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Evidence That a Metabolite of Equine Estrogens, 4-Hydroxyequilenin, Induces Cellular Transformation in Vitro

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Estrogen replacement therapy has been correlated with an increased risk of developing hormone-dependent cancers. 4-Hydroxyequilenin (4-OHEN) is a catechol metabolite of equilenin and equinol which are components of the estrogen replacement formulation marketed under the name of Premarin (Wyeth-Ayerst). Previously, we showed that 4-OHEN autodizes to potent cytotoxic quinoids which can consume reducing equivalents and molecular oxygen, 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 [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/genotoxic effects of equine estrogens in vivo. In the study presented here, we studied the oxidative and carcinogenic potential of 4-OHEN and the catechol metabolite of the endogenous estrogen, 4-hydroxyestrone (4-OHE), in the JB6 clone 41 5a and C3H 10T1/2 murine fibroblast cells. The relative ability of 4-OHEN and 4-OHE to induce oxidative stress was measured in these cells by oxidative cleavage of 2',7'-dichlorodiacetylfluoresceindiacetate to dichlorofluoroscein. 4-OHEN (1 μM) displayed an increase in the level of reactive oxygen species comparable to that observed with 100 μM H2O2. In contrast, 4-OHE demonstrated antioxidant capabilities in the 5–50 μM range. With both cell lines, we assessed single-strand DNA cleavage using the comet assay and the formation of oxidized DNA bases, such as 8-oxo-7,8-dihydro-2'-deoxyguanosine, utilizing the Trævigen Fpg comet assay. 4-OHEN caused single-strand breaks and oxidized bases in a dose-dependent manner in both cell lines, whereas 4-OHE did not induce DNA damage. Since oxidative stress has been implicated in cellular transformation, we used the JB6 clone 41 5a anchorage independence assay to ascertain the relative ability of 4-OHEN and 4-OHE to act as tumor promoters. 4-OHEN caused a slight but significant increase in the extent of cellular transformation at the 100 nM dose; however, in the presence of NADH, which catalyzes redox cycling of 4-OHEN, the transformation ability of 4-OHEN was dramatically increased. 4-OHE did not induce transformation of the JB6 clone 41 5a in the 0.1–10 μM range. The initiation, promotion, and complete carcinogenic transformation potentials of both metabolites were measured in the C3H 10T1/2 cells. 4-OHEN demonstrated activity in all stages of transformation at doses of 10 nM to 1 μM, whereas 4-OHE only demonstrated promotional capabilities at the 10 μM dose. These data suggest that oxidative stress could be partially responsible for the carcinogenic effects caused by 4-OHEN and that 4-OHEN is a more potent transforming agent than 4-OHE in vitro.

Introduction

The risk factors associated with postmenopausal estrogen replacement therapy are highly controversial (1–5). The many benefits of estrogen replacement therapy include a substantial reduction in the risk of osteoporosis (5) and the alleviation of menopausal symptoms. However, it is known that long-term high-dose estrogen replacement therapy is associated with a slight increase in the risk of developing breast cancer and a more significant increase in the risk of developing endometrial cancer (2, 6, 7). As a result, it is crucial to fully understand all the deleterious effects of estrogens, including their potential to initiate and/or promote the carcinogenic process.

Premarin (Wyeth-Ayerst) is the most widely prescribed estrogen replacement formulation, and yet there is very little information about the potential cytotoxic/genotoxic effects of the many different estrogens present in Premarin, which include the endogenous estrogens, estrone and estradiol, and the equine estrogens, equinol and equilenin. It is known that treating hamsters for 9

Figure 1. Redox cycling by 4-OHEN generating reactive oxygen species and DNA damage, and potentially initiating carcinogenesis.

months with either estrone, equilenin, or sulfatase-treated Premarin resulted in 100% tumor incidences and abundant tumor foci (8). Several possible pathways could be involved with estrogen-induced carcinogenesis, such as mitogenic stimulation of cells as well as various genotoxic effects induced by reactive estrogen metabolites (9). For example, several studies have shown that endogenous and estrogenic carcinogens can be oxidized by cytochrome P450 to catechols, which could be enzymatically or spontaneously converted to o-quinones (9-16). O-Quinones are Michael acceptors which cause damage in cells through alkylation of DNA, lipids, and proteins. In addition, they are potent redox cycling agents generating reactive oxygen species (ROS) leading to oxidation of cellular macromolecules. Oxidative stress and cellular damage from ROS have been implicated in both the initiation and promotion stages of carcinogenesis (17).

Previously, we showed that the major phase I metabolite of both equilenin and equilenin was 4-OHEN (13-15; Figure 1). This catechol estrogen was found to autoxidize to an o-quinone which caused depletion of reducing equivalents and molecular oxygen, alkylation of drug-metabolizing enzymes, and a variety of DNA lesions, including single-strand cleavage, oxidation of DNA bases, and formation of apurinic sites and bulky stable adducts (14). In addition, we recently reported that 4-OHEN induces DNA damage and apoptosis in breast cancer cell lines and that these effects could be potentiated by the presence of estrogen receptor alpha (18). In contrast, we determined that the carcinogenic catechol metabolite of estrone, 4-hydroxyestrone (4-OHE), did not induce these deleterious effects under similar conditions (14, 18).

In the investigation presented here, we examined the relative ability of 4-OHEN as well as 4-OHE to produce reactive oxygen species potentially leading to DNA damage in vitro using the JB6 clone 41 5a and the C3H 10T1/2 cell lines. We also studied the relative ability of these catechol estrogens to act as tumor promoters and/or complete carcinogens with transformation studies in the same cell lines. Our data suggest that 4-OHEN has the potential to be a much more effective tumor promoter and complete carcinogen than 4-OHE, perhaps due to the ability of 4-OHEN to generate considerably more reactive oxygen species and DNA damage in these cell lines.

Materials and Methods

Materials. Caution: The catechol estrogens were handled in accordance with NIH Guidelines for the Laboratory Use of Chemical Carcinogens (19). All chemicals were purchased from Aldrich (Milwaukee, WI), Fisher Scientific (Itasca, IL), or Sigma (St. Louis, MO) unless stated otherwise. 4-OHEN was synthesized by treating equilenin with Fremy's salt as described previously (20, 21) with minor modifications (13). Minimum essential media with Earle's salts, PSF, and nonessential amino acids were obtained from Gibco-BRL (Grand Island, NY). Fetal bovine serum was purchased from Atlanta Biologicals (Atlanta, GA). The Fgf FLARE Module for the detection of oxidative damage was purchased from Trevigen Inc. (Gaithersburg, MD).

Cell Culture Conditions. The C3H 10T1/2 and JB6 clone 41 5a cells were purchased from American Type Culture Collection (Rockville, MD). The C3H 10T1/2 cells were maintained in MEME supplemented with 10% FBS. Only cells at passage number 15 or lower were utilized for the transformation assays. The JB6 clone 41 5a cells were maintained in MEME amended with nonessential amino acids, PSF, and 5% FBS. All cell lines were cultured in humidified 5% CO2 and at subconfluence to minimize the risk of self-transformation. Test samples were freshly dissolved in DMSO and then diluted 1000-fold in medium for a final DMSO concentration of 0.01%.

Evaluation of the Cytotoxic Potential of Catechols. Cell viability was determined by the trypan blue exclusion assay (22, 23). Briefly, following the appropriate treatment, all floating cells were collected by centrifugation and attached cells were first trypsinized and then added to the floating cells for the determination of total cellular viability. The cells were pelleted, washed with PBS, stained with 0.4% trypan blue, and counted microscopically.

Assessment of Intracellular ROS (24). Cells were collected by scraping and were washed twice with PBS. A 50 μL aliquot (6 x 10⁶ cells/mL) was added to the center wells of a 96-well fluorescent plate. The cells were kept on ice until the addition of the test compounds and DCDF-DA. DCDF-DA, H₂O₂, 4-OHE, and 4-OHEN were initially dissolved in methanol and then diluted 100-fold in PBS. Test compounds (25 μL) and 40 μg/mL DCDF-DA (25 μL) were added to the cells. The plates were read (excitation at 485 nm, emission at 539 nm) on a Packard FluorCount (Meriden, CT) at 3 h. A standard curve of DCF was also treated in the same manner.

Evaluation of DNA Damage in Breast Cancer Cells Using the Alkaline Single-Cell Gel Electrophoresis Assay (comet assay) (25, 26). Cells were treated for 3 h and harvested in a manner similar to that described for the trypan blue exclusion assay. Following the PBS wash, a suspension of 30 μL of 2-4 x 10⁶ cells/mL was added to 80 μL of 1% low-melting agar. The cell/agar mixture was layered onto a 1% high-melting agar precoated frosted microscope slide. After the agar had been allowed to set at 4 °C, the slides were immersed in lysis buffer [100 mM Na₂EDTA, 2.5 M NaCl, 10 mM Tris (pH 10.0), and 1% Triton X-100] for 1 h at 4 °C. The slides were then transferred to alkaline electrophoresis buffer (300 mM NaOH and 1 mM Na₂EDTA) for 40 min at 4 °C. The slides were electrophoresed at 35 V for 30 min at 4 °C. The slides were incubated three times for 5 min with neutralizing buffer [0.4 M Tris (pH 7.5)] at 4 °C. The DNA was stained with EtBr (1 μg/mL) for 20 min at room temperature, and excess EtBr was removed by 10 mM MgSO₄. 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)

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1 Abbreviations: 4-OHEN, 4-hydroxyestrone, 3,4-dihydroxy-1,3,5-(10,8,6-estratres-17-one; 4-OHE, 4-hydroxyestrone, 3,4-dihydroxy-1,3,5(10,8,6-estratres-17-one; DCF, dichlorofluorescein; DMSO, dimethyl sulfoxide; EtBr, ethidium bromide; Fpg, E.coli formamidopyrimidine DNA glycosylase; MC, 3-methylcholanthrene; PBS, phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 0.115% Na₂HPO₄, and 0.02% KH₂PO₄); PSF, pantocillin-streptomycin-fungizone; ROS, reactive oxygen species; TPA, 12-O-tetradecanoyl phorbol 13-acetate.
Results

Evaluation of the Cytotoxic Potential of Catechol Estrogens. To establish appropriate concentrations of the catechol estrogens for the transformation assays, it was necessary to measure cytotoxicity. Trypan blue exclusion was chosen due to the ease of the procedure and interpretation of the data. We chose a 3 h time point for toxicity to correlate with the ROS induction and DNA damage experiments as well as a 24 h toxicity time point for the C3H 10T1/2 transformation study to observe any increases in toxicity over time and to correlate with the initiation regime of the C3H 10T1/2 study. In the C3H 10T1/2 cells, 4-OH-EN exhibited significant toxicity; the LC50 values were 5.4 and 3.2 μM at 3 and 24 h, respectively. In the JB6 clone 41 5a cells, 4-OH-EN was less toxic with an LC50 value of 10 μM at 3 h. The addition of NADH, which completes the redox couple, caused a slight increase in the toxicity observed with 4-OH-EN; the 3 h LC50 values were 5.0 and 8.1 μM for C3H 10T1/2 and JB6 clone 41 5a cells, respectively. 4-OH-DE did not cause any measurable toxicity at 10 μM following a 3 h incubation with these cell lines, and the addition of NADH did not increase toxicity.

Generation of Cellular ROS. The oxidant sensitive dye DCF-DA has been employed to directly assess ROS induction in several different cell types, including lung (30), liver (31), neurons (32), and granulocytes (24). In this study, both cell lines responded to a H2O2 challenge with an increase in the level of the oxidation product, DCF, indicating that the assay could be utilized for ROS measurements. Within 3 h, the positive control, H2O2 (100 μM), induced a 2–3-fold increase in fluorescence in both cell lines (data not shown). 4-OH-EN induced ROS formation in both cell lines in a dose-dependent manner (Figure 2A); C3H 10T1/2 and JB6 clone 41 5a cells treated with 10 μM 4-OH-EN exhibited a relative fluorescence 2 and 1.2 times greater than the background, respectively, which is more than half of that observed with H2O2. While this dose is within the toxic range, significant fluorescence was exhibited at 4-OH-EN concentrations as low as 100 nM in both cell lines. These results indicate that 4-OH-EN can produce cellular ROS species at toxic doses. Comparability to the toxic data was the increased response of the C3H 10T1/2 cells, relative to the JB6 cells, toward 4-OH-EN.

In contrast, the addition of 4-OH-E caused a suppression of native cellular ROS production at 3 h (Figure 2B), suggesting that this catechol estrogen functions as an antioxidant instead of as a prooxidant. Both cell lines showed a decrease of 30–40% in normalized fluorescence at 5–100 μM 4-OH-E. The antioxidant capabilities of 4-OH-E were further studied with H2O2 co-incubation; 4-OH-EN suppressed the H2O2-induced ROS formation in a dose-dependent manner in both cell lines with dose–response curves similar to those observed without the addition of H2O2 (data not shown).

4-OH-EN-Induced DNA Damage in Cell Lines. Cellular responses to ROS include lipid, protein, and DNA oxidation and transcription factor(s) activation (17). Single-strand DNA breaks and formation of 8-oxodeoxyguanosine have been extensively used as markers for DNA oxidation (17, 33). The comet assay measures the number of single-strand DNA breaks and alkali labile DNA sites in individual cells, and enzymatic modification of the standard comet assay allows for the qualitative
Figure 2. Modulation of reactive oxygen species by catechol estrogens. (A) Relative fluorescence of C3H 10T1/2 (●) and JB6 clone 41 (■) cells treated for 3 h with 4-OHEN. (B) Relative fluorescence of C3H 10T1/2 (C) and JB6 clone 41 (D) cells treated for 3 h with 4-OHE. Fluorescence was normalized to solvent-treated controls.

determination of oxidized bases (26). Results from the standard comet assay are shown in panels A and B of Figure 3, which demonstrated that 4-OHEN caused a dose-dependent increase in the number of DNA single-strand breaks in both cell lines and the addition of NADH caused an increase in the severity of the breaks. In contrast, 4-OHE did not cause single-strand breaks after 3 h up to a dose of 100 µM (data not shown). Similar to the toxicity and ROS data, C3H 10T1/2 cells displayed a slight increase in sensitivity toward 4-OHEN. The effects of the initiation concentration used in the transformation assay of MC (0.9 µM) or 4-OHEN (100 nM to 10 µM) following treatment for 24 h were also determined to observe any possible relationship between DNA damage and initiation effect. The results indicated that the damage caused by 1 µM 4-OHEN approximated that produced by MC; the scores were 33 ± 9 and 38 ± 4, respectively. These doses were at subtoxic levels for both compounds following treatment for 24 h. Using the Pgf FLARE comet assay, a slight, but significant, amount of oxidized DNA bases, which was dose-dependent, was observed in the cell lines treated with 4-OHEN (Figure 4). Again, C3H 10T1/2 cells were slightly more sensitive to 4-OHEN than the JB6 cells.

JB6 Neoplastic Transformation. The mouse epidermal JB6 clone 41 5a cell line is commonly used to study the effects of TPA and reactive oxygen species on tumor promotion (34, 35). The JB6 clone 41 5a cells undergo transformation to a tumorigenic, anchorage-independent phenotype, which is easily read as colony formation on agar basement (27). The cells responded to

Figure 3. Induction of DNA single-strand breaks by 4-OHEN in (A) C3H 10T1/2 cells and (B) JB6 clone 41 cells. Cells (2–4 × 10^6 cells/mL) were treated with NADH (15 µM) or DMSO followed by incubation with various concentrations of 4-OHEN for 3 h (● and ■) 4-OHEN and (○ and □) 4-OHEN and 15 µM NADH. Experimental details are given in Materials and Methods.

Figure 4. Modified comet assay quantifying the oxidized DNA bases produced after incubation with 4-OHEN for 3 h in C3H 10T1/2 cells (●) and JB6 clone 41 cells (■). Experimental details are given in Materials and Methods.

16 nM TPA with values similar to those observed by others with a transformation rate of 14.8% (27, 34, 35; Figure 5). The highest concentrations of 4-OHEN (1 µM) and 4-OHE (10 µM) were toxic to the cells over the 3 week incubation period, leading to a reduction in the number of colonies observed as compared to the control. Lower concentrations of 4-OHE (100 nM to 1 µM) and 10 nM 4-OHEN increased the number of colonies over background, but the transformations were not significant (p > 0.05). However, 4-OHEN at 100 nM caused a slight, but significant, increase in transformation potential
Figure 5. Anchorage independence transformation of JB6 cells. Asterisks indicate a significant difference ($p < 0.001$).

($p < 0.01$). All colonies observed with the catechols were smaller in size than those of the TPA positive control. The addition of 15 μM NADH with 100 nM 4-OHEN dramatically increased the number and size of the transformed colonies ($p < 0.001$) which suggests that redox cycling might play a major role in the mechanism of tumor promotion by 4-OHEN. NADH alone had no effect on transformation potential (data not shown).

C3H 10T½ Transformation. The C3H 10T½ murine fibroblast cells have been used extensively to study the relative ability of compounds to transform initiated cells to a malignant cell type (29, 30). Further development of the C3H 10T½ assay allows for an assessment of initiation, promotion, or complete carcinogenic potential in vitro. Cells treated in a schedule similar to that for initiation and promotion demonstrated phorbol ester mediated transformation of cells previously treated with polycyclic aromatic hydrocarbons (29). The transformation of the C3H 10T½ cells is assessed by the appearance of stained foci on the lightly stained background monolayer. The foci are classified into three types on the basis of appearance and on tumorigenic capability in immunosuppressed C3H mice. Type I foci resemble insignificant slight motting and dark staining of the monolayer that do not give rise to tumors. Type II foci are smooth-edged pileup of cells that stain darkly. Deeply staining type III foci exhibit criss-crossing at the edge of the focus. Approximately 50% of the type II and 80% of the type III foci produce tumors when injected into C3H mice (28).

Analysis of the ability of the catechol estrogens to exhibit activity similar to that of a complete carcinogen involved treating the cells with a regimen of either 4-OHE or 4-OHEN as the initiator and promoter. While lower concentrations of 4-OHE (100 nM to 1 μM) did not cause transformation, 10 μM 4-OHE resulted in a significant cellular transformation, as measured by the number of foci per well ($p < 0.005$; Table 1 and Figure 6A). In contrast, 4-OHEN (0.1–1 μM), at concentrations 10–100-fold lower than the active dose of 4-OHE, induced cellular transformation. A lower concentration of 4-OHEN (10 nM) did not significantly transform the cells as compared to the solvent control (Table 1 and Figure 6A).

Analysis of the promotional capabilities of 4-OHE and 4-OHEN with the C3H 10T½ cells involved the prolonged treatment of the cells with the catechol estrogens follow-

Figure 6. Carcinogenesis of catechol estrogen in C3H 10T½ cells. (A) Complete carcinogenesis experiment. Cells were treated with test samples as potential initiators and promoters. (B) Promotion experiment. Cells were treated with MC (0.9 μM) as the initiator followed by treatment for 6 weeks with test samples as potential promoters. (C) Initiation experiment. Cells were treated for 24 h with test samples as potential initiators followed by treatment for 6 weeks with TPA as the promoter (0.16 μM). One asterisk means $p < 0.001$. Two asterisks mean $p < 0.01$. Three asterisks mean $p < 0.05$.
number of foci with decreasing concentration, which is likely due to the toxic effects of the higher doses of 4-OHEN. While the difference between the promotion concentrations observed in the JB6 cells and C3H 10T_{1/2} cells is 10-fold, it is important to note that both occur at subtoxic and nanomolar levels.

The initiation potential of 4-OHE and 4-OHEN was tested with an initial 24 h treatment with the catechol estrogens followed by a prolonged treatment with TPA (0.16 μM). The highest concentration of 4-OHE that was tested (10 μM) demonstrated significant initiation potential (Table 3 and Figure 6C) as compared to the TPA control. As observed with the complete transformation study (Figure 6A), much lower doses of 4-OHEN (0.1–1 μM) were required to cause initiation. While the number of foci induced at 10 μM 4-OHEN was not significantly different from the number for the TPA control (p > 0.5), all the induced foci were tumorigenic type III, indicating that the cells that survived the toxicity of 4-OHEN were greatly mutated.

### Table 2. Evaluation of the Relative Ability of Catechol Estrogens To Act as Tumor Promoters in the C3H 10T_{1/2} Transformation Assay

<table>
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<th>treatment (initiator/promoter)</th>
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<th>no. of wells</th>
<th>no. of foci/well</th>
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<td>MC9/TPA</td>
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<td>4-OHE</td>
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<td>0.35</td>
<td>0.109</td>
<td>0.0113</td>
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* Total number of type II and III foci; cells treated with MC for initiation and test compounds as promoters. At 0.9 μM. At 0.16 μM.

### Table 3. Evaluation of the Relative Ability of Catechol Estrogens To Act as Tumor Initiators in the C3H 10T_{1/2} Transformation Assay

<table>
<thead>
<tr>
<th>treatment (initiator/promoter)</th>
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* Total number of type II and III foci; cells treated with compounds for initiation and TPA as the promoter. At 0.9 μM. At 0.16 μM.
previously with LD₅₀ values of >100 μM in several breast cancer cell lines (18). In contrast, 4-OHEN was a potent cytotoxin in the same breast cancer cell lines; a 3 h incubation with 4-OHEN gave LD₅₀ values of 30, 68, and 5.7 μM for MCF-7, MDA-MB 231, and S-30 cell lines, respectively (unpublished data). The lower 3 h LD₅₀ values seen in the C3H 10 T₁/2 and JB6 clone 41 5a cell lines (54 and 10 μM, respectively), as compared to the values of these breast tumor cell lines, may be attributed to the decrease in oxidation sensitivity in the breast cancer cells (33).

Assessment of intracellular ROS by membrane-permeable DCDF-DA is possible by cytoplasmic deacidification trapping of DCF. Intracellular hydrogen peroxide and other reactive oxygen species oxidize the deacylated product species to form the fluorescent, oxidation product, DCF, which correlates with reactive oxygen species production (41). The concentration-dependent increase in fluorescence produced by 4-OHEN indicates that this catechol estrogen functions as a prooxidant in vitro. The increase in the level of DCF even at toxic doses indicates that while the cell membrane may be disrupted, 4-OHEN is still producing ROS due to redox cycling. In contrast, the decrease in native and H₂O₂-induced fluorescence observed with 4-OHE suggests that 4-OHE acts as an antioxidant in these cells. These results correlate with previous reports which showed that 4-OHE caused a significant decrease in the extent of lipid peroxidation in macrophages (42) and in rat liver microsomes (43, 44).

Single-strand DNA breaks have been found in oxidatively stressed cells, and this has been used as a general indicator of DNA damage (33). The comet assay can be used to give an effective qualitative measure of single-strand breaks in individual cells (26). Subtoxic doses of 4-OHEN caused significant and sustained single-strand breaks in both cell lines, whereas no damage was observed with 4-OHE under similar conditions. Longer incubations demonstrated 4-OHEN induced DNA damage similar to that observed with an initiation concentration of MC. Further procedural modifications of the comet assay with the addition of the Escherichia coli enzyme Fpg, which catalyzes the excision of 8-oxodeoxyguanosine and other oxidized bases (45), increase the amount of information which can be obtained from the traditional comet assay. The combination of the comet assay with Fpg can qualitatively determine the extent of oxidized DNA bases. Dose-response curves of 4-OHEN in a breast cancer cell line (S-30) have demonstrated similarities between the qualitative information gathered from the Fpg FLARE comet assay and the quantitative data obtained from LC–MS/MS assessment of 8-oxodeoxyguanosine. In this study, 4-OHEN showed dose-dependent increases in the level of oxidized DNA bases with the Fpg FLARE comet assay (Figure 4) at doses similar to those which produced significant reactive oxygen species production in the DCDF-DA assay (Figure 2A).

An early study examined the role of reactive oxygen species in the transformation of JB6 clone 41 5a cells where it was observed that the oxidation of surface lipids led to transformation (46). Other studies have demonstrated the inhibitory effects of antioxidants (47) and superoxide dismutase (48, 49) on TPA-induced JB6 clone 41 5a transformation. 4-OHEN induced transformation of these cells at a subtoxic concentration (100 nM) which also induced significant reactive oxygen species production. The increase in the extent of transformation by the addition of NADH, which has previously been shown to increase the level of redox cycling with 4-OHEN (14), indicates that the promotion effects caused by 4-OHEN may be mediated by reactive oxygen species production.

Similarly, several studies have investigated the role of reactive oxygen species in the transformation of C3H 10 T₁/2 cells (50, 51). An interesting result from one study determined that reactive oxygen species are involved with possible mutations throughout postconfluent promotion (51). Another study determined that 17β-estradiol enhanced the genomic instability of X-ray-transformed C3H 10 T₁/2 cells, which did not persist after withdrawal (52). This investigation also showed that C3H 10 T₁/2 cells possess a small amount of the estrogen receptor: 1582 receptors/cell as compared to 36 700 receptors/cell for MCF-7 cells (52). The presence of the estrogen receptor in the C3H 10 T₁/2 cells may account for the slightly increased sensitivity of this cell line toward the effects of 4-OHEN as compared to JB6 where the estrogen receptor status is unknown. In addition, our work has shown that 4-OHEN demonstrated complete carcinogenic, initiation, and promotion activity in C3H 10 T₁/2 cells at subtoxic concentrations that also showed an increased level of reactive oxygen species production and oxidized DNA bases, indicating transformation may be dependent on these effects.

Oxidative DNA damage by reactive oxygen species likely represent an event of considerable importance in the early development of breast and endometrial cancer (53). It has been shown that mutations resulting from reactive oxygen species-mediated DNA damage can result in miscoding, conformational change, and multiplicative errors (17, 54). While previous reports demonstrate in vivo carcinogenesis and oxidative damage with endogenous catechol estrogens (65), our in vitro data suggest that 4-OHEN has the potential to be a much more potent carcinogen than the endogenous catechol estrogens. We have shown, utilizing the transformation assays described herein, that 4-OHEN is capable of initiation, promotion, and complete carcinogenesis at doses 10–100-fold lower than that of 4-OHE. In addition, our data strongly suggest that oxidative stress is responsible for the transformation effects caused by 4-OHEN in these cell lines and that this mechanism should be included with the other pathways of carcinogenesis noted for estrogen tumorigenesis.

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