

Modulatory effects of α - and γ -tocopherols on 4-hydroxyestradiol induced oxidative stresses in MCF-10A breast epithelial cells*

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Abstract

The elevated level of circulating estradiol increases the risk of breast tumor development. To gain further insight into mechanisms involved in their actions, we investigated the molecular mechanisms of 4-hydroxyestradiol (4-OHE₂) to initiate and/or promote abnormal cell growth, and of α - or γ -tocopherol to inhibit this process. MCF-10A, human breast epithelial cells were incubated with 0.1 μ M 4-OHE₂, either with or without 30 μ M tocopherols for 96 h. 4-OHE₂ caused the accumulation of intracellular ROS, while cellular GSH/GSSG ratio and MnSOD protein levels were decreased, indicating that there was an oxidative burden. 4-OHE₂ treatment also changed the levels of DNA repair proteins, BRCA1 and PARP-1. γ -Tocopherol suppressed the 4-OHE₂-induced increases in ROS, GSH/GSSG ratio, and MnSOD protein expression, while α -tocopherol up-regulated BRCA1 and PARP-1 protein expression. In conclusion, 4-OHE₂ increases oxidative stress reducing the level of proteins related to DNA repair. Tocopherols suppressed oxidative stress by scavenging ROS or up-regulating DNA repair elements.

Key Words: Breast cancer, DNA damage, 4-hydroxyestradiol, oxidative stress, tocopherol

Introduction

The etiology of breast cancer is related to multiple factors, a significant one of which is estrogen exposure (Colditz, 1998; Grodstein *et al.*, 1997; Service, 1998). 4-Hydroxyestradiol (4-OHE₂) is hydroxylated metabolite of 17 β -estradiol formed by CYP1B1. This catechol estradiol is further oxidized by sequential one-electron oxidations to produce the estrogen semiquinone radical and estrogen quinone. In rodent models, estrogens have been shown to act as carcinogens through a mechanism that involves oxidative stress in various tissues including kidney, liver, and breast (Han & Liehr, 1994; Harvell *et al.*, 2000; Yager & Liehr, 1996).

Several cellular defense molecules including superoxide dismutase (SOD) and reduced glutathione (GSH) protect cells from oxidative damage. Manganese superoxide dismutase (MnSOD) catalyzes the conversion of superoxide to hydrogen peroxide in mitochondria. It has been demonstrated that MnSOD levels are lowered in certain cancer cells (Hitchler *et al.*, 2006), while other studies have shown that the stimulated expression of MnSOD suppresses malignant phenotypes in mammalian

tumor cells, including breast (Li *et al.*, 1998), prostate (Liu *et al.*, 1997), and oral carcinoma cells (Liu *et al.*, 1998). The GSH/GSSG ratio is tightly regulated under normal circumstances, while the balance shifts towards a low GSH/GSSG ratio under conditions of oxidative stress. The ratio of GSH/GSSG measured in the blood of patients with colon and breast cancer has been found to be significantly decreased compared to that of control subjects (Pastore *et al.*, 2003).

Several proteins are involved in oxidative DNA damage repair, and therefore genetic defects in these proteins increase the risk of developing breast cancer. Breast cancer susceptibility gene 1 (BRCA1), for example, regulates cell cycle progression and DNA damage repair, and maintains genomic integrity (Rosen *et al.*, 2003). In addition, BRCA1 stimulates antioxidant response element-driven transcriptional activity and shifts the cellular redox balance to a higher ratio of reduced to oxidized glutathione (Bae *et al.*, 2004). Inherited mutations or deficiencies in BRCA1 predispose its carriers to higher risks of breast and ovarian cancers (King *et al.*, 2003; Walsh *et al.*, 2007). Poly (ADP-ribose) polymerase-1 (PARP-1), a nuclear member of the PARP superfamily, binds rapidly to DNA single and double strand

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breaks where its activation facilitates DNA repair (Ame *et al.*, 2004). In addition, PARP-1 is essential for the regulation of BRCA1 in response to DNA damage (Tong *et al.*, 2007). PARP-1 complementation partly rescues defective DNA damage response resulting from defective BRCA1. Hence, BRCA1 and PARP-1 are thought to respond to cellular oxidative stress including exposure to the oxidized estradiol metabolites.

Cancer patients have been shown to have low concentrations of tissue and plasma tocopherols compared to their healthy counterparts (Abiaka *et al.*, 2001; Goodman *et al.*, 2003; McMillan *et al.*, 2002). The functions of tocopherols in scavenging lipid peroxyl radicals in biological membranes are well documented (Baba *et al.*, 2005). A recent report has demonstrated that tocopherols can also directly suppress ROS production by interfering with the assembly of membrane-bound NADPH-oxidase complexes (Wittmann *et al.*, 2005). Tocopherols also regulate the expression of genes related to cell proliferation, cell cycle progression, and apoptosis in an antioxidant-independent way (Campbell *et al.*, 2003; Campbell *et al.*, 2006; Gysin *et al.*, 2002; Roy *et al.*, 2002).

In this study, we hypothesized that continuous exposure of normal breast epithelial cells to 4-OHE₂ increases oxidative stress and disrupts redox balance thereby altering the availability of repair elements. Effects of α - or γ -tocopherol on the markers of oxidative stress and the expression of DNA repair elements were also examined.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Cholera toxin was obtained from List Laboratories (Campbell, CA, USA). Culture media, epidermal growth factor, penicillin-streptomycin, horse serum and glutamine were purchased from Invitrogen (Carlsbad, CA, USA). Anti-MnSOD, BRCA1, and HRP-conjugated secondary antibody were obtained from Upstate (Lake Placid, NY, USA). The glutathione assay kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). PRO-PREPTM was purchased from iNtRON Biotechnology (Daejeon, Korea). The Bradford protein assay kit was purchased from BioRad laboratories (Hercules, CA, USA).

Cell culture

Human mammary epithelial cells (MCF-10A) obtained from ATCC (Manassas, VA, USA) were cultured in DMEM/F12 containing 10 μ g/ml bovine insulin, 100 ng/ml cholera toxin, 0.5 μ g/ml hydrocortisone, 20 ng/ml recombinant human epidermal growth factor, 2 mM L-glutamine, 100 μ g/ml penicillin/streptomycin, and 5% horse serum at 37°C in an atmosphere of 5% CO₂. The

non-tumorigenic MCF-10A cell line was originally derived from a patient with proliferative breast disease (Cuendet & Bolton, 2006).

Cell treatment

MCF-10A cells were incubated with 0.1 μ M 4-OHE₂, either with or without 30 μ M tocopherol for 24 to 96 h. 4-OHE₂ and tocopherols were replaced every day during the incubation period.

Measurement of intracellular ROS accumulation

MCF-10A cells were washed and treated for 20 min with 100 μ M 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a nonfluorescent probe that diffuses into cells. Cytoplasmic esterase hydrolyzes DCFH-DA to 2', 7'-dichlorodihydrofluorescein (DCFH) and then ROS generated within cells oxidize DCFH to the fluorescent dye, 2', 7'-dichlorofluorescein (DCF). The intensity of DCF was measured by a flow cytometry (FACS Calibur, Becton Dickinson, USA) at 530 nm using an FL1 filter.

Measurement of GSH/GSSG ratios

MCF-10A cells were collected by centrifugation at 1000 \times g for 5 min at 4°C. The cell pellet was sonicated in 1 mL PBS (pH 7.2) and centrifuged at 13,000 \times g for 20 min. Total GSH and GSSG were measured using a glutathione assay kit. The GSH disulfide (GSSG) content of cells was determined by adding 10 μ L 2-vinylpyridine solution per mL of sample in order to remove GSH according to the method described by Griffith (1980).

Protein extraction and western blot analysis

Cells were harvested by trypsinization, washed with PBS (pH 7.2) and the pellets were collected. The pellets were extracted using the PRO-PREPTM and protein concentration was determined. Protein (30 μ g) was added to each well of 10-12% SDS-PAGE. The gel was run at 100 V, 250 mA and transferred to a PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 80 min. The membrane was blocked in blocking buffer (0.2% Tween 20 in PBS, pH 7.2), containing 5% non-fat dry milk for 2 h at room temperature. Dilution of primary antibodies (MnSOD 1:2000, BRCA1 1:250, PARP-1 1:500) was made in blocking buffer. The blots were incubated with primary antibody for 2 h at room temperature. Following three washes with 0.2% Tween 20 in PBS, pH 7.2 (PBST), the blots were incubated with horseradish peroxidase-conjugated secondary antibodies in blocking buffer for 1 h at room temperature. The blots were then washed three times with PBST, and developed with ECL (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 min. Band intensity was measured by Versa Doc 5000 (Bio Rad, Hercules, CA, USA).

Statistical analysis

Data were expressed as the mean \pm SD. Differences in 4-OHE₂ treated and non-treated group were analyzed by Student's *t*-test. Comparisons among the treatments were tested by ANOVA followed Duncan's multiple range test. All statistical tests were performed using the SAS statistical package Version 9.0 (SAS Institute, Cary, NC).

Results

Changes in oxidative stress markers

Following the exposure of cells to 0.1 μ M 4-OHE₂ for 96 h, intracellular ROS was increased by 60% (Fig. 1A). Cellular

GSH/GSSG ratios were increased at 24 h and then decreased at 48 and 96 h (Fig. 1B). The expression of MnSOD, a key antioxidant enzyme involved in scavenging oxygen radicals was significantly decreased at 72 h and 96 h (Fig. 1C).

Changes in oxidative stress-related DNA damage response proteins

DNA damage response elements including BRCA1 and PARP-1 are closely related to the regulation of oxidative stress in breast cancer. As shown in Fig. 2A, BRCA1 protein levels were significantly suppressed at 48 h, while longer exposure (72 h) to 4-OHE₂ restored the level. Fig. 2B shows a significant decrease in PARP-1 level at 48 h when cells were treated with 4-OHE₂ treatment, while a considerable increase was observed

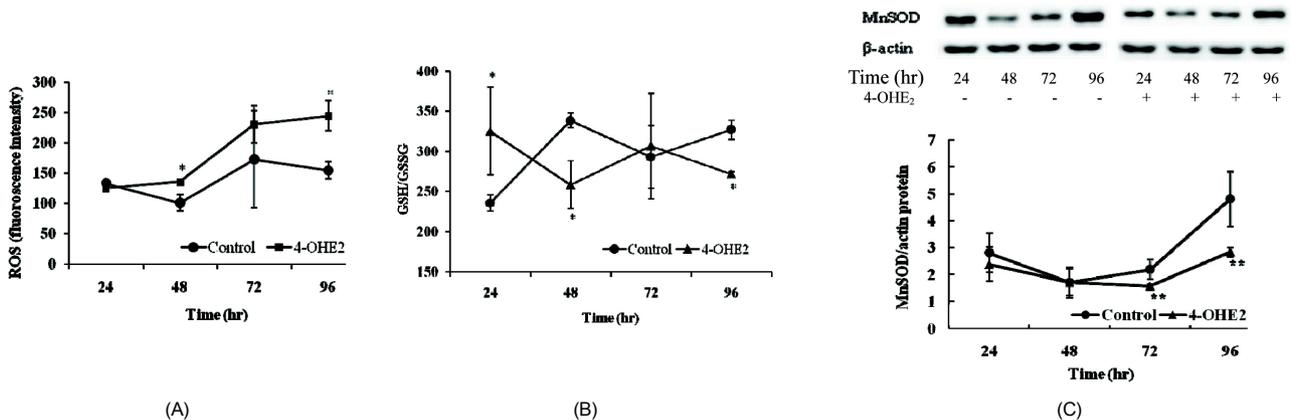


Fig. 1. Effect of 4-hydroxyestradiol (4-OHE₂) on oxidative markers in MCF-10A cells. A, Effect of 4-hydroxyestradiol (4-OHE₂) on intracellular ROS levels in MCF-10A cells. MCF-10A cells were treated with or without 0.1 μ M 4-OHE₂ for 24 h to 96 h. The ROS accumulation was measured using DCF-DA as described in materials and methods. * Significantly different from the control value ($P < 0.05$). B, Effect of 4-hydroxyestradiol (4-OHE₂) on GSH levels in MCF-10A cells. MCF-10A cells were incubated with or without 0.1 μ M 4-OHE₂ for 24 to 96 h. The level of GSH was measured as described in materials and methods. *Significantly different from the control value ($P < 0.05$). C, Effect of 4-hydroxyestradiol (4-OHE₂) on MnSOD protein expression in MCF-10A cells. MCF-10A cells were incubated with or without 0.1 μ M 4-OHE₂ for 24 to 96 h. MnSOD protein expression was detected by western blot analysis and normalized by β -actin as described in materials and methods. ** Significantly different from the control value ($P < 0.01$). Values present means \pm standard deviation ($n=3$ or 4).

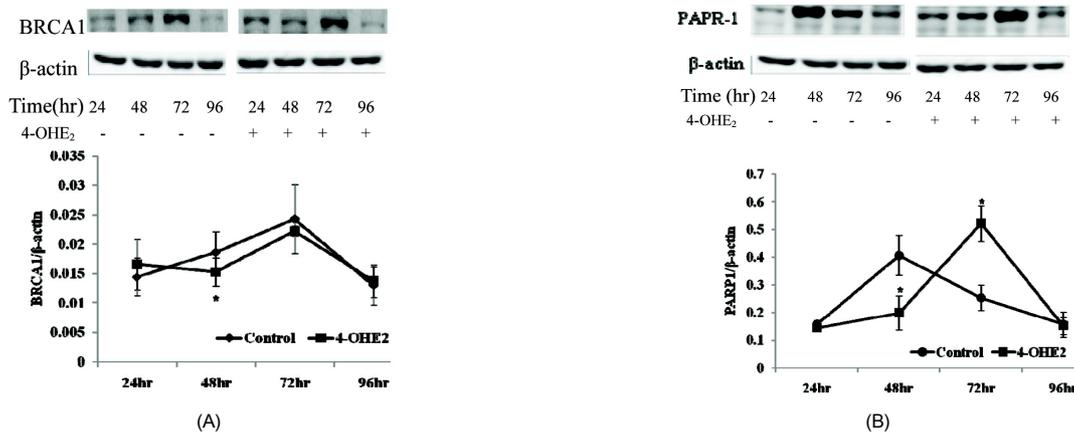


Fig. 2. Effect of 4-hydroxyestradiol (4-OHE₂) on BRCA1 and PARP-1 protein expression in MCF-10A cells. A, Effect of 4-hydroxyestradiol (4-OHE₂) on BRCA1 protein expression in MCF-10A cells. MCF-10A cells were incubated with or without 0.1 μ M 4-OHE₂ for 24 to 96 h. BRCA1 protein expression was detected by western blot analysis as described in materials and methods. * Significantly different from the control value ($P < 0.05$). B, Effect of 4-hydroxyestradiol (4-OHE₂) on PARP-1 protein in MCF-10A cells. MCF-10A cells were incubated with or without 0.1 μ M 4-OHE₂ for 24 to 96 h. PARP-1 protein was detected by western blot analysis and normalized by β -actin as described in materials and methods. * Significantly different from the control value, $P < 0.05$. Values present means \pm standard deviation ($n=3$ or 4).

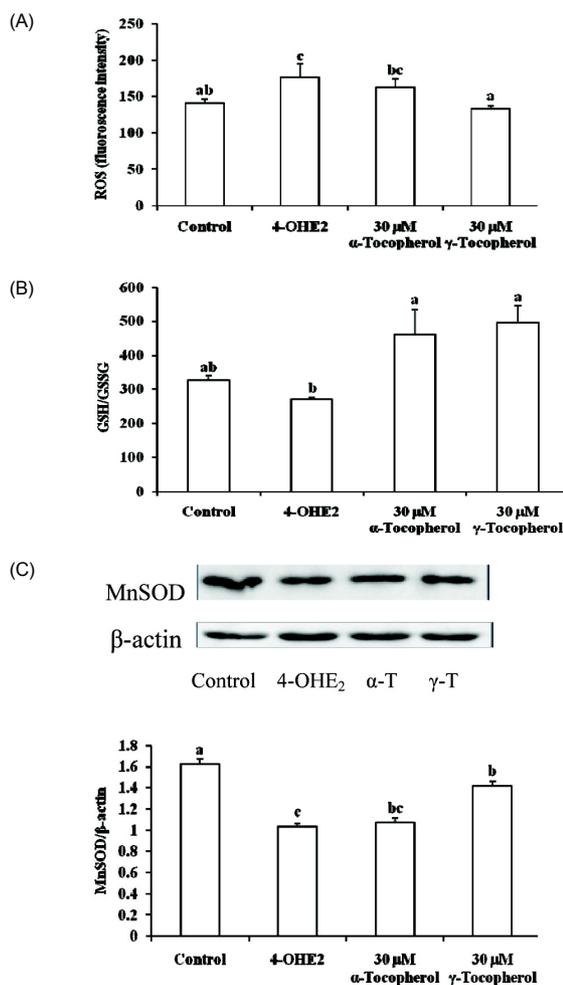


Fig. 3. Effect of α - and γ -tocopherol on oxidative stresses in MCF-10A cells. A, Effect of α - and γ -tocopherols on 4-OHE₂ induced ROS in MCF-10A. MCF-10A cells were incubated with or without 0.1 μ M 4-OHE₂ only with or without 30 μ M α - or γ -tocopherols for 96 h. Different superscripts (a, b, c) indicate significant differences ($P < 0.05$). B, Effect of α - and γ -tocopherol on GSH/GSSG levels in MCF-10A cells. MCF-10A cells were incubated with or without 0.1 μ M 4-OHE₂ only with or without 30 μ M α - or γ -tocopherols for 96 h. * Significantly different from 4-OHE₂ value, $P < 0.05$. Different superscripts (a, b) indicate significant differences ($P < 0.05$). C, Effect of α - and γ -tocopherol on MnSOD expression in MCF-10A cells. MCF-10A cells were incubated with or without 0.1 μ M 4-OHE₂ with or without 30 μ M α - or γ -tocopherols for 96 h. MnSOD protein expression was detected by western blot analysis and normalized by β -actin as described in materials and methods. Different superscripts (a, b, c) indicate significant differences ($P < 0.05$). Values present means \pm standard deviation ($n=3$ or 4).

at 72 h exposure.

Effects of tocopherols on modulating oxidative stress

Cells treated with 30 μ M γ -tocopherol showed a decreased level of intracellular ROS (Fig. 3A). γ -Tocopherol increased the GSH/GSSG ratio by reducing the level of GSSG (Fig. 3B). MnSOD protein level was increased with γ -tocopherol treatment (Fig. 3C). Although α -tocopherol did not exert a significant effect on these markers, it inhibited 4-OHE₂-induced BRCA1 and PARP-1 depletion (Fig. 4A, 4B).

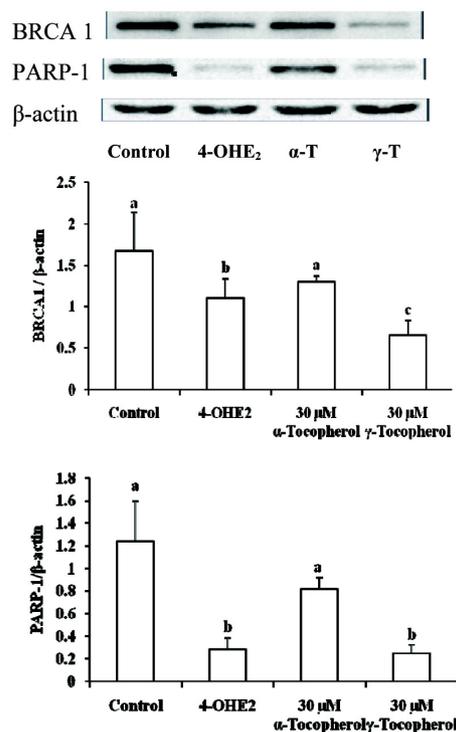


Fig. 4. Effect of α - and γ -tocopherol on BRCA1 and PARP-1 expression in MCF-10A cells. MCF-10A cells were incubated with or without 0.1 μ M 4-OHE₂ with or without 30 μ M α - or γ -tocopherols for 48 h. BRCA1 and PARP-1 protein expression was detected by western blot analysis and normalized by β -actin as described in materials and methods. Different superscripts (a, b, c) indicate significant differences ($P < 0.05$). Values present means \pm standard deviation ($n=3$ or 4).

Discussion

Carcinogenesis can be viewed as a stepwise process beginning with genotoxic events followed by enhanced cell proliferation and tumor progression. Evidence has accumulated supporting the theory that catechol estrogens are oxidized to quinones accompanied by the generation of ROS, capable of causing genotoxicity and tumorigenicity. Despite the importance of this process, a limited number of studies have attempted to evaluate oxidized estrogen as a possible compound affecting redox balance in the cellular environment and the possible effects of antioxidant intervention. Therefore, we investigated the molecular events involved in 4-hydroxyestradiol (4-OHE₂)-induced oxidative stresses as a possible event in cancer initiation process using normal breast epithelial cells. Protective roles of tocopherols were also determined.

In the present study, ROS levels, GSH/GSSG ratios, and manganese superoxide dismutase (MnSOD) protein levels were measured as indicators of oxidative stress in normal breast epithelial cells treated with 0.1 μ M 4-OHE₂, which is close to the physiological concentration in breast tissue. Changes in ROS level and the GSSG/GSH ratio indicate that intracellular ROS production caused by 4-OHE₂ was possibly alleviated by GSH.

In addition, reactive quinone formed from 4-OHE₂ can produce a conjugate with GSH causing depletion of cellular thiol levels, which renders the cells vulnerable to oxidative damage. Since H₂O₂-detoxifying enzymes were poorly expressed in breast cancer cells (Esworthy *et al.*, 1995), ROS accumulation may exert a greater effect in breast tumor development. We observed the restoration of GSH/GSSG balance in cells treated for 72 h. Cellular exposure to xenobiotics also results in a temporal increase in the total intracellular GSH content (Arrigo, 1999) indicating that cellular redox shift may stimulate GSH biosynthesis to overcome a redox shift, thereby stimulating GSH biosynthesis. Our study showed that MnSOD protein expression was decreased when cells were exposed to 4-OHE₂ for more than 72 h, suggesting MnSOD protein expression is responsive to increased ROS levels caused by 4-OHE₂ exposure. The decrease in MnSOD levels also leads to the accumulation of the superoxide anion (O₂⁻) possibly inducing further oxidative damage.

BRCA1 and Poly (ADP-ribose) polymerase-1 (PARP-1) are established as DNA repair elements especially in breast tissue (Ginestier *et al.*, 2009). Previous studies have reported that genetic polymorphisms in these genes are closely related to the increased risk of breast cancer (King *et al.*, 2003; Lane, 1992; Malkin *et al.*, 1990), indicating the functional importance of these genes in breast cancer development. It has been reported that estradiol and its metabolites induce mutation of chromosome 17 which contains the BRCA1 genes in human breast epithelial cells (Fernandez *et al.*, 2006). Deficiency of BRCA1 and PARP-1 results in the accumulation of DNA strand breaks by impairing DNA damage repair, and causing defects in cell cycle checkpoints, centrosome amplification, and genetic instability (Brodie & Deng, 2001; Cantor & Andreassen, 2006; Deng & Wang, 2003; Venkitaraman, 2002; Zhang & Powell, 2005), which eventually result in tumorigenesis. Even though the effect was a transient, 4-OHE₂ significantly decreased BRCA1 and PARP-1 protein expression. Since BRCA1 regulates the activity of nuclear factor (erythroid-derived 2)-like 2 (NFE2L2), an antioxidant response transcription factor, and is required for the repair of oxidative 8-oxoguanine lesions by transcription-coupled DNA repair (Bae *et al.*, 2004), BRCA1 depletion by 4-OHE₂ possibly accelerates carcinogenesis through inhibition of DNA damage repair and antioxidant enzyme expression.

Besides generating ROS during redox cycling, 4-OHE₂ reacts with DNA to form covalently bound adducts such as 4-OHE₂-N7-Gua and 4-OHE₂-N3-Ade (Belous *et al.*, 2007). ROS-induced oxidized bases are predominantly repaired via the base excision repair process (BER). It is generally believed that the repair of DNA single and double stranded breaks in mammalian cells is initiated by PARP-1 (de Murcia & Ménessier de Murcia, 1994; Plummer, 2006). Moreover, PARP-1 has a functional domain that includes the BRCA1 C-terminal domain (Plummer, 2006), and therefore PARP-1 deficiency impairs BRCA1 recruitment to the site of DNA damage (De Soto & Deng, 2006), which may facilitate cancer development. A reduction in PARP-1 protein

expression and an increase in its activity at 48 and 96 h were possibly accompanied by 4-OHE₂-induced ROS production. Chen *et al.* (2005) also showed that treatment with 4-OHE₂ at 25 μM for 24 h causes PARP-1 cleavage. Depletion of both PARP-1 and BRCA1 at 48 h can therefore cause an overload of 4-OHE₂ induced DNA damage.

Since exogenous antioxidants may regulate 4-OHE₂-induced cellular oxidative stress, we next evaluated the effects of tocopherols on oxidative stress markers and DNA repair protein expression. In these experiments, we used tocopherols at 30 μM which falls within the normal range of blood level in human. For measurement of oxidative stress markers, 4-OHE₂ and/or tocopherols were treated for 96 h, because our pretests indicated that the disturbance of antioxidative system was induced within 96 h. Study results indicated that in 4-OHE₂ treated MCF-10A cells, the accumulation of intracellular ROS and depletion of GSH and MnSOD were significantly attenuated by γ-tocopherol. The antioxidant activity of tocopherols is rooted in their ability to donate phenolic hydrogens to lipid radicals. Because it lacks electron-donating methyl groups on the chromanol ring, γ-tocopherol is somewhat less potent in donating electrons than α-tocopherol and thus a slightly less powerful antioxidant. However, the unsubstituted C-5 position of γ-tocopherol appears to make it as a better trap for lipophilic electrophiles such as reactive nitrogen oxide species (RNOs). Jiang *et al.* (2002) showed that *in vivo* γ-tocopherol supplementation, not α-tocopherol, resulted in inhibition of protein nitration and ascorbate oxidation in kidney. Li *et al.* (1999) also reported that γ-tocopherol was more potent than α-tocopherol in enhancing MnSOD protein expression in arterial tissues affected by cardiovascular disease. Mense *et al.* (2009) showed that antioxidant vitamin C reduces the incidence of estrogen-induced mammary tumors and increases tumor latency through changes in the activities of antioxidant enzymes superoxide dismutase and glutathione peroxidase. The cellular functions of α-tocopherol that are independent of this antioxidant/radical scavenging ability have been well characterized. A recent report suggests that vitamin E isomers possess anti-cancer activity by regulating DNA repair gene and apoptosis via signaling pathways (Aiub *et al.*, 2009; Theriault *et al.*, 1999). Therefore, we determined the effects of tocopherols on DNA repair elements in 4-OHE₂ induced oxidative stress in breast epithelial cells. We treated 4-OHE₂ and/or tocopherols for 48 h because our pretests indicated that 4-OHE₂ induced the depletion of BRCA1 and PARP-1 protein expression at 48 hr. Our results showed that α-tocopherol stimulates the up-regulated expression of DNA repair proteins, including BRCA1 and PARP-1. Yu *et al.* (2003) and Gu *et al.* (2008) reported that human cancer cells undergo apoptosis stimulated by α-tocopherol succinate in a concentration- and time-dependent manner by restoring PARP-1 levels.

In conclusion, the estrogen metabolite, 4-OHE₂ affects cellular redox status, regulates the expression of proteins closely related DNA repair and promotes breast cancer cell growth. As far as

we know, this is one of few studies to examine the involvement of oxidized estradiol in the impaired DNA repair. Exogenous α - and γ -tocopherol played a role as anti-cancer compounds by modulating 4-OHE₂ induced oxidative stresses.

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