The Effects of Catechol-O-Methyltransferase Inhibition on Estrogen Metabolite and Oxidative DNA Damage Levels in Estradiol-treated MCF-7 Cells

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ABSTRACT

Many of the major identified risk factors for breast cancer are associated with exposure to endogenous estrogen. In addition to the effects of estrogen as a growth factor, experimental and epidemiological evidence suggest that catechol metabolites of estrogen also contribute to estrogen carcinogenesis by both direct and indirect genotoxic mechanisms. O-Methylation catalyzed by catechol-O-methyltransferase (COMT) is a Phase II metabolic inactivation pathway for catechol estrogens. We and others have found that a polymorphism in the COMT gene, which codes for a low activity variant of the COMT enzyme, is associated with an increased risk of developing breast cancer; therefore, the goal of the current study was to investigate the role of decreased COMT activity on estrogen catechol levels and on oxidative DNA damage, as measured by 8-hydroxy-2-deoxyguanosine (8-oxo-dG) levels. MCF-7 cells were pretreated with dioxin as a means to increase estrogen metabolism to catechol estrogens, then treated with estradiol (E₂) ± Ro 41-0960, a COMT-specific inhibitor. After extraction from culture medium, estrogen metabolites were separated using high-performance liquid chromatography-electrochemical detection. As expected, dioxin dramatically increased E₂ oxidative metabolism, primarily to its 2-OH and 2-methoxy metabolites. The COMT inhibitor blocked 2-methoxy E₂ formation. This was associated with increased 2-hydroxy E₂ (2-OH E₂) and 8-oxo-dG levels. In the presence of COMT inhibition, increased oxidative DNA damage was detected in MCF-7 cells exposed to as low as 0.1 μM E₂, whereas in the absence of COMT inhibition, no increase in 8-oxo-dG was detected at E₂ concentrations ≥10 μM. This study is the first to show that O-methylation of 2-OH E₂ by COMT is protective against oxidative DNA damage caused by 2-OH E₂, a major oxidative metabolite of E₂.

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women in the United States (1). The major risk factors that have been identified implicate a role for endogenous E₂ (2). However, recent evidence suggests that in addition to the parent molecule, the oxidative metabolites of estrogen, including the CEs (2-OH-E₂/E₁ and 4-OH-E₂/E₁) and 16α-OH-E₁, may also contribute to estrogen-induced tumors in certain animal models and to the development of human breast cancer (3–8). Of the two CEs, 4-OH-E₂ had greater carcinogenicity than 2-OH-E₂ in the Syrian hamster kidney and mouse uterine tumor models (3, 4, 8). In addition, recent studies using the Syrian hamster embryo cell model have shown that the CEs induce cell transformation, somatic mutations, and chromosome aberrations, with the 4-OH being more potent than the 2-OH estrogen catechols (9). In vitro and in vivo studies have demonstrated that CE metabolites can bind to DNA via their quinone metabolites (10–13) and cause oxidative damage through redox cycling processes (5, 6, 14–18). Redox cycling could contribute to the increased oxidative DNA damage that has been detected in human breast cancer tissue (19–22), though these findings have been called into question (23). Moreover, the CEs, like the parent molecule, bind to the estrogen receptor and have been shown to be estrogenic, with 4-OH-E₂ reported to have greater affinity and effects than 2-OH-E₂ (24–26).

Human breast tumor tissue microsomes have been shown to metabolize E₂ to catechol metabolites (27). In tissues and cells derived from the human breast, studies indicate that CEs are generated by hydroxylation of E₂ or E₁ at the 2- or 4- positions by specific CYP450s, including CYP450s 1A1 and 1B1 (28). CYP1A1 hydroxylates E₂ primarily at the C-2 position, whereas CYP1B1 primarily hydroxylates E₁ at the C-4 position (28–32). The binding of CEs to the estrogen receptor, their additional metabolism to quinones, and their redox cycling can be blocked via several detoxication pathways including O-methylation by COMT (33–38). Both rat (39) and human (40) COMT have been shown to have a higher catalytic activity toward 2-OH compared with 4-OH-E₂, which may contribute to the comparatively weaker carcinogenicity of 2-OH-E₂. 2-MeOE₂, which is formed by COMT, has been shown to increase apoptosis, inhibit growth, and inhibit angiogenesis (41–45). Thus, 2-MeOE₂ may be a protective metabolite and COMT an important protective enzyme.

COMT is polymorphic in the human population. Twenty-five percent of United States Caucasians are homozygous for a val108met polymorphism in the COMT gene (46–48). This polymorphism results in 3–4-fold less enzyme activity (46, 47) and could, therefore, result in decreased CE detoxication. We conducted previously a genetic epidemiology study using a nested case-control study design from a large cohort from Washington County, Maryland, to explore the hypothesis that women homozygous for the low activity allele (COMT<sup>LL</sup>) would be at increased risk for developing breast cancer (49). We found that in postmenopausal women with a body mass index >24.47 kg/m², COMT<sup>LL</sup> women exhibited a significantly increased risk for developing breast cancer (odds ratio, 3.58; confidence interval, 1.07–11.98). This was the first study to provide evidence consistent with the hypothesis that estrogen catechol metabolites contribute to the increased risk of breast cancer in humans. Three of four additional published studies have also suggested that certain COMT<sup>LL</sup> individuals have an increased risk for developing breast cancer (50–53), although in one of these studies, the high-risk population was premenopausal women (50). In addition, Matsui et al. (54) reported recently that in a population of 140 breast cancer patients,
COMT<sup>L2</sup> was associated with advanced clinical stage and extent of regional lymph node metastasis, providing additional evidence that the COMT<sup>L2</sup> allele may contribute to estrogen carcinogenesis.

The goal of the present study was to begin to explore the effects of alterations in COMT activity on cellular levels of estrogen catechol metabolites and oxidative DNA damage in estrogen-treated cells. Whereas human breast tissue constitutively expresses CYP450 enzymes that metabolize E<sub>2</sub> to catechols, MCF-7 breast tumor epithelial cells do not unless induced by TCDD (29, 30, 55). Thus, in this study, we pretreated MCF-7 cells with TCDD to get measurable levels of 2- and 4-OH catechols in this cell line (29–31, 55). Then, to assess the effects of altered COMT activity on E<sub>2</sub> metabolism and on formation of oxidative DNA damage attributable to CEs, we used the COMT-specific inhibitor Ro 41-0960 (56) to inhibit COMT in the presence of E<sub>2</sub>. The results demonstrate that inhibition of COMT blocks the formation of the methylated E<sub>2</sub> catechol metabolites and dramatically enhances E<sub>2</sub>-induced 8-oxo-dG levels.

**MATERIALS AND METHODS**

**Chemicals and Reagents.** 2- and 4-OH E<sub>2</sub>, 2- and 4-MeO E<sub>2</sub>, E<sub>1</sub>, 2-, and 4-MeO E<sub>1</sub>, and 16α-OH E<sub>1</sub> were purchased from Steraloids, Inc. (Newport, RI). Ascorbate, DMSO, BSA, and fluorescein dyes were purchased from Sigma Chemical Co. (St. Louis, MO). Acetic acid and ethyl acetate were from J. T. Baker (Phillipsburg, NJ). TCDD was purchased from ULTRA Scientific (North Kingston, RI), and the COMT inhibitor, Ro 41-0960 (52), was purchased from Research Biochemicals International (Natick, MA). 8-oxo-dG, 2′dG, DFAM, nuclease P1 (EC 3.1.30.1) from *P. citrinum*, and alkaline phosphatase (EC 3.1.3.1) from *Escherichia coli* were purchased from Sigma Chemical Co. All of the other chemicals and solvents used in this study were of analytical reagent grade and purchased from various commercial sources.

**Preparation of Estrogen Standards.** Individual estrogen standards were prepared in EtOH or MeOH and quantified from their UV spectral absorbance using molar absorptivity data available from Sigma Chemical Co. or from the literature. Four levels of standard mixtures were prepared by adding appropriate aliquots of each standard together such that 20-μL injections yielded 300, 100, 300, or 1000 pmol on-column of each standard in the mixture.

**Cell Culture.** MCF-7 cells, obtained from American Type Culture Collection (Manassas, VA), were maintained in culture with phenol red-free IMEM (28), 100 μM sodium phosphate, 20 mM citric acid, 0.15 mM Na<sub>2</sub>EDTA, 2 mM sodium octanesulfonic acid in 14% MeOH (pH 3.2) and separated on a 5-μm, 4.6 × 250 mm Phenomenex C18 (2) column (Torrance, CA) that had been equilibrated with mobile phase A (75 mM citric acid, 25 mM ammonium acetate, and 20% acetonitrile). The estrogens and metabolites were eluted using the following gradient method: 0–3.5 min, 100% mobile phase A; 3.5–43.5 min, linear gradient from 0 to 50% mobile phase B (75 mM citric acid, 25 mM ammonium acetate, and 70% acetonitrile) and 43.5–45.5 min, linear gradient from 50 to 100% mobile phase B. The estrogens and their metabolites were detected by their oxidation on eight electrodes in series. They were then identified and quantified by comparison with the authentic standards. Cell potentials were set at 0–560 mV with increments of 80 mV.

**Determination of COMT Activity.** COMT activity was ascertained by modifying a method published previously (57). Briefly, cells were plated on 35-mm culture dishes and grown to ~90% confluence. On the day of assay, culture medium was removed and fresh medium added containing either vehicle control or a known concentration of Ro 41-0960. At the time of interest, the COMT substrate 3,4-DHBA was added to the cells, and incubation continued for varying times. The medium was then harvested and filtered through a 30,000 molecular weight cutoff filter (Millipore, Bedford, MA) before analysis. Samples were then either frozen at −20°C for ≤4 days or analyzed immediately for methylated products by HPLC with ECD [LKB; Pharmacia, Uppsala, Sweden (HPLC) and BAS; West Lafayette, IN (ECD)]. Filtered medium (20 μL) was diluted to 100 μL in running buffer consisting of 100 mM sodium phosphate, 20 mM citric acid, 0.15 mM Na<sub>2</sub>EDTA, 2 mM sodium octanesulfonic acid in 14% MeOH (pH 3.2) and separated on a 5-μm, 4.6 × 250 mm Supelco LC-18S column (Bellefonte, PA) essentially as described previously (57). Culture dishes with attached cells were frozen at −20°C for protein determination, and the amount of 3,4-DHBA metabolites formed was normalized to total protein/dish.

**Determination of Cellular Protein.** Protein concentrations were determined using fluorescein dye (Molecular Probes, Eugene, OR). HBSS (8 mL) was added to each 100-mm culture dish (estrogen metabolism experiments) or 1 mL to each 35-mm dish (COMT activity assay). The cells were then subjected to three rounds of freezing at −80°C and thawing at 37°C for 30 min each. The cells in HBSS were then scraped off the plate, homogenized with a Tissue Tearor (BioSpec Products, Inc.), and 1 mL of homogenate was transferred to one well of a 6-well microplate for protein analysis. Then 500 μL of stock fluorescein dye (3 mg/ml acetonitrile) was added and the fluorescence determined using a PerSeptive Biosystems plate reader (Framingham, MA) at 360-nm excitation and 460-nm emission and compared with a standard curve of BSA in HBSS. A 100-mm plate contained, on average, 6–7 mg of protein.

**Determination of 8-oxo-dG in Cellular DNA.** Samples suspended previously in high potassium medium and frozen were thawed on ice and briefly spun down. The pellets were resuspended in 1 mL of lysis buffer [0.5 M Tris-HCl (pH 8.0); 20 mM EDTA; 10 mM NaCl; and 1% SDS]. One-tenth volume of freshly prepared proteinase K (5 mg/ml stock) was added, and the tubes were incubated overnight at 37°C. After addition of 0.25 volume of saturated NaCl, the contents of the tubes were mixed thoroughly by gentle inversion until all of the salt was dissolved. It was necessary to heat briefly at 55°C to dissolve the precipitate completely. After brief cooling on ice, the tubes were centrifuged for 30 min (or longer if necessary) at 15,000 × g until a compact precipitate formed. The supernatants (containing DNA) were transferred to clean tubes. The DNA was precipitated by adding 2 volume of EtOH.
The DNA digests were passed through Ultra Free MC which were typically in the range of 8 conditions. Using this method, we obtained low baseline levels of 8-oxo-dG, C on to a reversed-phase HPLC ° injected using an autosampler maintained at 5 °C for 1 h, after which 20 μl of 1 m M EDTA was added to each sample followed by 6-18 % had been converted to E2. 18% was found in TE (10 μl aliquots from each sample prepared as described by Shigenaga et al. (58). A 40-μl aliquot of the metal ion chelator DFAM [0.1 m M stock in 20 m M sodium acetate (pH 5.0)] was added. In contrast, in cells pretreated with TCDD (Table 1), metabolism of E2 was primarily to 2-OH E2 (34%) and 2-MeO E2 (30%). Formation of the corresponding 4-OH estrogens was very low; 4-OH E2 and 4-MeO E2 combined were <5% of the total, and no 16a-OH E2 formation was detected. Other minor metabolites detected included E1 and its 2- and 4-hydroxy metabolites. The sum of all of the estrogen metabolites recovered in this analysis (10.3 ± 1.5 nmol after correction for dilution factor, time, and mg protein/plate) was not statistically different from the total 10 nmol (1 μl, 10 ml medium) E2 added at the beginning of the experiment (by Student’s t test).

In preliminary toxicity experiments, cell growth was not affected by treatment with 10 μM of the COMT-specific inhibitor Ro 41-0960 for up to 48 h (data not shown). This concentration of inhibitor reduced COMT activity 87 and 90% at 6 and 24 h, respectively, as assessed by methylation of the COMT substrate DBHA (data not shown). To determine the effect of COMT inhibition on E2 metabolism, TCDD pretreated MCF-7 cells were exposed to 1 μM E2 for 15 h in the presence of 10 μM Ro 41-0960 (Table 1). COMT inhibitor treatment decreased the concentration of 2-MeO E2 by 99% and 4-MeO E2 to undetectable levels as compared with cells not treated with the inhibitor. This was accompanied by a 2.6-fold increase in 2-OH E2 concentration, whereas no significant change was seen in 4-OH E1 levels. Therefore, 2-OH E2, which represented 34% of the total estrogens and stored overnight at −20°C. After centrifugation for 10 min at 1,100 × g, the DNA pellet was dried with argon gas and resuspended in TE (10 mm Tris-HCl (pH 8.0) and 1 mm EDTA) using a Beckman 160 detector (Beckman Instruments, Brea, CA) connected to the analyzer. The procedure was carried out on the CoulArray Model 5600 HPLC System described earlier. Aliquots (20 μl) of filtered DNA digests were injected using an autosampler maintained at 5°C on to a reversed-phase HPLC column (C18, 3 μm; 4.6 × 150 mm; YMCBasic; YMC, Inc., Wilmington, NC) and eluted with 100 m M sodium acetate-MeOH [95:5 (v/v), (pH 5.0; adjusted with phosphoric acid)] at a flow rate of 1.0 ml/min. The 8-oxo-dG was detected on two electrodes with applied potentials of 250 and 400 mV (versus palladium). The dG was detected and quantified by UV absorbance at 254 nm with a Beckman 160 detector (Beckman Instruments, Brea, CA) connected to the fourth electrode channel of the coulometric array cell module. Data were acquired and analyzed using ESA CoulArray software. Results are reported as the ratio 8-oxo-dG/dG × 10^9 calculated from peak areas based on calibration curves of authentic standards of 8-oxo-dG and dG under identical experimental conditions. Using this method, we obtained low baseline levels of 8-oxo-dG, which were typically in the range of 8–12 8-oxo-dG/10^9 dGs (see Table 2), values similar to those obtained by others using DNA extraction and analytical techniques optimized to reduce artificial oxidation of DNA (23). Statistical Analysis of the Data. The data presented are representative of results obtained from one of two or more separate experiments. The statistical analyses were performed as described in the legends of the figures and tables and in the text. Differences were considered significant when P < 0.05.

RESULTS

E2 Metabolites ± COMT Inhibition. To examine the profile of estrogen metabolism in MCF-7 cells, an HPLC method with CoulArray ECD was developed to quantify the relative concentrations of estrogen metabolites. A chromatogram showing the retention times of an equimolar (300 pmol) mixture of 11 estrogen standards is shown in Fig. 1. It can be seen that each metabolite is distinct in regard to retention time and that each is oxidized as a characteristic cluster of oxidation peaks that are observed on two or more adjacent channels (59). Standard solutions of each compound were combined to generate equimolar mixtures containing varying concentrations of each estrogen standard and injected onto the column. These standard solutions were then used to generate calibration curves. Standard curves were linear between 10 and 500 pmol (data not shown). The limit of detection under the conditions of analysis was ~10 pmol on column. The spectrum of metabolites produced was first assessed in control or TCDD-pretreated MCF-7 cells subsequently treated with 1 μM of E2. In non-TCDD-treated MCF-7 cells, metabolism of E2 in control cells was very limited. After 15 h, 80% of the estrogen recovered was unmetabolized E2, 18% had been converted to E1, and the combination of other metabolites represented <2% of the total (data not shown). In contrast, in cells pretreated with TCDD (Table 1), metabolism was primarily to 2-OH E2 (34%) and 2-MeO E2 (30%). Formation of the corresponding 4-OH estrogens was very low; 4-OH E2 and 4-MeO E2 combined were <5% of the total, and no 16a-OH E2 formation was detected. Other minor metabolites detected included E1 and its 2- and 4-hydroxy metabolites. The sum of all of the estrogen metabolites recovered in this analysis (10.3 ± 1.5 nmol after correction for dilution factor, time, and mg protein/plate) was not statistically different from the total 10 nmol (1 μM, 10 ml medium) E2 added at the beginning of the experiment (by Student’s t test).

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| Table 1 The effect of COMT inhibition on estrogen metabolite levels in TCDD-pretreated MCF-7 cells exposed to 1 μM E2 for 15 h* |
|-----------------|-------------|-------------|-------------|
| Estrogen/metabolite | TCDD pretreated | TCDD + Ro 41-0960 | |
| E2 | 191.4 ± 39.2* (28.6) | 100.8 ± 20.1 (13.8) | |
| 2-OH E2 | 225.3 ± 23.9 (33.6) | 394.3 ± 46.2 (81.5) | |
| 4-OH E2 | 11.0 ± 9.8 (1.6) | 8.1 ± 2.4 (1.1) | |
| 2-MeO E2 | 203.3 ± 23.1 (30.3) | 24 ± 2.7 (0.3) | |
| 4-MeO E2 | 20.7 ± 2.3 (3.1) | — | |
| E1 | 3.2 ± 1.4 (0.5) | 5.7 ± 2.7 (0.8) | |
| 2-OH E1 | 4.0 ± 1.0 (0.6) | 14.3 ± 0.6 (2.0) | |
| 4-OH E1 | 10.2 ± 0.2 (5) | 3.9 ± 3.2 (5) | |

* Cells were plated in 100 mm culture dishes and allowed to come to ~40% confluence. Cells were then preincubated with 10 nm TCDD or DMSO vehicle for 72 hours, after which TCDD was removed. After overnight incubation in fresh culture medium, cultures received either 10 μM Ro 41-0960 or vehicle control 30 min before and incubated along with 1 μM E2. After 15 hours, medium was removed, and the culture plates with attached cells were frozen for protein analysis. Medium was then extracted subsequent to overnight β-glucuronidase treatment. Concentrations of metabolites were determined by comparison to known concentrations of standards after analysis by HPLC/ECD as described in “Materials and Methods.” The metabolite levels detected were corrected for recovery and normalized to cellular protein concentration.

Data represent the mean ± range of two determinations.

* p < 0.05.
recovered without COMT inhibition, represented 82% of the total recovered with COMT inhibited. Corresponding 2-MeO E2 concentrations were 30 and 0.3% of the total, respectively. The concentration of unmetabolized E2 remaining in cells treated with COMT inhibitor was ~60% of that in cells without inhibitor, suggesting that E2 metabolism was increased in the presence of the inhibitor. However, this effect was not seen when E2 metabolism was analyzed after only 9 h (see below). As in noninhibitor-treated cells, the amount of E1 and its metabolites was very low, although it was observed that 2-MeO E1 concentrations were decreased and 4-OH E1 concentrations were increased in the presence of inhibitor. (The concentration of 2-OH E1 could not be determined, because it coeluted with and oxidized at a potential similar to that of the inhibitor.) The level of inhibition and profile of metabolites observed using 10 or 3 μM COMT inhibitor was similar (data not shown); therefore, the 3 μM concentration of inhibitor was used in subsequent experiments.

**Effect of COMT Inhibition on Oxidative DNA Damage.** Initial experiments demonstrated that in TCDD-pretreated MCF-7 cells exposed to 10 μM of E2 for up to 9 h, 8-oxo-dG levels were unchanged compared with non-E2 treated controls (data not shown). However, when cells were treated with E2 while COMT enzyme activity was concurrently inhibited, 8-oxo-dG levels continued to rise for the entire duration of treatment (data not shown). Fig. 2 shows 8-oxo-dG levels in TCDD-pretreated cells exposed to 1 or 10 μM of E2 for 9 h, with or without 3 μM of COMT inhibitor. There were no significant differences in 8-oxo-dG levels in cells treated with 3 μM COMT inhibitor, TCDD alone, 10 μM E2, TCDD plus 10 μM E2, or TCDD plus 3 μM inhibitor. In contrast, statistically significant, ~3-fold and 5-fold increases in 8-oxo-dG levels were seen when TCDD-pretreated cells were treated with either 1 or 10 μM E2, respectively, in the presence of 3 μM COMT inhibitor. Preliminary analysis of the estrogen catechol metabolite levels in the medium was done to determine their association with the levels of 8-oxo-dG. The results indicated that the increased 8-oxo-dG concentrations in cellular DNA were associated with high 2-OH catechol levels and low 2-MeO E2 levels (data not shown).

To more clearly elucidate the relationship between 8-oxo-dG and estrogen catechol metabolite concentrations, both were next assessed using lower concentrations of E2 and either 3 or 0.3 μM COMT inhibitor (Table 2). There were no statistically significant differences in 8-oxo-dG levels in cells treated with DMSO, TCDD, TCDD plus 1 μM E2, or TCDD plus 3 μM COMT inhibitor (by ANOVA analysis). On the other hand, after TCDD pretreatment and in the presence of 3 μM inhibitor, as the concentration of E2 increased from 0 μM to 1.0 μM, the amount of 8-oxo-dG in cellular DNA also increased significantly. This increase in 8-oxo-dG was associated with an increase in the amount of 2-OH E2 present. A graph of the amount of 8-oxo-dG versus the amount of 2-OH plus 4-OH E2 present yielded a line with a slope of 0.63 (P < 0.0001 by linear regression analysis) and a correlation coefficient of 0.84 (by the least squares method), suggesting a direct relationship between CE metabolite levels and oxidative DNA damage in the absence of COMT activity and O-methylated metabolites (Fig. 3).

MCF-7 cells were also treated with a lower concentration of the COMT inhibitor (0.3 μM) plus 1 μM E2 and the results compared with those treated with 3.0 μM inhibitor plus 1 μM E2. There were significantly less catechol (2-OH plus 4-OH E2) metabolites (90.9 versus 158.2 pmol/mg protein; P < 0.001 by Student’s t test) and significantly less 8-oxo-dG (56 ± 12 versus 116 ± 21 8-oxo-dG/106; P < 0.012 by Student’s t test) in the cells incubated with 0.3 μM inhibitor (Table 2). On the basis of the relationship between catechol metabolite and 8-oxo-dG levels shown in Fig. 3, this level of 8-oxo-dG is somewhat less than the expected value of 78.8 ± 7.5 in the

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![Graph showing the effect of COMT inhibition on oxidative DNA damage](image)
COMT is protective against adverse cellular effects mediated through activity. the role of COMT in breast cancer. In light of this, it is important to environmental factors that modulate this pathway could have an impact on indicate that COMT is a low penetrance allele, the overall risk cancer. Although the calculated odds ratios from the positive studies DISCUSSION breast cancer in certain women (49) has now received support from low activity allele of COMT confers an increased risk for developing increased risk for developing breast cancer. Our original finding that the homozygous for the low activity allele of COMT would be in-
metabolism to quinone metabolites, we hypothesized that women -methylation of CEs by COMT blocks their additional oxidative O
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through metabolism to quinones that can form adducts to DNA and processes (15), it is possible that in this microsome system, COMT catalyzed the specific metabolites were not determined in the Han and Liehr study be done to elucidate this finding.

In our study, whereas E2 was metabolized to predominately 2-OH catechol the majority of which was methylated by COMT, we observed no increase in oxidative DNA damage unless COMT was inhibited by Ro 41-0960 (Table 2; Fig. 2). Ro 41-0960 is a specific, tight-binding inhibitor that has been shown to competitively inhibit catecholamine O-methylation by COMT (56). In cells exposed to 3 µm inhibitor, the amount of 2-OH E2 and 8-oxo-dG increased with increasing concentrations of E2. Analysis of 2- plus 4-OH E2 and 8-oxo-dG levels in the presence of 3.0 µm COMT inhibitor, where O-methylated metabolites were undetectable, indicated the relationship to be linear (Fig. 3). However, this was not the case in the complete absence of COMT inhibition. Although 2-OH E2 levels in cells treated with 1 µM E2 in the absence of COMT inhibition were higher (45.4 ± 3.9 pmol/mg protein) than in cells treated with 0.1 µM E2 plus 3 µM Ro 41-0960 (25.7 ± 7.6 pmol/mg protein), 8-oxo-dG levels were lower (8 ± 2 versus 36 ± 5 8-oxo-dG/10^6 dG, respectively; Table 2). Furthermore, as pointed out in the results, in the presence of 0.3 µM COMT inhibitor, the amount of oxidative DNA damage was less than expected given the amount of catechol metabolites present. At this point, an explanation for these differences is not apparent, although it can be seen that the 2-MeO E2 concentrations were higher under those conditions where levels of 8-oxo-dG were lower although 2-OH E2 was present. Additional studies will need to be done to elucidate this finding.

The results from our study clearly show that 2-OH E2 levels are associated with 8-oxo-dG levels in E2-treated MCF-7 cells in which COMT is inhibited. Han and Liehr (17), using a rat liver microsome system plus hamster liver DNA, observed that 4-OH E2 but not 2-OH E2 caused a significant increase in 8-oxo-dG levels. COMT is found in both the cytosol and microsomes (38). In addition, as mentioned in the “Introduction,” COMT has a greater catalytic activity toward 2-OH E2 as compared with 4-OH E2. Thus, whereas the levels of the specific metabolites were not determined in the Han and Liehr study (15), it is possible that in this microsome system, COMT catalyzed the inactivation of 2-OH E2 to a greater extent than 4-OH E2. Furthermore, other studies have observed that 2-OH and 4-OH E2 have similar abilities to participate in redox cycling processes. Moley et al. (18) determined that 2-OH and 4-OH E2 have similar electrochemical properties. They also showed that in the presence of copper, both estrogen catechols caused the formation of similar amounts of 8-oxo-dG in calf thymus DNA caused by the production of superoxide through redox cycling (18). In addition, we observed that 2-OH and 4-OH E2, in the presence of copper, caused similar amounts of DNA strand breaks (15), although electron spin resonance studies indicated presence of 90.9 pmol/mg protein of catechol metabolites. However, in the presence of 0.3 µM inhibitor, 2-OH methylation was not completely inhibited as shown by the presence of some 2-MeO E2, although the amount of unmetabolized E2 present was similar. The only other treatment condition under which 2-OH methylation was detected was the one in which cells were treated with 1 µM E2 without COMT inhibitor (Table 2). Here, too, based on the relationship shown in Fig. 3, 8-oxo-dG levels were far lower than would otherwise be expected given the amount of 2-OH E2 present. These results suggest that the presence of 2-MeO E2 may reduce 8-oxo-dG formation.

DISCUSSION

Experimental evidence suggests that the catechol metabolites of E2, particularly 4-OH E2 may contribute to estrogen carcinogenicity through metabolism to quinones that can form adducts to DNA and cause oxidative DNA damage through participation in redox cycling processes (3, 5, 6, 11–13, 17). On the basis of the knowledge that O-methylation of CEs by COMT blocks their additional oxidative metabolism to quinone metabolites, we hypothesized that women homozygous for the low activity allele of COMT would be at increased risk for developing breast cancer. Our original finding that the low activity allele of COMT confers an increased risk for developing breast cancer in certain women (49) has now received support from three of four subsequent studies (50–53). Together, these genetic epidemiology studies provide support for the hypothesis that catechol metabolites of E2 contribute to the development of human breast cancer. Although the calculated odds ratios from the positive studies indicate that COMT is a low penetrance allele, the overall risk attributable to this polymorphism could be significant. In addition, other polymorphisms in the estrogen metabolic pathway and/or environmental factors that modulate this pathway could have an impact on the role of COMT in breast cancer. In light of this, it is important to understand the effects and biological consequences of altered COMT activity.

The goal of the present study was to determine the extent to which COMT is protective against adverse cellular effects mediated through the estrogen catechol metabolites. Accordingly, we chose oxidative DNA damage, represented by the 8-oxo-dG levels, as an end point to investigate, though it is important to realize that other cellular end points such as quinone DNA adducts may also be altered in response to decreased COMT activity. We exploited the ability of TCDD to increase E2 metabolism to catechol metabolites in MCF-7 cells via induction of CYP450 enzymes involved in estrogen oxidative metabolism, and we developed an HPLC/ECD method that allowed us to analyze the concentration of various estrogens and estrogen oxidative metabolites in a single injection. Using this method, analysis of medium isolated from MCF-7 cells revealed that TCDD pretreatment caused an increase in E2 metabolism, primarily to its 2-OH and 2-MeO metabolites (Table 2). It was shown previously that TCDD induction increases E2 metabolism to its 2- and 4- hydroxyxatechols in association with increased CYP450 1A1 and CYP450 1B1 mRNAs (29–31). We, too, found that these RNA levels were increased by 10 nM TCDD pretreatment in our MCF-7 cells (data not shown); however, we detected little E2 metabolism to 4-OH catechol. This suggests that our MCF-7 cells responded somewhat differently from the ones used in other studies on TCDD and CYP450 1B1 induction (30–32).

The line was obtained by linear regression analysis and the correlation coefficient determined by the least squares method.
that in the presence of copper, 4-OH E₂ generated somewhat greater amounts of hydroxyl radical than 2-OH E₂ (16). These findings suggest that at least in some systems, 2-OH and 4-OH E₂ have similar potential to participate in redox cycling processes and cause oxidative damage. Thus, differences observed in the carcinogenicity of 2-OH and 4-OH E₂ may be attributable, at least in part, to differences in their inactivation in vivo.

In summary, the results of this study demonstrate that in MCF-7 cells, under conditions where estrogen metabolism to catechol has been enhanced, an increase in oxidative DNA damage is not observed unless COMT activity is inhibited. In the absence of COMT inhibition, no increased oxidative DNA damage is observed at E₂ levels as high as 10 μM, whereas with COMT inhibited so that no O-methylated catechol metabolites are detected, a 4-fold increase above control levels of oxidative DNA damage is detected in cells treated with 0.1 μM E₂. A decreased level of COMT inhibition was associated with a decrease in oxidative DNA damage. These results demonstrate that COMT activity is protective against oxidative DNA damage associated with CE metabolite levels. The results also suggest that in breast tissue of women with low COMT activity, an increase in oxidative DNA damage mediated through the estrogen catechols may be a contributory factor to the development of breast cancer. Moreover, it is possible that the increased catechol levels may also be associated with other adverse affects such as increased quinone DNA adduct levels, which we did not measure. Additional mechanistic studies and perhaps the development of an appropriate mouse model are warranted to provide more insight into the role of estrogen catechol metabolites and polymorphisms affecting enzymes that determine their levels in breast tissues and in breast cancer.

**REFERENCES**


The Effects of Catechol-O-Methyltransferase Inhibition on Estrogen Metabolite and Oxidative DNA Damage Levels in Estradiol-treated MCF-7 Cells

Jackie A. Lavigne, Julie E. Goodman, Tekum Fonong, et al.