6
estrone	16 ± 4	40
estradiol	6.5 ± 0.6	100
EN	3.6 ± 0.2	178
17β-OHEN	1.3 ± 0.3	509

[a] The reaction mixture consisted of 5 μL of test samples in DMSO, 5 μL of pure human recombinant diluted ERα in 85 μL of ER binding buffer, and 5 μL of 'Hot Mix'. Samples were incubated for 2 h. Experimental details are described under Materials and Methods. [b] Relative binding affinity was calculated with respect to estradiol set at 100.

Table 3. Induction of Alkaline Phosphatase Activity in the Ishikawa Cell Line[a]

compounds	ED_{50} (μM)	relative induction ability[b]
4-OHE	16.8 ± 0.7	0.22
4-OHE_2	3.9 ± 0.4	0.95
4-OHEN	5.7 ± 0.2	0.64
4,17β-OHEN	1.8 ± 0.7	2.00
estradiol	0.037 ± 0.002	100

[a] Cells (5 × 10^4/well) were treated with compounds for 2 h. Experimental details are described under Materials and Methods. [b] Relative induction ability calculated with respect to estradiol set at 100.

Estrogen Receptor Competitive Binding Assay. Since the estrogen receptor appears to play a role in the induction of DNA damage (*15*), we measured the relative binding affinity of the catechol estrogens and phenols for ERα (Table 2). The data showed that the binding affinity of ERα for 4,17β-OHEN is about 4-fold higher compared to 4-OHEN. The parent estrogens, equilenin (EN) and 17β-equilenin (17β-EN), showed higher affinities for both receptors compared to 4-OHEN and 4,17β-OHEN likely due to the rapid oxidation of the catechols to *o*-quinones which are unlikely to have affinity for the receptor. Interestingly, both 4-OHE and 4-OHE_2 have higher binding affinity compared to 4-OHEN and 4,17β-OHEN but slightly lower than those of estrone and 17β-estradiol.

Induction of Alkaline Phosphatase Activity in Ishikawa Cells. The ER binding assay measures the relative affinity of compounds for the estrogen receptor; however, this assay gives no information concerning the relative estrogenic activity of compounds in cells. The Ishikawa cell line has been used extensively to determine estrogenic activity (*29*). Ishikawa is an ER positive endometrial adenocarcinoma cell line derived from a glandular epithelial cell line which is a stable human endometrial cancer cell line that displays estrogen-inducible alkaline phosphatase activity (*28*, *35*). This cell line responds to estrogens and antiestrogens at concentrations approximating physiological levels (*36*). Induction of alkaline phosphatase activity in Ishikawa cells indicates an estrogenic response (*28*). This cell line was used to investigate the estrogenic effects of the catechol estrogens. The results (Table 3) showed that in contrast to the ER binding assay, 4,17β-OHEN gave the highest induction of alkaline phosphatase activity compared to the other catechols. However, 4-OHE_2 and 4,17β-OHEN were 3−4-fold more effective in producing an estrogenic

Table 4. Evaluation of the Cytotoxic Potential of Catechol Estrogens in Breast Cancer Cell Lines[a]

	LC_{50} (μM)			
	MDA-MB-231		S30	
compounds	3 h	18 h	3 h	18 h
4-OHEN	18.5 ± 0.6	10.5 ± 0.2	10.2 ± 0.7	6.80 ± 0.5
4,17β-OHEN	19.4 ± 2.9	10.9 ± 1.2	11.3 ± 0.5	6.90 ± 0.1

[a] Cells (10^5 cells/mL) were incubated with various concentrations of 4,17β-OHEN or vehicle for 3 or 18 h. Values are expressed as the mean ± SD of four determinations. Experimental details are described under Materials and Methods.

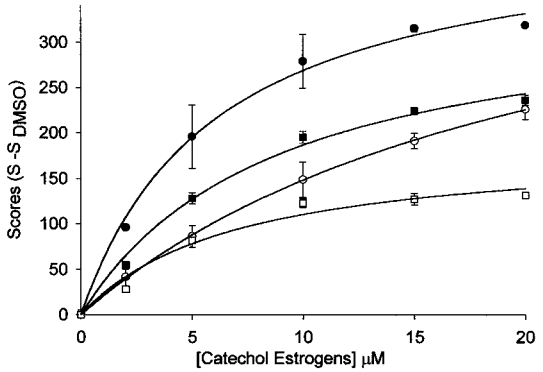


Figure 3. Induction of DNA single strand breaks by 4,17β-OHEN, 4-OHEN in S30 and MDA-MB-231 cells measured using the alkaline single-cell gel electrophoresis assay. Cells (3×10^5 cells/mL) were treated with compounds or vehicle for 90 min. Closed squares, 4-OHEN in S30 cells; open squares, 4-OHEN in MDA-MB-231 cells; closed circles, 4,17β-OHEN in S30 cells; open circles, 4,17β-OHEN in MDA-MB-231 cells.

response compared to their 17-one analogues, which was consistent with the ER binding data.

Evaluation of the Cytotoxic Potential of 4-OHEN and 4,17β-OHEN in Breast Cancer Cells. To establish the appropriate concentration range of the catechol estrogens for comet assays, cytotoxicity was measured using the trypan blue exclusion assay. The data (Table 4) showed that 4-OHEN and 4,17β-OHEN were equally cytotoxic in both cell lines; however, the estrogen receptor positive cells (S30) were more sensitive to the toxic effects of both equine catechol estrogens compared to the estrogen receptor negative cell line (MDA-MB-231). 4-OHE and 4-OHE_2 as well as the four parent phenols had no effect on the cell viability of both MDA-MB-231 and S30 cells (LC_{50} >100 μM, data not shown).

Induction of DNA Single Strand Breaks in Breast Cancer Cells. Our previous studies have shown that 4-OHEN can cause dose- and time-dependent DNA single strand breaks in breast cancer cells and that the damage could be enhanced by agents which induce redox cycling or could be reduced by scavengers of reactive oxygen species (*15*). Since 4,17β-OHEN showed higher binding affinity to ERα and was more estrogenic in the Ishikawa cells than 4-OHEN, we compared the relative ability of these catechol estrogens to induce DNA damage in the S30 and MDA-MB-231 cell lines (Figure 3). As observed previously (*15*), the S30 cells were considerably more sensitive to induction of DNA damage in experiments with either 4-OHEN or 4,17β-OHEN. However, 4,17β-OHEN induced twice as much DNA damage in both cell lines compared to 4-OHEN. This effect was expected in the ER^+ S30 cells since 4,17β-OHEN was found to be more estrogenic, and since the ER appears to play a role in enhancing catechol estrogen-mediated DNA damage.

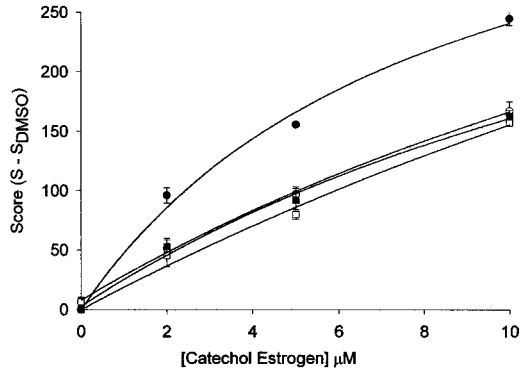


Figure 4. Induction of DNA single strand breaks by 4,17β-OHEN in S30 and MDA-MB-231 cells measured using the alkaline single-cell gel electrophoresis assay. Cells (3×10^5 cells/mL) were treated with various concentrations of 4,17β-OHEN for 90 min. Closed circles, 4,17β-OHEN in S30 cells; open circles, 4,17β-OHEN + tamoxifen (1 nM) in S30 cells; closed squares, 4,17β-OHEN in MDA-MB-231 cells; open squares, 4,17β-OHEN + tamoxifen in MDA-MB-231 cells.

We expected that both catechols would induce similar amounts of DNA damage in the ER^- MDA-MB-231 cells, and the data shown in Figure 3 supported our hypothesis for concentrations less than 10 μM. However, at higher concentrations, 4,17β-OHEN was a more effective inducer of DNA damage in MDA-MB-231 cells, which may not be receptor-mediated. It is possible that pharmacokinetic differences between the catechols account for these observations. Finally, we explored the effect of blocking the estrogen receptor with tamoxifen on DNA damage caused by 4,17β-OHEN in S30 and MDA-MB-231 cells (Figure 4). The data showed that tamoxifen significantly reduced the DNA single strand breaks in the ER^+ S30 cells; however, as expected, no effect of tamoxifen on DNA damage was observed with the ER^- MDA-MB-231 cells.

Generation of Cellular ROS. The DCF-DA oxidation assay was used to determine the generation of cellular ROS. DCF-DA easily crosses the cellular membrane into the cytoplasma where it is de-acetylated and trapped; reaction with cellular ROS produces the fluorescent oxidation product DCF. In this study, both cell lines (S30 and MDA-MB-231) responded to an H_2O_2 challenge with an increase in the level of DCF, indicating that the assay could be utilized for ROS measurements. Within 3 h, H_2O_2 (100 μM) induced a 3-fold increase in fluorescence in both cell lines (data not shown). Similar experiments with 4-OHEN and 4,17β-OHEN induced ROS formation in both cell lines in a dose-dependent manner (Figure 5); both compounds showed a maximum increase of 2.5-fold at the 10 μM dose in both cell lines. These results indicated that both 4-OHEN and 4,17β-OHEN could produce cellular ROS species at subtoxic doses. There was no difference between the ER^+(S30) and ER^-(MDA-MB-231) cells, indicating ROS production was estrogen receptor independent.

Discussion

The relationships among DNA damage, apoptosis, necrosis, and carcinogenesis are poorly understood (*37*). We previously reported that 4-OHEN induced DNA single strand breaks using the comet assay, which is a simple and reliable method for detecting DNA damage (*15*). The objective of the current study was to examine the relative ability of equine catechol estrogens to induce

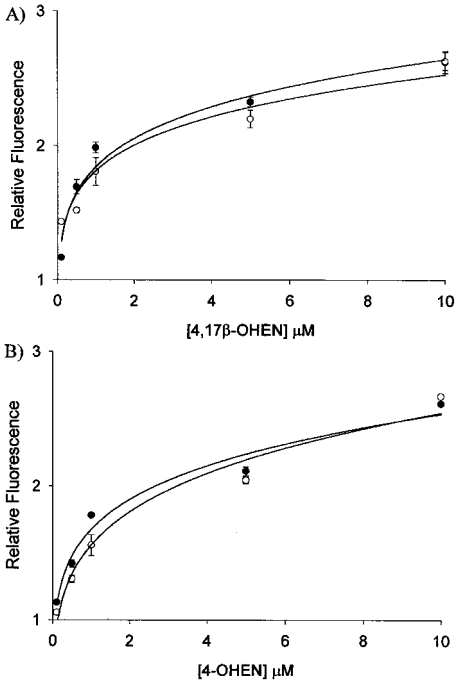


Figure 5. Modulation of reactive oxygen species by catechol estrogens. (A) Relative fluorescence of S30 (closed circles) and MDA-MB-231 (open circles) cells treated for 3 h with 4,17β-OHEN. (B) Relative fluorescence of S30 (closed circles) and MDA-MB-231 (open circles) cells treated for 3 h with 4-OHEN.

oxidative DNA damage in breast cancer cells. Oxidative damage to DNA is thought to play a significant role in mutagenesis, cancer, and aging (*17*). The use of 8-oxo-dG as a marker for oxidative stress is well-documented (*38*, *39*). 8-Oxo-dG is mutagenic, causing G→T transversions (*39*); this type of mutation has been found in the p53 gene in hepatic cells exposed to chronic inflammation (*40*). In addition, it has been shown that elevated levels of 8-oxo-dG have been detected in the breast tissue of cancer patients compared to controls (*41*).

In the present study, the formation of 8-oxo-dG was monitored using LC-MS-MS, which gives a quantitative measure of this lesion. The data showed that the estrogen receptor seems to play a role since 4-OHEN and 4,17β-OHEN induced concentration-dependent increases in 8-oxo-dG formation in S30 (ER^+) cells whereas no effect was observed in the MDA-MB-231 (ER^-) cell line. The estrogen receptor antagonist tamoxifen blocked 8-oxo-dG formation, suggesting ERα was involved in formation of this lesion. Furthermore, the formation of 8-oxo-dG could be enhanced by agents that either stimulate redox cycling (NADH) or deplete GSH (DEM) in S30 cells. 8-Oxo-dG was also monitored by the Fpg FLARE comet assay which is a qualitative assessment of oxidized DNA bases. It has the advantage over the LC-MS-MS method in that samples can be processed rapidly. Very similar data were obtained with both methods, which shows that the Fpg comet assay can be used as a powerful tool for mechanistic studies involving oxidative damage to cellular DNA.

The estrogen receptor competitive binding assay demonstrated that the 17β-OH group significantly increased the binding affinity for all the compounds studied (Table 1). Both the endogenous 4-OHE_2 and 4-OHE had a higher binding affinity compared to 4,17β-OHEN and 4-OHEN. The parent estrogens, equilenin (EN) and 17β-equilenin (17β-EN), had a higher affinity for ERα compared to estrone and estradiol. The ER RBA values of 4-OHE_2, 20, and estrone, 40, obtained in this study are within the range of determinations reported by other laboratories which are 13 and 60, respectively (*42*, *43*). Experiments using the Ishikawa cell line gave more information on the estrogenic activity of these compounds in cells. Interestingly, the data showed that 4-OHEN and 4,17β-OHEN were more estrogenic than endogenous 4-OHE and 4-OHE_2, although both of the equine estrogens' binding affinities were lower in the competitive binding assay. As observed with the competitive binding data, the 17β-OH group enhanced the estrogen-mediated induction of alkaline phosphatase in Ishikawa cells; the relative order of induction was 4,17β-OHEN > 4-OHE2 $\approx$ 4-OHEN > 4-OHE (Table 3).

The lack of toxicity by 4-OHE and 4-OHE_2 in S30 cells was comparable to levels reported previously with LC_{50} values greater than 100 μM in breast cancer cell lines (*15*). In contrast, 4-OHEN and 4,17β-OHEN were potent cytotoxins in breast cancer cell lines (Table 4). Unlike 4-OHEN and 4,17β-OHEN, 4-OHE and 4-OHE_2 cannot autoxidize to *o*-quinones (*14*) and thus require catalysis by oxidative enzymes or metal ions. As a result, 4-OHE and 4-OHE_2 caused very little DNA damage, cytotoxicity, or cellular transformation in vitro (*15*, *16*). There are some other examples of quinone metabolites that induce DNA damage. Hydroquinone also induced oxidative damage to DNA catalyzed by metal ions producing both single strand cleavage and oxidation of DNA bases (*44*−*46*). Several naphthoquinones also have been shown to induce DNA damage through a mechanism involving oxidative stress (*47*, *48*).

The results of the ER binding assay and estrogenic experiments in Ishikawa cells correlated with the comet assay for single strand breaks. Both 4,17β-OHEN and 4-OHEN significantly induced more damage in the ER^+ S30 cells as compared to the ER^- MDA-MB-231 cells. In addition, 4,17β-OHEN, which has a higher affinity for the estrogen receptor, was also more effective at induction of DNA single strand breaks in S30 cells. At low concentrations, no differences were observed between these two catechol estrogens in the ER^- MDA-MB-231 cells; however, 4,17β-OHEN was more effective at higher doses in this cell line perhaps due to pharmacokinetic differences. Formation of ROS by these catechol estrogens showed that there was no significant difference between the structurally similar 4-OHEN and 4,17β-OHEN. These data suggest that the mechanism of DNA damage induced by equine estrogens and their metabolites involves oxidative stress.

In summary, we have shown that metabolites of equine estrogens induce oxidation of DNA bases as well as single strand breaks in breast cancer cells likely through a redox cycling mechanism forming reactive oxygen species (Scheme 1). In addition, DNA damage could be enhanced by agents which stimulate redox cycling or by agents which reduce the levels of cellular GSH. The estrogen receptor also appears to play a major role, since cells, which contain ERα, are much more sensitive to catechol estrogen-induced DNA damage. The extent of DNA damage correlated with the estrogenicity of the catechol estrogens, and estrogen receptor antagonists could protect cellular DNA. At present, the exact mechanism of how the estrogen receptor stimulates catechol estrogen-induced DNA damage is unknown and will be the subject of future investigations.

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