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A Metabolite of Equine Estrogens, 4-Hydroxyequilenin, Induces DNA Damage and Apoptosis in Breast Cancer Cell Lines

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Estrogen replacement therapy has been correlated with an increased risk of developing breast or endometrial cancer. 4-Hydroxyequilenin (4-OHEN) is a catechol metabolite of equilenin which is a minor component of the estrogen replacement formulation marketed under the name of Premarin (Wyeth-Ayerst). Previously, we showed that 4-OHEN autoxidizes to quinoids which can consume reducing equivalents and molecular oxygen, are potent cytotoxins, and cause a variety of damage to DNA, including formation of bulky stable adducts, apurinic sites, and oxidation of the phosphate—sugar backbone and purine/pyrimidine bases [Bolton, J. L., Pisha, E., Zhang, F., and Qiu, S. (1998) Chem. Res. Toxicol. 11, 1113–1127]. All of these deleterious effects could contribute to the cytotoxic and genotoxic effects of equilenin in vivo. In the study presented here, we examined the relative toxicity of 4-OHEN in estrogen receptor (ER) positive cells (MCF-7 and S30) compared to that in breast cancer cells without the estrogen receptor (MDA-MB-231). The data showed that 4-OHEN was 4-fold more toxic to MCF-7 cells (LC_{50} = 6.0 ± 0.2 μM) and 6-fold more toxic to S30 cells (LC_{50} = 4.0 ± 0.1 μM) than to MDA-MB-231 cells (LC_{50} = 24 ± 3 μM). Using the single-cell gel electrophoresis assay (comet assay) to assess DNA damage, we found that 4-OHEN causes concentration-dependent DNA single-strand cleavage in all three cell lines, and this effect could be enhanced by agents which catalyze redox cycling (NADH) or deplete cellular GSH (diethyl maleate). In addition, the ER+ cell lines (MCF-7 and S30) were considerably more sensitive to induction of DNA damage by 4-OHEN than the ER- cells (MDA-MB-231). 4-OHEN also caused a concentration-dependent increase in the amount of mutagenic lesion 8-oxo-dG in the S30 cells as determined by LC/MS-MS. Cell morphology assays showed that 4-OHEN induces apoptosis in these cell lines. As observed with the toxicity assay and the comet assay, the ER+ cells were more sensitive to induction of apoptosis by 4-OHEN than MDA-MB-231 cells. Finally, the endogenous catechol estrogen metabolite 4-hydroxyestrone (4-OHE) was considerably less effective at inducing DNA damage and apoptosis in breast cancer cell lines than 4-OHEN. Our data suggest that the cytotoxic effects of 4-OHEN may be related to its ability to induce DNA damage and apoptosis in hormone sensitive cells in vivo, and these effects may be potentiated by the estrogen receptor.

Introduction

Epidemiology studies have established that exposure to long-term high-dose estrogen replacement therapy (ERT) increases the risk of women developing breast or endometrial cancer (1–6). Premarin (Wyeth-Ayerst)

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1 Abbreviations: 4-OHEN, 4-hydroxyequilenin, 3,4-dihydroxy-1,2,5(10),6,8-estratetraen-17-one; 4-OHE, 4-hydroxyestrone, 3,4-dihydroxy-1,3,5(10)-oestratrien-17-one; estrone, 3-hydroxy-1,2,5(10)-oestratrien-17-one; equilenin, 1,3,5(10),6,8-estra-1,3,5(10)-triene-3,17-one; equilin, 1,3,5(10),7-estratetraen-3,17-one; E2, estrogen receptor; P450, cytochrome P450; o-quinone, 3,5-cyclohexadiene-1,2-dione; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DEM, diethyl maleate.

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enzymatically or spontaneously converted into o-quinones (8–13). As o-quinones are Michael acceptors as well as potent redox cycling agents, damage in cells could occur to DNA, lipids, and proteins.

We previously synthesized the major phase I metabolite of equilenin, 4-hydroxyequilenin (4-OHEN, Figure 1), and examined how aromatization of the B ring affects the formation and reactivity of the equilenin quinones (11). Unlike the endogenous catechol estrogens, 4-OHEN rapidly autoxidizes to the long-lived 4-OHEN-o-quinone (t1/2 = 2.3 h (11)) which readily enters into a redox couple with the semiquinone radical catalyzed by NAD(P)H, P450 reductase, or quinone reductase (11). Significant oxygen consumption was also detected, consistent with in vitro models that have shown that 4-OHEN-o-quinone significantly increases the amount of oxidative damage to DNA bases (14, 15). 4-OHEN also induced DNA single-strand breaks, a process that could be enhanced by redox cycling and inhibited by scavengers of reactive oxygen species (15). Finally, we showed that the 4-OHEN-semiquinone radical forms very unusual cyclic adducts with deoxyribonucleosides and DNA which may represent one mechanism for equilenin carcinogenesis (16, 17).

Since 4-OHEN could cause damage to naked DNA either through formation of reactive oxygen species or through alkylation of DNA bases, we examined whether similar effects could be observed in breast cancer cell lines. In addition, we investigated whether 4-OHEN could induce apoptosis in breast cancer cells since it has been shown that agents that produce reactive oxygen species in particular are very effective apoptotic inducers (18). These data suggest that agents that stimulate redox cycling as well as GSH-depleting agents can enhance 4-OHEN-mediated DNA damage and inhibition of cell cycle progression leading to apoptosis. In addition, the estrogen receptor positive cell line (MCF-7) is more sensitive than the ER negative cell line MDA-MB-231 to both DNA damage and apoptosis induction by 4-OHEN which suggests DNA damage and cell death could be influenced by the estrogen receptor. This was further confirmed by using the ERA stable transfectant S30 cell line provided by V. C. Jordan's laboratory which was constructed from the MDA-MB-231 cells as described previously (19). These cells were considerably more sensitive than the MDA-MB-231 cell line to 4-OHEN-mediated cytotoxicity, DNA damage, and induction of apoptosis. Finally, the endogenous catechol estrogen metabolite 4-hydroxyestrone (4-OHE) was much less effective at inducing both DNA damage and apoptosis in breast cancer cells.

**Materials and Methods**

Materials. Caution: The catechol estrogens were handled in accordance with NIH guidelines for the Laboratory Use of Chemical Carcinogens (20). All chemicals were purchased from Aldrich (Milwaukee, WI), Fisher Scientific (Hanover, IL), or Sigma (St. Louis, MO) unless stated otherwise. 4-OHEN was synthesized by treating equilin with Freeny's salt as described previously (14, 21) with minor modifications (11). 8-OhDA was synthesized as described previously (15). Stable isotopically labeled 8-hydroxydeoxyguanosine (every carbon and nitrogen atom labeled with 13C or 15N) for use as an internal standard in the LC/MS-MS studies was synthesized from isotopically labeled deoxyguanosine (Cambridge Isotope Laboratories, Andover, MA) using the published procedure (22). Stock solutions of catechol estrogens were prepared fresh daily in DMSO, and the concentration of DMSO in cultivation media was 0.1%.

**Cell Culture Conditions**

The MCF-7 and MDA-MB-231 cells were obtained from American Type Culture Collection (Rockville, MD). The S30 cell line was a generous gift from V. C. Jordan's laboratory (Northwestern University, Evanston, IL). MCF-7 cells were maintained in MEME medium supplemented with 1% penicillin, streptomycin, fungizone, 10 mg/L insulin, 1% nonessential amino acids, 10% fetal bovine serum (Gibco-BRL, Grand Island, NY), and 5% CO2. MDA-MB-231 cells were maintained in Liebovitz L-15 medium supplemented with 1% penicillin, streptomycin, fungizone, 10% fetal bovine serum, and 5% CO2. The S30 cells were maintained in MEME medium supplemented with 1% penicillin, streptomycin, fungizone, 6 mg/L insulin, 1% nonessential amino acids, 5% charcoal–dextran–treated fetal bovine serum, 500 μg/mL G418, 1% glutamax, and 5% CO2. The medium was routinely changed every 3 or 4 days, and it was changed every 24 h prior to beginning any experiments to maintain logarithmic growth.

**Evaluation of the Cytotoxic Potential of Catechols in Breast Cancer Cells**

Cell viability was assessed by trypan blue exclusion (23, 24). Briefly, the cells (104 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 then 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 LC₅₀ values were obtained by linear regression analysis, and the data represent the average ± SD of triplicate determinations.

Evaluation of DNA Damage in Breast Cancer Cells Using the Alkaline Single-Cell Gel Electrophoresis Assay (Comet Assay (25)). Briefly, the cells (3 × 10⁶ cells/mL) were incubated with various concentrations of catechol estrogens or DMSO for 3 h. In some cases, the cells were pretreated for 1 h with DEX (1 μM), NADH (15 μM), tamoxifen (1 nM), or vehicle (2 μL) prior to addition of 4-OHEN. After incubation, the cells were washed with PBS and suspended in 1 mL of PBS. The cell suspension (30 μL) was mixed with 80 μL of 1% low-melt agarose and added to a fully frosted slide precoated with 1% agarose. The gel was covered with a cover slip and incubated in the dark at 4 °C for 5 min. The slides were immersed in a lysis solution (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, and 1% Triton X-100 (pH 10.0)) for 1 h and then incubated in an electrophoresis buffer (300 mM NaOH and 1 mM EDTA) at 4 °C for an additional 40 min. After electrophoresis, the slides had been washed at 25 °C for 30 min. After electrophoresis, the slides were washed three times with a neutralizing buffer (0.4 M Tris (pH 7.5)), stained with 1 μg/mL ethidium bromide for 20 min, and destained with 2 M NaCl (10 mM). Comets were observed by fluorescence microscopy. Using DNA from at least 100 cells, 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 N₀ (intact DNA) and Nₚ–N₀ (completely damaged DNA) were the number of different kinds of comets.

\[
\text{score (S)} = \left( N₀ + 2N₁ + 3N₂ + 4Nₚ \right) / \left( N₀ + N₁ + N₂ + Nₚ + Nₚ \right) \times 100
\]

4-OHEN-Mediated Oxidation of DNA in Breast Cancer Cells. The breast cancer cells (5 × 10⁶ cells) were incubated with 4-OHEN (10 μM) or DMSO (10 μL) for 90 min or 18 h at 37 °C. At the conclusion of the incubation, floating cells were collected by centrifugation at 3000 rpm for 5 min and attached cells were first trypsinated and then harvested by centrifugation. Floating cells and attached cells were combined and washed with PBS. To isolate DNA, the cell pellets were homogenized in 35 mL of lysing buffer at 4 °C containing 320 mM sucrose, 10 mM Tris (pH 7.4), 5 mM MgCl₂, 10 mM Tris Triton X-100, and 50 mM mannitol. After centrifugation, the nuclei pellet was treated for 30 min at 37 °C with RNase T1 (20 units) and RNase A (0.2 mg/mL EDTA, 10 mM Tris (pH 7.4), and 0.45 M NaCl. The DNA content was measured at 260 nm, and the DNA was treated for 30 min at 37 °C with protease K (0.64 mg/mL). NaCl and additional Tris buffer were then added until the solution was 0.62 M and 20 mM in each, respectively. An equal volume of n-butanol was added; the solution was centrifuged, and the bottom layer was isolated. After ethanolic precipitation, the DNA pellet was washed twice with 70% ethanol at 4 °C and then dissolved in 100 mL of ammonium acetate (25 mM, pH 5.3) containing 0.1 mM ZnCl₂. The DNA was hydrolyzed using nuclease P1 (3.9 units) and alkaline phosphatase (5 units) at 37 °C for 90 min. The enzymes were removed prior to LC/MS-MS analysis using ultrafiltration with a 10,000 molecular weight cut-off membrane. The ultrafiltrate solution was analyzed using LC/MS-MS on a triple-quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with negative ion electro spray ionization according to the method of Wainhaus et al. (26). Stable isotope-labeled 8-oxo-dG was used as an internal standard. Samples were separated using an ODS C18 column (2.0 mm × 250 mm, 5-μm, YMC, Wilmington, NC) at a rate of 0.2 mL/min with a gradient mobile phase consisting of 18% methanol in water for 20 min and then increasing to 25% methanol in water over 7 min. 8-Oxo-dG and 8-Oxo-dA were detected using multiple-reaction monitoring with collision-induced dissociation for the fragmentation pathways of m/z 282 → 192 and m/z 266 → 176, respectively. In each case, the deproteinized molecules were used as the precursor ions.

Under these conditions, the limit of detection and retention times of the oxidized deoxyribonucleosides were as follows: 8-oxo-dG, 7.4 fmol and 26 min; and 8-oxo-dA, 3.9 fmol and 37 min.

Acridine Orange/EthBr Staining Assay. To quantify apoptosis in breast cancer cell lines, the procedure of Mercille and Massie was followed (27). Briefly, the cells (10⁶ 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 trypsinated and then harvested by centrifugation. Floating cells and attached cells were combined, washed with PBS, and harvested by centrifugation at 3000 rpm for 4 min. The supernatant was removed, and the cells were stained with 2 μL of acridine orange/ethidium bromide stain (100 μg/mL acridine orange and 100 μg/mL ethidium bromide in PBS). An aliquot (10 μL) of the stained cell suspension was placed on a microscope slide and covered with a coverslip. Cell morphology of at least 100 cells was examined with a fluorescence microscope.

DAPI Nuclear Staining Assay (28). The breast cancer cells (10⁶ cells/mL) were incubated with various concentrations of catechol estrogens or vehicle only for 18 h. At the conclusion of the incubation, floating cells were collected by centrifugation at 3000g for 15 min, and attached cells were first trypsinated and then harvested by centrifugation. Floating cells and attached cells were combined and washed with PBS before being fixed in a solution of MeOH and acetic acid (3:1) for 30 min. Fixed cells were placed on slides and, after evaporation of the fixing solution, were stained with 1 μg/mL DAPI for 15 min. The nuclear morphology of the cells was observed by fluorescence microscopy.

Instrumentation. HPLC experiments were performed on a Shimadzu LC-10A gradient HPLC system equipped with an SIL-10A autoinjector, an SPD-M10AV UV/VIS photodiode array detector, and an SPD-10AV UV detector. Peaks were integrated with Shimadzu EZ-Chrom software and a 486-33 computer. LC/MS-MS experiments were carried out using a Micromass Quattro II electrospray triple-quadrupole mass spectrometer equipped with a HP series 1050 HPLC system consisting of a binary pump and autosampler. Operating parameters for MS/MS included a cone voltage of 20–25 V, an argon collision gas pressure of 2.5 × 10⁻⁴ mbar, and a source temperature of 110 °C. LC/MS-MS analysis was carried out using multiple-reaction monitoring (as described above), and the peak areas were calculated using Micromass MassLynx software. The fluorescence microscope used for morphological quantitation during the acridine orange/ethidium bromide staining assay was the Axioskop universal microscope with 10 × 40 Plan-Neofluar objectives (Zeiss). The filter for visualizing cell morphology was the FITC/P1 filter with BP485 (Excitation), FT510 (Dichroic), LP520 (Barrier) filter from Zeiss. The same fluorescence microscope was used for the comet assay except the lenses were 10 × 10 Plan-Neofluar objectives. The filter for visualizing DNA was the BP546 (Excitation), FT580 (Dichroic), LP590 (Barrier) filter from Zeiss. The fluorescence microscope used for the DAPI nuclei staining assay was the Axioskop microscope with 10 × 40 Plan-Neofluar objectives (Zeiss). The filter for visualizing DAPI-stained nuclei was the BP365 (Excitation), FT395 (Dichroic), LP397 (Barrier) filter from Zeiss.

Results

Effect of 4-OHEN on Cell Viability in Breast Cancer Cells. Previously, we have shown that 4-OHEN is a potent cytotoxin in hormone sensitive cells using the protein-binding dye sulforhodamine B (SBR) assay which assesses the ability of compounds to inhibit the growth of cells (11, 29, 30). Although useful for comparative purposes, this assay is not able to give a good indication of the relative ability of the test compound to cause cell death. In this study, we employed the trypan blue dye
**Figure 2.** Cytotoxic potency of 4-OHEN to MDA-MB-231 cells (■), MCF-7 cells (●), and S30 cells (○). The cells (10^5 cells/mL) were incubated with various concentrations of 4-OHEN for 18 h as described in Materials and Methods. The data represent the average ± SD of triplicate determinations. For MDA-MB-231, LC₅₀ = 24 ± 0.3 μM. For MCF-7, LC₅₀ = 6.0 ± 0.2 μM. For S30, LC₅₀ = 4.0 ± 0.1 μM.

exclusion assay which gives an accurate assessment of cell death since living cells exclude the dye whereas dead cells with ruptured cell membranes do not (23). The data, shown in Figure 2, suggest that the estrogen receptor positive cell lines (MCF-7 and S30) are more sensitive to induction of cell death by 4-OHEN than the estrogen receptor negative cell line (MDA-MB-231); the LC₅₀ for 4-OHEN in MCF-7 cells after 18 h was 6.0 ± 0.2 μM, whereas the LC₅₀ for the MDA-MB-231 cell line was 24 ± 0.3 μM which is 4-fold higher than in MCF-7 cells. The S30 cell line was the most sensitive to the toxic effects of 4-OHEN (LC₅₀ = 4.0 ± 0.1 μM). This cell line is the MDA-MB-231 cell line stably transfected with ERα which means we are now directly comparing the effects of the estrogen receptor on cell viability. Finally, similar experiments with the endogenous catechol estrogen 4-OHE revealed that the LC₅₀ in MDA-MB-231 cells is 640 ± 1 μM (data not shown) which is 27-fold higher than the LC₅₀ for 4-OHEN.

**4-OHEN-Induced DNA Single-Strand Cleavage in Breast Cancer Cells.** Our previous in vitro study showed that 4-OHEN can cause dose- and time-dependent DNA single-strand breaks in phage DNA, and the extent of damage can be increased by agents that induce redox cycling and diminished by scavengers of reactive oxygen species (18). In the study presented here, it was of interest to examine the relative ability of 4-OHEN to cause DNA damage in breast cancer cell lines. The “comet assay” (or single-cell gel electrophoresis) is a simple, sensitive, and reliable method of micro-electrophoresis which allows assessment of DNA fragmentation in individual cells (25). The amount of DNA that is able to migrate in an electric field depends on the number of strand breaks and the size of the DNA fragments. Recently, the comet assay has been widely used to study DNA damage (31). For example, single-strand DNA breaks were detected immediately after H₂O₂ treatment in the hamster V79 and human CaCo2 cell lines using the comet assay (32).

The breast cancer cell lines were treated with 4-OHEN, and DNA damage was assessed using the comet assay. As shown in Figure 3A, the DNA from MDA-MB-231 cells treated with DMSO migrated as spherical dots with no evidence of tails, indicating the DNA was intact. In contrast, increasing amounts of 4-OHEN lead to a corresponding increase in the size of the tails (comets) up to the 50 μM dose where very little intact DNA remains (Figure 3C). The results of the comet assay are displayed graphically in Figure 4. A dose-dependent increase in the extent of DNA damage was observed in all three cell lines; however, MCF-7 cells (Figure 4A) which are from a cell line which contains the estrogen receptor were considerably more sensitive to induction of DNA single-strand cleavage than MDA-MB-231 cells (ER−, Figure 4B). Even more dramatic are comparisons between MDA-MB-231 cells (Figure 4B) and the ERα-transfected cells (S30, Figure 4C) where 3–3.5-fold increases in DNA damage were observed in the latter cell line. Including NADH in the incubations enhanced DNA damage in all three cell lines (Figure 4), confirming our previous work which showed that agents that promote redox cycling enhanced the amount of DNA damage to
Table 1. LC/MS-MS Analysis of 8-Oxo-dG Formed in S30 Cells Treated with 4-OHEN

<table>
<thead>
<tr>
<th>reaction conditions</th>
<th>90 min</th>
<th>18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0.26 ± 0.03</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td>4-OHEN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 µM</td>
<td>0.33 ± 0.04</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>5 µM</td>
<td>0.38 ± 0.04 b</td>
<td>0.43 ± 0.05</td>
</tr>
<tr>
<td>7.5 µM</td>
<td>0.41 ± 0.04 b</td>
<td>0.77 ± 0.12 b</td>
</tr>
<tr>
<td>10 µM</td>
<td>0.44 ± 0.08 b</td>
<td>0.94 ± 0.8</td>
</tr>
<tr>
<td>15 µM</td>
<td>0.51 ± 0.06 b</td>
<td>1.1 ± 0.08 b</td>
</tr>
</tbody>
</table>

* Cells (5 × 10⁶ cells) were incubated with various concentrations of 4-OHEN or DMSO (10 µL) for 90 min or 18 h. Experimental details are described in Materials and Methods. Data represent the average ± SD of duplicate determinations. * Significantly different from DMSO (p < 0.05).

4-OHEN induces concentration-dependent increases in the extent of 8-oxo-dG formation up to a maximum of 3.5-fold at 15 µM 4-OHEN over DMSO-treated controls (Table 1). At the 90 min time point, less dramatic increases in the level of 8-oxo-dG were detected (2-fold at the 15 µM dose) which may reflect the action of DNA repair enzymes in these live cells. At the 18 h time point, the trypan blue data (Figure 2) clearly show that the S30 cells are no longer viable above 10 µM and DNA repair enzymes could not operate to repair the oxidized bases. At both time points, the levels of 8-oxo-dA were below the level of detection in the study presented here in contrast to studies with calf thymus DNA, where significant increases in the amount of this oxidized base were observed (15).

**Induction of Apoptosis in Breast Cancer Cell Lines by 4-OHEN.** Since 4-OHEN can induce DNA single-strand cleavage, oxidation of DNA bases, and cell death in breast cancer cell lines, it was of interest to examine if cell death occurred through a necrotic and/or apoptotic mechanism. To characterize the mechanism of cell death, the morphology of the breast cancer cells which had been treated with 4-OHEN was examined under a fluorescence microscope using the fluorescent intercalating DNA-binding agents acridine orange and ethidium bromide. Cells that were treated with the vehicle displayed only normal morphology. In contrast, after these cells were treated with 4-OHEN for 18 h, a dose-dependent increase in cell death was observed. Morphological evaluation of the cells after staining with acridine orange and ethidium bromide revealed that the mechanism of cell death for some of these cells proceeded through apoptosis as opposed to necrosis since condensation or fragmentation of the DNA could be observed, as well as the formation of apoptotic bodies (data not shown). A graphical representation of the data is shown in Figure 5. Dose-dependent increases in the extent of apoptosis were observed in the MCF-7 (●) and MDA-MB-231 (■) (Figure 5A) and S30 cell lines (Figure 5B) up to a maximum of 10 µM. Above this concentration, there was an increase in cell death by necrosis at the expense of apoptosis. As observed with the relative ability of 4-OHEN to induce DNA damage in these cell lines, the ER+ cells (MCF-7 and S30) were more sensitive to induction of apoptosis than the MDA-MB-231 cells (ER-). At the 10 µM dose, 26% of the S30 cells were apoptotic as compared to 7% of the MDA-MB-231 cells. Finally, including NADH which complements the redox couple leading to an increase in production of reactive oxygen species enhanced the ability of 4-OHEN to induce...
apoptosis in all three cell lines (Figure 5, open symbols).

To further confirm that cell death was occurring through apoptosis, we employed another morphological assay called the DAPI nuclear staining assay (Figure 6). In this assay, cells are fixed and then the nuclei stained with the DNA binding agent DAPI. Like what was observed with the acridine orange/ethidium bromide assay, all three cell lines showed a dose-dependent increase in the level of apoptosis after 4-OHEN treatment. In addition, MCF-7 cells were more sensitive to induction of apoptosis than MDA-MB-231 cells and NADH was able to enhance the level of apoptotic cells compared to that of cells treated with 4-OHEN alone (Figure 6A). Further confirmation of the importance of the estrogen receptor to 4-OHEN-mediated induction of apoptosis in breast cancer cells was obtained in S30 cells using the DAPI nuclear staining assay. As can be seen in Figure 6B, this ERα-transfected cell line was approximately 3 times more sensitive to induction of apoptosis than the ER− MDA-MB-231 cells. This increase in sensitivity in the S30 cells corresponds to the increases (3-fold) in the extent of DNA damage observed in this cell line using the comet assay. Like the data described above for MCF-7 and MDA-MB-231 cells, NADH and DEM increased the apoptotic percentage in the S30 cells to approximately 35–40% at the 7 μM dose. Above this dosage, the extent of apoptosis rapidly declined in a concentration-dependent manner, indicating a change in the cell death mechanism from apoptosis to necrosis. Finally, as observed with the DNA damage experiments,

physiological concentrations of tamoxifen protected the S30 cells from induction of apoptosis (Figure 6B) presumably by blocking the estrogen receptor. In contrast, tamoxifen had no effect on 4-OHEN-mediated induction of apoptosis in the ER− MDA-MB-231 cells (Figure 6A).

**DNA Damage and Induction of Apoptosis by 4-OHEN Compared to Apoptosis by 4-OHE.** It was of interest to compare the relative apoptotic induction ability of the carcinogenic endogenous catechol estrogen, 4-OHE, with that of 4-OHEN in the MCF-7 cell line. As shown in Figure 7, while 4-OHEN caused dose-dependent DNA damage as determined by the comet assay (Figure 7A) and induction of apoptosis as determined by the DAPI assay (Figure 7B), very little DNA damage or increase in apoptotic percentage was observed with 4-OHE. These data suggest that like what was observed with the trypan blue exclusion assay for cell death, 4-OHEN should be considerably more effective at inducing DNA damage and apoptosis than 4-OHE in vivo.

**Discussion**

Previously, we showed that 4-OHEN was capable of causing a variety of detrimental effects to naked DNA, including the formation of stable bulky adducts, apurinic sites, single-strand breaks, and oxidation of the DNA bases (13). In the study presented here, 4-OHEN-induced DNA damage was assessed in breast cancer cell lines using the comet assay which is a simple, sensitive, and
oxidized base, 8-oxo-dG, after treating S30 cells with 4-OHEN (Table 1). The extent of the increase was significantly more modest (2–3.5-fold) than that detected with calf thymus DNA and 4-OHEN [100-fold (15)]; however, it must be emphasized that whole cells are considerably more complex than reaction of 4-OHEN with DNA in vitro. The organization of nuclear DNA into nucleosomes and higher structures, the numerous reaction sites in cells, and cellular DNA repair mechanisms likely account for the slight increases in the level of oxidative damage to cellular DNA bases as compared to that for naked DNA. These data are particularly intriguing since 8-oxo-dG (33) is a mutagenic lesion associated with a heightened cancer risk. In addition, elevated levels of 8-oxo-dG have been detected in the breast tissue of cancer patients compared to controls, consistent with a major role of reactive oxygen species in cancer initiation and/or progression (34, 35).

Since we had detected single-strand DNA cleavage and oxidation of DNA bases in breast cancer cells, it was of interest to ascertain if 4-OHEN could induce apoptosis in these cell lines. Initially, we performed the trypan blue dye exclusion assays to determine the LC50 for 4-OHEN which would give us an accurate assessment of total cell death (apoptosis and necrosis). As observed with the DNA damage experiments (Figures 3 and 4), MCF-7 cells were 4 times more sensitive and the S30 cells 6 times more sensitive to the cytotoxic effects of 4-OHEN than MDA-MB-231 cells (Figure 2). Two independent experiments which included the arccride orange/ethidium bromide cell morphology assay (Figure 5) and the DAPI nuclear staining assay (Figure 6) verified that the mechanism of cell death proceeded through a combination of apoptosis and necrosis in all three cell lines. The maximum apoptotic percentage (approximately 11%) in MCF-7 cells occurred at 10 μM where the percent of total cell death is approximately 65%. As a result, the majority of MCF-7 cells die through a necrotic mechanism. Similar effects were observed with the MDA-MB-231 cells, although this cell line was less sensitive to induction of apoptosis (7%) and necrosis (13%) by 4-OHEN (10 μM, 18 h). With the S30 cell line, maximum apoptosis induction was observed at the 7 μM dose (24%) where 86% of total cell death occurs in 18 h. Therefore, the estrogen receptor appears to play a role in 4-OHEN-mediated induction of both apoptotic and necrotic cell death.

We found that the addition of NADH which promotes redox cycling of 4-OHEN and the generation of reactive oxygen species (11) enhanced the apoptotic percentage of cells in all three cell lines, suggesting a crucial role for oxidative stress in the induction of apoptosis. As mentioned earlier, NADH also increased the level of DNA single-strand breaks, implying that the generation of reactive oxygen species leads to DNA damage which triggers apoptosis and necrosis in these cells. Similarly, DNA damage, apoptosis, and necrosis were enhanced when the cells were depleted of GSH by pretreatment with diethyl maleate. GSH acts to scavenge the 4-OHEN-o-quinone in addition to maintaining the redox balance within the cells (36); thus, depletion of this protective nucleophile and/or reducing agent leads to increased production of reactive oxygen species, DNA damage, and cell death. Finally, we compared the relative ability of the endogenous catechol estrogen, 4-OHE, to induce DNA damage and apoptosis in MCF-7 cells (Figure 7). Unlike 4-OHEN, 4-OHE does not autoxidize (11) and...
thus requires catalysis by oxidative enzymes or metal ions to generate the o-quinone. As a result, 4-OHE caused very little DNA damage or cytotoxicity through either apoptosis or necrosis in the MCF-7 cells. These data suggest that 4-OHEN would be much more likely than 4-OHE to cause these deleterious effects in vivo.

There are other examples of quinone metabolites that induce DNA damage and/or apoptosis which may contribute to the cytotoxic and genotoxic effects of the parent compounds. For example, hydroxyquinone, a potentially carcinogenic metabolite of benzene, was found to induce apoptosis in HL-60 cells (38). Hydroquinone also induces oxidative damage to DNA catalyzed by metal ions, producing both single-strand cleavage and oxidation of DNA bases (38-40). Similarly, the benzquinone metabolite of butylated hydroxytoluene, 2,6-di-tert-butyl-1,4-benzquinone, induced both DNA damage and apoptosis in human leukemic cell lines in the presence of NADH (41), although the doses required to produce these effects were considerably higher (2 mM) than the 4-OHEN dose (1-10 μM). Finally, several naphthoquinones, including menadione, have been shown to induce DNA damage and/or apoptosis through a mechanism involving oxidative stress (42, 43).

The relationships between DNA damage, apoptosis, necrosis, and carcinogenesis are poorly understood (44). Cells that undergo apoptosis following DNA damage do not contribute to the induction of carcinogenesis. However, at concentrations lower and time points earlier than those required for apoptosis, DNA damage could occur, leading to mutations in these surviving cells. 4-OHEN appears to be a potent inducer of both DNA damage and apoptosis (Figure 1) at concentrations far below (1-10 μM) the concentrations of classical apoptotic inducers such as etoposide (50-100 μM range (45)). To the best of our knowledge, the concentration of 4-OHEN or equilenin in the breast or endometrium is not known. However, on the basis of estimates for the concentrations of endogenous estrogens, we would predict they would be in the nanomolar range. As a result, it is quite possible that at physiologically relevant concentrations, 4-OHEN is capable of causing DNA damage, mutations, and carcinogenesis in these hormone sensitive target tissues.

In summary, on the basis of these data, it may be concluded that the involvement of quinoids in 4-OHEN-mediated DNA damage and triggering of apoptosis and necrosis depends on a combination of the rate of formation of the o-quinone, the rate of disappearance of the o-quinone, and the electrophilic and/or redox reactivity of the quinoids. The formation of reactive oxygen species through redox cycling between the o-quinone and the catechol and semiquinone radical (Figure 1) likely plays a crucial role in initiating all of these effects. For the endogenous catechol estrogen 4-OHE, oxidative enzymes are necessary to form the o-quinone prior to DNA damage, induction of apoptosis, and cell death, and as a result, 4-OHE is considerably less effective than 4-OHEN. All of these critical factors are closely linked to structure as well as microenvironment, and the details are just beginning to be elucidated. Given the direct link between excessive exposure to estrogens, metabolism of estrogens, and increased risk of breast cancer, it is crucial that factors which affect the formation, reactivity, and cellular targets of estrogen quinoids be explored.

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