Resveratrol inhibits TCDD-induced expression of CYP1A1 and CYP1B1 and catechol estrogen-mediated oxidative DNA damage in cultured human mammary epithelial cells

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Resveratrol (3,5,4'-trihydroxystilbene), a naturally occurring phytoalexin present in grapes and other foods, has been reported to possess chemopreventive effects as revealed by its striking inhibition of diverse cellular events associated with tumor initiation, promotion and progression. In our present study, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), when treated with the cultured human mammary epithelial (MCF-10A) cells, induced the expression of cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1) that are responsible for the oxidation of 17β-estradiol to produce catechol estrogens. Resveratrol strongly inhibited the TCDD-induced aryl hydrocarbon receptor (AhR) DNA binding activity, the expression of CYP1A1 and CYP1B1 and their catalytic activities in MCF-10A cells. It also reduced the formation of 2-hydroxyestradiol and 4-hydroxyestradiol from 17β-estradiol by recombinant human CYP1A1 and CYP1B1, respectively. Furthermore, resveratrol significantly attenuated the intracellular reactive oxygen species (ROS) formation and oxidative DNA damage as well as the cytotoxicity induced by the catechol estrogens. Our data suggest that CYP1A1- and CYP1B1-catalyzed catechol estrogen formation might play a key role in TCDD-induced oxidative damage, and resveratrol can act as a potential chemopreventive against dioxin-induced human mammary carcinogenesis by blocking the metabolic formation of the catechol estrogens and scavenging the ROS generated during their redox cycling.

Introduction

Breast cancer has become one of the most important malignant disorders among women all over the world. It has been reported that one in 10 women in the US develops breast cancer, and over 40 000 per year are expected to die from this disease (1, 2). Etiology of breast carcinogenesis depends on several parameters, such as the absence or presence of the estrogen receptor, exposure to xenobiotics and/or genetic factors. The accidental human exposure to ubiquitous environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other halogenated aromatic hydrocarbons has been well documented (3–5). It is widely accepted that the majority of biological effects of TCDD in higher organisms are mediated through activation of cytosolic aryl hydrocarbon receptor (AhR). Upon ligand binding, the activated AhR translocates into the nucleus where it interacts with the xenobiotic responsive element (XRE) (6, 7), responsible for regulating expression of several isoforms of cytochrome P450 (CYP) enzymes, including CYP1A1, CYP1A2 and CYP1B1, and some of the phase II detoxification enzymes.

Estrogen and estradiol can stimulate cancer cell growth by triggering estrogen receptor-mediated signal transduction, resulting in increased DNA synthesis and cell proliferation (8). Estrogen metabolites formed by cytochrome P450s may also play a role in the initiation of cancer. CYP1A1 and CYP1B1 are responsible for hydroxylation of 17β-estradiol (E2) at the C-2 and C-4 positions, respectively, resulting in the formation of 2-hydroxyestradiol (2-OHE₂) and 4-hydroxyestradiol (4-OHE₂). These catechol estrogens can be oxidized to quinones, which are putative tumor initiators (9–13). 2,3-Estradiol quinone derived from 2-OHE₂ reacts with DNA to form stable adducts, which do not seem to generate mutations. In contrast, 3,4-estradiol quinone, derived from 4-OHE₂, forms depurinating adducts, which readily leads to genotoxic events (10–15). Therefore, the extent of CYP1B1 expression appears to be a critical determinant of the metabolism and toxicity of estrogens in mammary cells and may hence serve as a potential biomarker for hormonal carcinogenesis (13, 16).

Resveratrol (3,5,4'-trihydroxystilbene), a naturally occurring compound present in grapes and other foods, has been reported to exert chemopreventive effects in diverse in vitro and in vivo systems (17–19). Our previous studies also demonstrated some chemopreventive and chemoprotective activities of resveratrol (20–23). Recently, resveratrol has been reported to act as an AhR antagonist and inhibits TCDD-induced CYP1A1 expression (24–28). It also has inhibitory effects on the catalytic activities of certain CYP isoforms (29–32). In this work, we have explored the possible inhibitory effects of resveratrol on TCDD-induced expression and activities of CYP1A1 and CYP1B1 responsible for catechol estrogen formation in the human mammary epithelial MCF-10A cell line. We also investigated the protective effects of resveratrol on 2-OHE₂- and 4-OHE₂-induced cytotoxicity, intracellular reactive oxygen species (ROS) accumulation and oxidative DNA damage. Our data suggest that resveratrol, by inhibiting both expression and catalytic activity of CYP1A1 and CYP1B1, inhibits the formation of catechol estrogens and their subsequent oxidation, thereby attenuating oxidative DNA damage and possibly neoplastic transformation in human mammary epithelial cells.
Materials and methods

### Materials

- Resveratrol (99% purity), E2, 2-OHE2, 4-OHE2, 2',4'-deoxyoxygenase (dGuo) and 8-oxo-7,8-dihydro-2'-deoxyoxygenase (8-oxo-dGuo) were purchased from Sigma Chemical (St Louis, MO). TCDD was a product of Pierce Biotechnology (Rockford, IL). Eagle’s medium (DMEM), fetal bovine serum and horse serum were obtained from Gibco BRL (Grand Island, NY).
- Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum and horse serum were analyzed by real-time PCR methods.
- Western blot analysis for CYP1A1 and CYP1B1
- After treatment, cells were washed with PBS, centrifuged and resuspended in ice-cold isotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)].
- After treatment, cells were washed with PBS, centrifuged and resuspended in ice-cold isotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT and 0.2 mM PMSF] and incubated on ice for 20 min. After vortex-mixing and centrifugation, the supernatant was stored at −70°C for the AhR DNA binding assay.
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### Cell culture

- The MCF-10A cell line, originally established at the Michigan Cancer Foundation in the US, was a generous gift from Dr Aree Moon of Dukung Women’s University, Seoul, South Korea. Cells were cultured with medium containing DMEM/F12, 10% fetal bovine serum, 20 ng/ml recombinant human epidermal growth factor, 0.5% hydrocortisone, 20 ng/ml recombinant human epidermal growth factor, 0.5% N2-supplemented DMEM, and 5% horse serum at 37°C (5% CO2).
- Bands were developed after exposure of the X-ray film at −80°C with a final concentration of 1 M 7-ethoxyresorufin and 10 µM dexamethasone for 5 min and cycled immediately 35 times.
- To monitor intracellular accumulation of ROS, the fluorescent probe DCF-DA (5',6'-dichloro-2',7'-dihydrofluorescein diacetate) was used. Following treatment with 5 µM DCF-DA, cells were exposed to TCDD (2 nM) or DMSO (0.1% v/v). After 72 h, the media were replaced with 50 mM MgCl2, 5 µM 7-ethoxyresorufin and 10 µM dexamethasone.

### Determination of cell proliferation

- Cells were cultured in 6-well dishes and co-treated with [methyl-3H]thymidine at a final concentration of 1 µCi/ml and varying concentrations of catechol estrogens in the presence or absence of resveratrol.
- After 24 h incubation, cells were trypsinized, washed with PBS and centrifuged. The supernatant was removed, and ice-cold 10% trichloroacetic acid (TCA) solution (20 µl of 0.2 N NaOH (with 10% N HCl) and 1 ml cocktail solution. Radioactivity in TCA-precipitable material was measured by liquid scintillation counting.

### Intracellular ROS accumulation

- To monitor intracellular accumulation of ROS, the fluorescent probe DCF-DA was used. Following treatment, cells were washed with Kreb’s ringer solution, and 10 µM DCF-DA was added. After a 15-min incubation at 37°C, the cells were examined under a confocal microscope equipped with an argon laser (488 nm, 200 mW).

### RT-PCR analysis for CYP1A1 and CYP1B1 mRNA expression

- Cells were cultured in 6-well dishes and co-treated with [methyl-3H]thymidine at a final concentration of 1 µCi/ml and varying concentrations of catechol estrogens in the presence or absence of resveratrol.

### Northern blot analysis for CYP1A1 and CYP1B1 mRNA expression

- Total RNA was isolated from cells using TRIzol® Regent (Gibco BRL, Life Technologies). Each RNA sample (10 µg) was electrophoresed through a 1.0% agarose-formalin/ethylenediamine gel in 1× MOPS buffer. The electrophoresed gel was stained with 0.05% ethidium bromide for 30 min with an acridine orange (final concentration 3 µg/ml) solution diluted with autoclaved distilled water, washed twice with distilled water for 20 min, and photographed under UV light to visualize 28S and 18S rRNA as size markers. The gel was then cross-linked with a final concentration of 1 M 7-ethoxyresorufin and 10 µM dexamethasone for 5 min and cycled immediately 35 times.

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### Intracellular ROS accumulation

- To monitor intracellular accumulation of ROS, the fluorescent probe DCF-DA was used. Following treatment, cells were washed with Kreb’s ringer solution, and 10 µM DCF-DA was loaded. After a 15-min incubation at 37°C, the cells were examined under a confocal microscope equipped with an argon laser (488 nm, 200 mW).

### HPLC analysis of 8-oxo-dGuo formation

- DNA isolation was performed by the conducting protocol supplied with the DNA extraction kit.

### Dose-effect analysis

- After 24 h incubation, cells were trypsinized, washed with PBS and centrifuged. The supernatant was removed, and ice-cold 10% trichloroacetic acid (TCA) solution (20 µl) was added to the pellet, followed by vortex-mixing. The mixture was centrifuged, and the resulting pellet was mixed with 400 µl of 0.2 N NaOH (with 10% N HCl) and 1 ml cocktail solution. Radioactivity in TCA-precipitable material was measured by liquid scintillation counting.

### Intracellular ROS accumulation

- To monitor intracellular accumulation of ROS, the fluorescent probe DCF-DA was used. Following treatment, cells were washed with Kreb’s ringer solution, and 10 µM DCF-DA was loaded. After a 15-min incubation at 37°C, the cells were examined under a confocal microscope equipped with an argon laser (488 nm, 200 mW).

### HPLC analysis of 8-oxo-dGuo formation

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of 10 μl of 1 M Tris-HCl buffer (pH 8.5), 2 U of calf intestine alkaline phosphatase was added, and the incubation was continued at 37°C for an additional 1 h. The reaction was terminated by the addition of 250 mM sodium acetate (pH 5.0) and 4 mM EDTA. The hydrolyzates were centrifuged at 15 000 g for 30 min at 4°C and analyzed by HPLC coupled with the electrochemical detector (ESA, Chelmsford, MA) using a C18 reverse-phase column (4 μm; 3.9 × 150 mm). Products were eluted with 10% methanol in 50 mM sodium phosphate buffer (pH 5.0), at a flow rate of 0.5 ml/min. The amounts of 8-oxo-dGuo and dGuo were calculated from the corresponding peak areas, and the results were expressed as the ratio of 8-oxo-dGuo to 105 dGuo.

Statistical analysis
When necessary, data were expressed as means ± SD, and statistical analysis for single comparison was performed using the Student’s t test. The criterion for statistical significance was P < 0.05.

Results
Inhibitory effects of resveratrol on TCDD-induced CYP1A1 and CYP1B1 expression
TCDD-induced CYP1A1 and CYP1B1 expression in MCF-10A cells was assessed by western blot assay, northern blot assay and RT-PCR. Western blot analysis showed that treatment of MCF-10A cells with 1 or 10 nM TCDD for 6, 24 or 72 h resulted in a progressive induction of CYP1A1 and CYP1B1, which was time- and concentration-dependent (Figure 1A). Resveratrol co-treated with TCDD for 3 days significantly suppressed the TCDD-induced expression of CYP1A1 and CYP1B1 (Figure 1B) and their mRNA transcripts (Figure 1C), which was concentration-dependent. TCDD-induced expression of CYP1A1 was almost completely suppressed by resveratrol at 20 μM as assessed by western and northern blot analyses, while the CYP1B1 expression was affected to a lesser extent. We also verified the inhibitory effects of resveratrol on transcription of CYP1A1 and CYP1B1 by RT-PCR (Figure 1D), although the extent of inhibition was less prominent compared with that observed in the northern blot analysis. Furthermore, resveratrol at 50 μM alone reduced the constitutive expression of CYP1B1 and its mRNA transcripts (Figure 1B and D).

TCDD-induced AhR DNA binding was suppressed by pre-treatment of resveratrol
Resveratrol was found recently to act as an AhR antagonist (24–28). We, therefore, assessed the possible inhibitory effects of resveratrol on TCDD-induced DNA binding activity of AhR in MCF-10A cells. In TCDD-stimulated MCF-10A cells, the AhR DNA binding activity peaked at ~1 h (Figure 2A), which was suppressed by resveratrol (Figure 2B). Resveratrol at 50 μM alone did not influence the AhR DNA binding activity.

Resveratrol inhibited the CYP enzyme activities
The catalytic activities of CYP1A1 and CYP1B1 were measured by the EROD assay. As illustrated in Figure 3, treatment of MCF-10A cells with 2 nM TCDD for 3 days significantly increased the EROD activity (∼5-fold induction). Resveratrol exerted a concentration-dependent inhibition of the TCDD-induced EROD activity. Resveratrol at 50 μM abolished the enzyme activity completely (Figure 3).

Inhibitory effects of resveratrol on the catechol estrogen formation by recombinant human CYP1A1 and CYP1B1
Catechol estrogen formation from E2 by CYP1A1 and CYP1B1 was analyzed by HPLC equipped with an electrochemical detector. Incubation of 10 μM E2 together with 50 μg recombinant human CYP1A1 and CYP1B1 predominantly produced 2-OHE2 and 4-OHE2, respectively (Table I). Addition of resveratrol to the incubation solution significantly inhibited the formation of both 2-OHE2 (Figure 4A) and 4-OHE2 (Figure 4B). The IC50 values for inhibition of catalytic activities of CYP1A1 and CYP1B1 by resveratrol were ~2 and 25 μM, respectively.

Resveratrol inhibited ROS accumulation induced by TCDD and E2
Intracellular ROS accumulation can be measured by the use of DCF-DA, which is freely permeable to the cell membrane. Once inside cells, the compound is hydrolyzed to DCF by the cellular esterase activity, which interacts with peroxides to form fluorescent 2′,7′-dichlorofluorescin. Treatment of MCF-10A cells with 1 μM 17β-estradiol alone for 6 h failed to cause...
an appreciable increase in ROS accumulation (Figure 5B). Stimulation with TCDD alone, exhibited slight enhancement of intracellular ROS formation (Figure 5C). However, treatment of MCF-10A cells with 10 nM TCDD for 3 days followed by exposure to 1 μM 17β-estradiol for an additional 6 h dramatically enhanced ROS accumulation (Figure 5D), which was significantly reduced by 20 μM resveratrol (Figure 5E). Resveratrol at this concentration alone did not influence an intracellular ROS level (Figure 5F).

**TCDD in combination with 17β-estradiol induced oxidative DNA damage**

8-Oxo-dGuo is recognized as the hallmark of oxidative DNA damage. Treatment of MCF-10A cells with 10 nM TCDD for 3 days in combination with 1 μM 17β-estradiol treatment for 6 h significantly increased the 8-oxo-dGuo level, which was abrogated by resveratrol (Figure 6). Either TCDD or 17β-estradiol alone did not cause 8-oxo-dGuo formation, which was consistent with the ROS accumulation data (Figure 5).

**Resveratrol protected MCF-10A cells against catechol estrogen-induced cytotoxicity**

In order to further confirm the possible involvement of catechol estrogens in TCDD-induced oxidative damage, we examined the inhibitory effects of resveratrol on the formation of catechol estrogens (Figures 4A and 4B).

### Table 1. Relative rates of catechol estrogen formation from 17β-estradiol catalyzed by recombinant human CYP1A1 and CYP1B1

<table>
<thead>
<tr>
<th>Catechol estrogen formation (pmol/mg protein/h)</th>
<th>Enzyme source</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CYP1A1</td>
</tr>
<tr>
<td>2-OHE2</td>
<td>211.54 ± 21.04</td>
</tr>
<tr>
<td>4-OHE2</td>
<td>51.56 ± 7.78</td>
</tr>
</tbody>
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Incubation conditions and other experimental details are described in Materials and methods. Data are means ± SD of three independent analyses.
the cytotoxicity induced by 2-OHE$_2$ and 4-OHE$_2$. 2-OHE$_2$ was found to be more toxic than 4-OHE$_2$ according to the results of the [methyl-3H]thymidine incorporation assay (Figure 7). Thus, 2-OHE$_2$ (20 μM) treatment for 24 h caused ~60% cell death (Figure 7A) while the same concentration of 4-OHE$_2$ induced only 20% decrease in the cell viability (Figure 7B). Resveratrol protected MCF-10A cells against catechol estrogen-induced cytotoxicity in a concentration-dependent manner.

Resveratrol inhibited the catechol estrogen-induced intracellular ROS accumulation

MCF-10A cells treated with 20 μM catechol estrogen for 6 h displayed intense fluorescence after staining with DCF dye. Intracellular ROS accumulation was significantly elevated after catechol estrogen treatment (Figure 8B and D). The ROS accumulation was strongly reduced when resveratrol was present in the media (Figure 8C and E).

Resveratrol had a protective effect on the catechol estrogen-induced DNA damage

Oxidative damage resulting from redox cycling between catechol estrogens and their quinoid forms is believed to be a critical event in carcinogenesis induced by estrogen. Figure 9 shows 8-oxo-dGuo formation induced by catechol estrogens and the protective effect of resveratrol. Treatment of MCF-10A cells with 2-OHE$_2$ and 4-OHE$_2$ at 20 μM each for 6 h increased the 8-oxo-dGuo level ~8- and 5-fold, respectively. Again, catechol estrogen-induced 8-oxo-dGuo formation was significantly attenuated by co-treatment with 20 μM resveratrol (Figure 9).

Discussion

Although many studies have shown the role of AhR-mediated CYP induction in estrogen metabolism in human breast cancer
Oxidative stress is considered as one of the plausible mechanisms underlying TCDD-induced carcinogenesis (38,39). We hypothesized that TCDD-induced oxidative stress could be mediated by catechol estrogens. In the MCF-10A cell line, 10 nM TCDD treatment alone for 3 days did not cause substantial oxidative stress. However, subsequent treatment with 1 μM E2 for 6 h dramatically increased both ROS accumulation and the 8-oxo-dGuo level while 1 μM E2 treatment alone did not cause appreciable oxidative stress (Figures 5 and 6), suggesting that TCDD-induced oxidative stress may be mediated via CYP-catalyzed catechol estrogen formation. Tritscher et al. (40) reported that TCDD increased oxidative DNA damage only in intact but not ovariectomized rats. Wyde and colleagues (41) have demonstrated that TCDD-induced 8-oxo-dGuo formation was female-specific and estrogen-dependent. The findings from these studies further support our hypothesis that the catechol metabolites of estradiol may mediate TCDD-induced oxidative stress. The ROS generated through redox cycling of metabolically formed catechol estrogens or their quinoid products can damage DNA to initiate the carcinogenesis. Alternatively, mild ROS may stimulate the growth signaling cascades, thereby inducing the promotion of neoplastic transformation. Thus, it is likely that catechol metabolites can contribute to estrogen-mediated carcinogenesis by both genotoxic and epigenetic mechanisms. Resveratrol inhibition of oxidative stress mediated by TCDD in combination with E2 is considered to be partly due to its free radical scavenging ability (45) as well as suppression of de novo synthesis of CYP1A1 and CYP1B1 and their catalytic activity responsible for the catechol estrogen formation.
The implication of estradiol in breast tumorigenesis is well documented (46,47). Multiple lines of evidence support that hormonal carcinogenesis is related to not only growth stimulatory effects of estrogens, but also the formation of catechol estrogens, particularly 4-OHE2 (10–13), which is generated mainly by CYP1B1 activity. On the other hand, CYP1A1 catalyzes the formation of primarily 2-OHE2, which is known to have less toxicity (14–16,48,49). However, our results suggest that, at least in the MCF-10A cell line, 2-OHE2 is likely to exert more cytotoxicity than 4-OHE2 by generating a larger amount of ROS and more oxidative DNA damage. Our observation is corroborated with an earlier report of Lavigne and colleagues on the ability of TCDD to induce oxidative E2 metabolism, primarily via the 2-hydroxylation (50). Mobley et al. (51) investigated the electrochemical properties of both 2-OHE2 and 4-OHE2, and found that both catechol estrogens exhibited nearly equal oxidative potentials under physiological conditions. Based on these findings, it is likely that the different oxidation rate of these two catechol estrogens in vivo would be determined by enzymatic rather than chemical reactions.

O-Methylation catalyzed by catechol-O-methyltransferase (COMT) represents a major phase II detoxification pathway for catechol estrogens (52,53). When MCF-7 cells were pre-treated with dioxin to stimulate the oxidative estrogen metabolism to catechol estrogens, followed by exposure to E2 with and without Ro 41-0960, a COMT-specific inhibitor, the pharmacologic inhibition of O-methylation led to increased formation of both 2-OHE2 and 8-oxo-dG (50). A genetic polymorphism in the COMT gene has been associated with a different risk of developing several types of human malignancy (54,55). The possible stimulation by resveratrol of expression or catalytic activity of COMT as an alternative mechanism responsible for its inhibition of catechol estrogen-mediated oxidative damage merits further investigation.
Bioavailability and blood and tissue levels of polyphenols are important in extrapolating results from studies in cell lines to animal models and humans (56). Several groups of investigators have assessed the pharmacokinetics of trans-resveratrol after oral or systemic administration (57,58). Although resveratrol appears to be poorly absorbed, there occurs enterohepatic recirculation of the glucuronide conjugate of resveratrol as well as the aglycone in rats (59), which may contribute significantly to its bioavailability. Although the inhibitory effects of resveratrol on oxidative estrogen metabolism and subsequent DNA damage and cytotoxicity were achievable at the supraphysiologic concentrations, our present study still provides important insights into the mechanistic basis for the estrogen carcinogenesis and also for the previously reported gender differences in the susceptibility to the TCDD-induced oxidative damage (40,41).

In conclusion, this study shows that TCDD can induce oxidative stress, which is mediated, at least in part, through the catechol estrogen formation. The ROS generated from redox cycling of catechol estrogens may play a key role in the TCDD-induced oxidative DNA damage and tumorigenesis. Resveratrol inhibits not only TCDD-induced CYP1A1 and CYP1B1 expression, but also their catalytic activities in MCF-10A cells, thereby protecting these cells against oxidative damage as schematically proposed in Figure 10. Further studies will be necessary to elucidate the possible cellular signaling events that resveratrol targets in attenuating TCDD-induced CYP1A1 and CYP1B1 expression and subsequent oxidative stress.

Acknowledgements

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References


Fig. 10. Schematic representation of resveratrol protection against oxidative DNA damage induced by TCDD and 17β-estradiol in MCF-10A cells.


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