Catechol metabolites of endogenous estrogens induce redox cycling and generate reactive oxygen species in breast epithelial cells

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Introduction

Human and animal studies suggest that exposure to hormone replacement therapies and other estrogens is a major risk factor for the development of idiopathic postmenopausal mammary cancer (1–8). Meta-analysis of development of idiopathic postmenopausal mammary cancer (1–8), metabolism and other estrogens is a major risk factor for the development of breast cancer patients versus women who had mammoplasties (12–14). Animal studies have also confirmed a dose-dependent link between oxidative DNA damage and catechol estrogen exposure (13,15–20).

The carcinogenic potential of equine estrogens is widely attributed to the metabolism of equilin (3-hydroxyestra-1,3,5,7-tetraen-17-one), to redox active catechol metabolites, via cytochrome P450 isozymes CYP1A1 and CYP1B1 (21–24). The endogenous estrogen, estradiol (E2), is also a substrate for CYP1A1 and CYP1B1, generating 2-hydroxyestradiol (2OHE2) and 4-hydroxyestradiol (4OHE2) (Figure 1) (25). These catechol metabolites have the capacity to undergo one-electron oxidation by reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase (POR) to form reactive SQ intermediates (26), which can modify DNA and are potential mutagens (18,21,27). Alternatively, the SQs can pass their unpaired electron to molecular oxygen, forming superoxide anion and restoring the catechol in a process referred to as redox cycling. Superoxide anion can be metabolized to other reactive oxygen species (ROS), including hydrogen peroxide (H2O2) and, in the presence of transition metals, highly mutagenic hydroxyl radicals (26,28). Excessive ROS cause lipid peroxidation, protein oxidation and DNA damage, which have been observed in both animals and humans exposed to estrogens (14,22,24,26,29,30).

The link between endogenous estrogens and breast cancer suggests that they may also be metabolized to reactive intermediates that contribute to the carcinogenic process (31). However, few studies have characterized redox cycling of endogenous estrogens or their metabolites. Earlier work has proposed that the SQ formed from the endogenous catechol estrogen, 2OHE2 may form a quinone in the presence of NADPH or cumene peroxidase and rat liver microsomes; however, these studies failed to demonstrate any signs of redox cycling (including ROS generation, reducing agent depletion and an increase in oxygen consumption) by this endogenous catechol (26). Furthermore, the stimulation of ROS generation that typifies redox cycling has never been characterized using an endogenous estrogen. In the present studies, we demonstrate that four catechol metabolites of estrone (E1), E2 and estriol (E3) are highly redox active and generate ROS. These data support the idea that redox cycling by endogenous catechol estrogens may contribute to breast tumorigenesis.

Materials and methods

Chemicals and reagents

Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) was obtained from Invitrogen (Eugene, OR). The estrogens, NADPH, terphenyl and all other chemicals were from Sigma–Aldrich (St Louis, MO).

Cell culture and preparation of cell lysates

MCF-7, MDA-MB-231 and MCF-10A breast epithelial cells were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 and MDA-MB-231 are transformed cells and were maintained in Dubecco’s modified Eagle medium supplemented with 10% fetal bovine serum (Mediatech, Manassas, VA), 100 U/ml penicillin/100 µg/ml streptomycin (Gibco BRL, Grand Island, NY). MCF-10A is a non-tumorigenic cell line and was grown in Mammary Epithelial Basal Medium supplemented with Lonza’s BulletKit™ (Walkersville, MD), containing insulin, hydrocortisone, recombinant human epidermal growth factor, bovine pituitary extract, gentamycin and amphotericin B. Cells which overexpress cytochrome P450 reductase (CHO-OR) and wild-type control cells (CHO-WT cells) were obtained and maintained as described previously by our laboratory (32–34). All cell lines were maintained at 37°C in a humidified incubator with 5% CO2. To prepare lysates, cells were trypsinized, washed and resuspended in phosphate-buffered saline (106 cells/ml). Cells were then disrupted on ice using a probe sonicator (Artek Systems, Farmingdale, NY).

Abbreviations: E1, estrone; E2, estradiol; E3, estriol; ER, estrogen receptor; DPL, diphenyleinediiodonium; H2O2, hydrogen peroxide; HRT, hormone replacement therapy; NADPH, reduced nicotinamide adenine dinucleotide phosphate; 2-OH-TPT, 2-hydroxyterephthalate; 2OHE2, 2-hydroxyestradiol; 2OHE3, 2-hydroxyestradiol; 4OHE1, 4-hydroxyestrone; 4OHE2, 4-hydroxyestradiol; POR, cytochrome P450 reductase; ROS, reactive oxygen species; SQ, semiquinone.
Protein concentrations were quantified using the DC protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

Analysis of ROS production
All assays for ROS were performed in 96-well black microtiter plates at 37°C. H2O2 production in redox-cycling enzyme assays was measured using the Amplex red/horseradish peroxidase method as described previously (35,36). Reactions in 100 μl volumes contained 70 mM NaCl in potassium phosphate buffer (30 mM, pH 7.8) supplemented with 100 μM NADPH, 100 μM Amplex red, 1 U/ml horseradish peroxidase, 50 μg/ml cell lysate protein and various concentrations of estrogens or estrogen metabolites. The fluorescence of the reaction product, resorufin (excitation 540 nm/emission 595 nm), was recorded using a SpectraMax M5 fluorescent microplate reader (Molecular Devices, Sunnyvale, CA). The terephthalate assay was used to measure hydroxyl radical generation (37). In this assay, hydroxyl radicals are scavenged by the non-fluorescent substrate terephthalate, producing the fluorescent product 2-hydroxyterephthalate (2-OH-TPT, excitation 315 nm/emission 425 nm). Fluorescence was measured using a spectrofluorometric microplate reader. Reactions were analyzed in 100 μl of phosphate-buffered saline containing 100 μM NADPH, 50 μg/ml cell lysate protein, 100 μM FeCl3, 110 μM ethylenediaminetetraacetic acid, 1 mM terephthalate and appropriate concentrations of either estrogens or estrogen metabolites. Because estrogens are sparingly soluble in buffer systems and most organic solvents are hydroxyl radical scavengers, stock solutions of estrogens (100 mM in isopropanol) were added to the wells of the microplates and evaporated overnight prior to the addition of other assay reagents.

H2O2 release was quantified using platinum amperometric microsensors as described previously with minor alterations (38). Briefly, the culture medium from 60 to 80% confluent monolayers of cells was replaced with Krebs-Ringer bicarbonate buffer (4 mM glucose, 140 mM NaCl, 30 mM N-2-hydroxymethylpiperazine-N’-2-ethanesulfonic acid, 4.6 mM KCl, 1 mM MgSO4, 0.15 mM Na2HPO4, 0.4 mM KH2PO4, 5 mM NaHCO3, 2 mM CaCl2 and 0.05% bovine serum albumin, pH 7.4, osmolarity 284 mosM) just prior to analysis (39). After measuring basal H2O2, increasing volumes of a stock solution of estrogens or estrogen metabolites (10 mM in isopropanol) were added to the buffer. Each addition was directly followed by electrochemical H2O2 quantification using the microsensors. All experiments were performed at room temperature. Each microsensor was hand-manufactured before each experiment and therefore displayed unique noise characteristics that were evident in the tracings. The microsensors used in our experiments had a linear response in the range of 0.3–30 μM H2O2 (Figure 2).

Results
In initial studies, we characterized redox cycling by estrogens and estrogen metabolites in lysates of MCF-7, MDA-MB-231 and MCF-10A cells by quantifying H2O2 and hydroxyl radical generation. MCF-7 and MDA-MB-231 are estrogen receptor (ER)-positive and ER-negative tumorigenic cell lines, respectively, whereas MCF-10A is an ER-negative non-tumorigenic transformed cell line. We found that the catechol estrogen metabolites 2OHE2 and 4OHE2, but not E2 or the methoxy-estrogen metabolite 2-methoxyestradiol (2MeOE2), readily generated H2O2 in lysates from each cell type (Figure 3). Similar redox cycling was observed with 4-hydroxyestrone (4OHE1) and 2-hydroxyestradiol (2OHES), but not E1 or E3 (data not shown). H2O2 formation by all redox active catechol estrogen metabolites was linear with respect to time and concentration (Figure 3A) and was inhibited by the flavin inhibitor diphenyleneiodonium (DPI) (supplementary Figure 1 is available at Carcinogenesis Online). The antioxidant enzyme catalase also prevented the accumulation of H2O2 in enzyme assays (supplementary Figure 1 is available at Carcinogenesis Online). It has been previously suggested that POR mediates catechol estradiol redox cycling (26,28). By comparing...
CHO cells overexpressing cytochrome P450 reductase (CHO-OR cells) with wild-type controls (CHO-WT cells), we found that the rate of 2OHE2-stimulated H2O2 production is dependent on POR expression (supplementary Figure 2 is available at Carcinogenesis Online).

The rates of H2O2 generation in the three breast epithelial cell lines by 2OHE2, 4OHE2, 4OHE1 and 2OHE3 were further analyzed using Michaelis–Menten kinetics; the apparent \( K_M \)'s ranged from 2.29 to 18.9 \( \mu \)M and \( V_{max} \)'s from 2.67 to 6.42 pmol H2O2/min/mg protein (Figure 3B and Table I). The redox active catechol estradiol metabolites also stimulated hydroxyl radical formation in cell lysates; this reaction was both time and concentration dependent (Figure 4A). Accumulation of hydroxyl radicals was prevented by DPI and catalase as well as the hydroxyl radical scavenger dimethyl sulfoxide (supplementary Figure 3 is available at Carcinogenesis Online). The apparent \( K_M \)'s and \( V_{max} \)'s for catechol estrogen redox cycling ranged from 8.85 to 250 \( \mu \)M and 9.29 to 102 pmol 2-OH-TPT/min/mg protein (Figure 4B and Table I). The kinetic constants for hydroxyl radical formation were lowest with 4OHE1 and highest with 2-OHE2. E1, E2, E3 and 2MeOE2 had no effect on the generation of hydroxyl radicals by the cell lysates (Figure 4B and data not shown).

H2O2 is membrane permeable and can readily diffuse from cells into their culture medium. We next determined whether catechol estrogen redox cycling-stimulated H2O2 release by intact cells. For these experiments, extracellular H2O2 production was measured using an amperometric platinum microsensor (38). Each of the four redox active catechol estrogens was found to stimulate H2O2 release by MCF-7, MDA-MB-231 and MCF-10A cells (Figure 5). H2O2 release was not observed with E1, E2, E3 or 2MeOE2 (Figures 5A and 6A). Catechol estrogen metabolite-stimulated cellular H2O2 release was evident at concentrations up to 30 \( \mu \)M, the highest concentrations tested (Figures 5A and 6A). H2O2 release by the cells was also inhibited by DPI. Pretreatment of MDA-MB-231 cells with DPI (10–100 \( \mu \)M) prevented 2OHE3-stimulated H2O2 release. H2O2 release was also reduced by DPI after catechol estrogen treatment and inhibited by catalase (Figure 5B). Michaelis–Menten kinetics was used to compare H2O2 release by the cells. We found that the catechol estrogen metabolites were generally similar in their ability to stimulate H2O2 production by the breast epithelial cells (Figure 6B).

**Discussion**

It is generally accepted that exposure to estrogens is an important risk factor for the development of mammary cancer (40,41). The carcinogenic process is thought to involve many factors including the promotion of random mutations due to ER-mediated changes in transcription (42), stimulation of non-ER-mediated signal transduction (43–45) and the generation of estrogen metabolites. Estrogen metabolism to catechols and subsequent methylation by catechol-O-methyltransferase is generally considered to be a detoxifying pathway because these metabolites can be readily excreted. However, increases in the metabolism of estrogens to catechols or decreases in the rate of methylation can result in the formation of reactive estrogen-SQs, which alkylate DNA (15,18–20,46,47). Recent evidence suggests that redox cycling by exogenous catechol estrogen SQs may also contribute to their carcinogenic activity (15,21). However, no studies have ever characterized ROS generation resulting from endogenous catechol estrogen-stimulated redox cycling. In the present studies, we characterized this activity in three different breast epithelial cell lines...
with varying tumorigenic potential. In each of the cell types, the endogenous catechol estrogens were found to redox cycle and generate ROS, including H₂O₂ and hydroxyl radicals; redox cycling was not observed with the parent estrogens or the methoxy metabolite. These findings are consistent with reports that the catechol structure is an important structural requirement for redox cycling activity (48). While all estrogens tested display the high degree of conjugation needed to stabilize the SQ radical intermediate, only the catechol...
Catechol estrogen redox cycling

Table I. Kinetic constants for catechol estrogen redox-cycling in breast cell lysates

<table>
<thead>
<tr>
<th>Cell type</th>
<th>2OH2</th>
<th>4OH2</th>
<th>4OH1</th>
<th>2OH3</th>
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<tr>
<td>MCF-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2O2</td>
<td>6.42 ± 0.76</td>
<td>4.28 ± 0.79</td>
<td>5.57 ± 0.39</td>
<td>4.01 ± 0.22</td>
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<td>KM</td>
<td>18.9 ± 6.6</td>
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<td>MCF-10A</td>
<td>4.37 ± 0.36</td>
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<td>KM</td>
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<td>3.20 ± 3.43</td>
<td>7.93 ± 2.73</td>
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<td>MDA-MB-231</td>
<td>5.02 ± 0.65</td>
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<tr>
<td>KM</td>
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Hydroxyl radical formation

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<th>Cell type</th>
<th>Vmax</th>
<th>Km</th>
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<td>MCF-7</td>
<td>102 ± 2</td>
<td>50.9 ± 2.6</td>
</tr>
<tr>
<td>KM</td>
<td>196 ± 11</td>
<td>41.0 ± 8.7</td>
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<td>MCF-10A</td>
<td>76.4 ± 4.1</td>
<td>51.9 ± 2.3</td>
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<td>KM</td>
<td>122 ± 21</td>
<td>73.1 ± 12.1</td>
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<td>MDA-MB-231</td>
<td>87.4 ± 8.4</td>
<td>51.4 ± 3.4</td>
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<tr>
<td>KM</td>
<td>175 ± 50</td>
<td>250 ± 45</td>
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Hydroxyl radical formation

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<td>175 ± 50</td>
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Catechol estrogen redox cycling

- Estrogens contain unprotected oxygen atoms on adjacent carbons.
- Each of these oxygen atoms exists in either a carbonyl (oxidized) or a hydroxyl (reduced) form. In partially oxidized or reduced SQ radical, the oxidized carbonyl oxygen atom contributes to the overall stability of the molecule by additional conjugation of its π-bonding electrons. This electrophilic conjugation provides enough of a dipole moment along the remaining hydroxyl bond for deprotonation to occur, forming the radical. The same conjugation also stabilizes the unpaired electron, thus the radical formed can function as a reactive metabolite rather than as a transient intermediate (22,48).

- MCF-7, MDA-MB-231 and MCF-10A cells represent human mammary epithelial cells at various stages of breast cancer development. Although MCF-7 and MDA-MB-231 cells both originate from the plural effusion of human breast adenocarcinomas, they have distinct properties. Thus, whereas MCF-7 cells retain some characteristics of differentiated mammary epithelium, such as the expression of cyto-plasmic ER alpha (ERα) (49), and are commonly used to model low-grade breast epithelial adenocarcinomas (52–54), MDA-MB-231 cells are poorly differentiated, do not express ERα and are insensitive to anti-estrogens (49). Originating from the fibrocystic lesion of a premenopausal woman, MCF-10A cells express a number of mammary epithelial markers (49) and are distinct in that they are transformed, but not tumorigenic. We found that the capacity of each of these breast cell lines to mediate redox cycling, the amount of ROS generated, the rate of ROS production, the reversibility of the redox cycling reaction and the maximal reaction rates, were generally comparable for each catechol estrogen. Thus, any measurable differences between the antioxygen potentials of the three cell lines are rendered insignificant by the magnitude of catechol estrogen-stimulated ROS generation. These data also suggest that redox cycling is independent of ERα expression, state of differentiation and tumorigenic potential. It may be that the ability to mediate catechol estrogen redox cycling and ROS production is a fundamental property of the breast epithelial cells; moreover, redox cycling by catechol metabolites of endogenous estrogens may precede the carcinogenic process.

The present studies demonstrate that breast tumor cell lysates have the capacity to generate ROS during catechol estrogen redox cycling, including both H2O2 and hydroxyl radicals. It is generally thought that redox cycling generates ROS via the univalent reduction of oxygen resulting in the formation of superoxide anion (55). In this reaction, one molecule of oxygen oxidizes two molecules of NADPH generating two molecules of superoxide anion (56). The further reduction of superoxide anion produces H2O2 and hydroxyl radicals in the presence of transition metals. The Vmax’s and apparent Km’s for the formation of H2O2 ranged from 2.67 to 6.42 pmol H2O2/min/mg protein and 2.29 to 18.9 μM, respectively. Of interest were our findings that the Vmax’s and Km’s for hydroxyl radical formation were ~5- to 10-fold higher than for H2O2 formation (Vmax’s = 9.29–102 pmol 2-OHTPT/min/mg protein and Km’s = 8.85–250 μM). This may be due to the fact that the assay for hydroxyl radicals is less sensitive than the assay for H2O2 because of the short half-life and high reactivity of the hydroxyl radicals and the fact that Amplex red is a more sensitive fluorescent ROS indicator than 2-OH-TPT (36,37). We also noted that the maximal reaction rate of 2OHE2 was greater than other catechol estrogens in the 2-OH-TPT assay; the lowest activity was evident with 4OHE1. The reasons for these differences are not clear but may be due to availability of redox active iron in the hydroxyl radical assays. Catechols are known to chelate iron (57) and this may alter the conversion rate of H2O2 to hydroxyl radicals in the reaction mixes.

- Also of interest was our finding that redox cycling by catechol estrogens stimulated the release of H2O2 from viable breast epithelial cells. Kinetic analysis of H2O2 release by the cells showed that the responses to each of the endogenous catechol estrogens was similar, as a percentage of Vmax and were generally consistent with the similarities in the kinetic constants of catechol estrogen-stimulated H2O2 generation in breast epithelial cell lysates (Table I). Catechol estrogen-stimulated release of H2O2 showed that redox cycling also occurs in intact cells. It also indicates that cellular ROS detoxification enzymes, such as various peroxidases and catalase, as well as antioxidants, are unable to limit increased intracellular H2O2 production formed during redox cycling (58). One can speculate that redox cycling may also cause the release of H2O2 by breast cells in vivo. If this is the case, then H2O2 formed during redox cycling has the potential to affect many cells in the tissue microenvironment. Depending on the localized concentrations of transition metals, highly mutagenic hydroxyl radicals may be generated which can further damage cells in breast tissue and may contribute to the development of cancer (59,60).

A question arises as to whether there are sufficient concentrations of the catechol metabolites of endogenous estrogens in human breast epithelial tissue to mediate redox cycling and generate cytotoxic ROS in vivo. Kinetic analysis of H2O2 production reveals that the rates of enzyme-mediated SQ formation are catechol estrogen concentration dependent. Because the concentration of cell lysate protein, and therefore number of catalytic sites, was held constant for each cell line, the measured Vmax is proportional to the turnover number (kcat) for catechol estrogen oxidation. This value is small, indicating poor catalytic efficiency at high substrate concentrations. Instead, the rate of this reaction during redox cycling is highly dependent on the value of the KM constant, indicating a high dependence on the catechol estrogen metabolite concentration. Since the apparent KM values of the catechol estrogen metabolites are in the micromolar range, we speculate that submicromolar concentrations of these endogenous metabolites generate significant quantities of H2O2. Although intracellular concentrations of estrogens are not known, they would be expected to be much higher in breast epithelial cells than in serum due to the presence of ERs. These high affinity-binding proteins would be expected to concentrate estrogens and their metabolites (15,42). Normal circulating serum estrogen levels in premenopausal females ranges from 75 to 2000 pg/ml (depending on the estrogen, the individual and the phase of the menstrual cycle) (61). As a lipophilic molecule, higher concentrations of estrogens may also be present in lipid compartments in cells including the microsomes where estrogen metabolism to catechols and redox cycling probably occurs (21).

It is also possible that breast epithelial cells synthesize their own catechol estrogens, as the enzyme which catalyzes the final step of estrogen synthesis (aromatase), is colocalized with enzymes governing estrogen metabolism to catechols (cytochromes P450 1A1 and 1B1) on the microsomal electron transport chain (28,62–65). Thus, the localized concentrations of catechol estrogens in the endoplasmic
reticulum may be significantly greater than in other compartments of breast epithelial cells or in the serum and therefore, within a plausible range to generate ROS. The production of ROS by these cells is also necessarily dependent on the presence of NAD(P)H oxidase enzymes which have the capacity to mediate redox cycling (28,62). Our findings that redox cycling in the breast epithelial cells was inhibited by...
DPI confirm that flavoenzymes indeed mediate catechol estrogen redox cycling. Further studies are needed to characterize these enzymes and to determine if they play a role in generating ROS in breast tissues.

In summary, our studies demonstrate that endogenous catechol estrogens can redox cycle and generate ROS in breast epithelial cells. Production of ROS is not dependent on ERs or tumorigenic potential of the cells and may be an inherent property of epithelial cells in the breast. Catechol estrogen-stimulated ROS production by both cell lysates and intact cells is also independent of the species of catechol estrogen. Intact cells also generate ROS during catechol estrogen redox cycling. Sufficient levels of ROS can be generated to saturate intracellular antioxidant defense resulting in their release into the microenvironment of the breast. Our data provide further support for the idea that metabolism of endogenous estrogens to catechols and subsequent redox cycling may contribute to breast cancer development.
Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

References


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