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Reduction of estrogen-induced transformation of mouse mammary epithelial cells by *N*-acetylcysteine

Divya Venugopal[§,1], **Muhammad Zahid**[§,1], **Paula C Mailander**[1], **Jane L. Meza**[2], **Eleanor G. Rogan**[1], **Ercole L. Cavalieri**[1], and **Dhrubajyoti Chakravarti**[*,1]

[1] Eppley Institute for Research in Cancer and Allied Diseases, 986805 Nebraska Medical Center, Omaha, NE 68198-6805

[2] Preventive and Societal Medicine, 984350 Nebraska Medical Center, Omaha, NE 68198-4350

Abstract

A growing number of studies indicate that breast cancer initiation is related to abnormal estrogen oxidation to form an excess of estrogen-3,4-quinones, which react with DNA to form depurinating adducts and induce mutations. This mechanism is often called estrogen genotoxicity. 4-catechol estrogens, precursors of the estrogen-3,4-quinones, were previously shown to account for most of the transforming and tumorigenic activity. We examined whether estrogen-induced transformation can be reduced by inhibiting the oxidation of a 4-catechol estrogen to its quinone. We demonstrate that E6 cells (a normal mouse epithelial cell line) can be transformed by a single treatment with a catechol estrogen or its quinone. The transforming activities of 4-hydroxyestradiol and estradiol-3,4-quinone were comparable. *N*-acetylcysteine, a common antioxidant, inhibited the oxidation of 4-hydroxyestradiol to the quinone and consequent formation of DNA adducts. It also drastically reduced estrogen-induced transformation of E6 cells. These results strongly implicate estrogen genotoxicity in mammary cell transformation. Since *N*-acetylcysteine is well-tolerated in clinical studies, it may be a promising candidate for breast cancer prevention.

Introduction

In 1896, Beatson reported that removal of the ovary regressed breast cancer in women, suggesting a role of endogenous hormones in the disease [1]. Initial studies identified this hormone as estrogen, and found it to act by a receptor-mediated mechanism to cause breast cancer [2]. Later studies suggested that oxidative metabolites, formed as a result of abnormal estrogen metabolism in the breast, are also a major cause of this disease [3,4]. The abnormal metabolism primarily involves the formation of high levels of carcinogenic 4-catecholestrogens and their quinones (estrogen-3,4-quinones) [4,5]. A significant number of breast cancer patients appear to suffer from it. For example, in one study with 77 patients (49 controls and 28 breast cancer cases), 4-hydroxyestrogens, estrogen-3,4-quinones and their derivatives were found elevated in 54% of cases and 10% of controls [5]. This breast cancer phenotype can be the result of polymorphisms in estrogen-metabolizing genes, and/or

* Address correspondence to: Dr. D. Chakravarti, Eppley Institute for Research in Cancer and Allied Diseases, 986805 Nebraska Medical Center, Omaha, NE 68198-6805, Tel: (402) 559-2951, Fax: (402) 559-8068, E-mail: dchakrav@unmc.edu.
[§]Equal contributors.

alterations in the expression of the enzymes that favor the oxidation of estrogens to form these metabolites [4,6,7].

The 4-catecholestrogens and the estrogen-3,4-quinones may initiate breast cancer by a genotoxic mechanism [4]. The estrogen-3,4-quinones are the ultimate carcinogens, as they react with DNA to form depurinating adducts, which are detectable in the breast tissue [8] and in the urine [4]. Estrogen-DNA depurinating adducts show a strong association with breast cancer. For example, one study indicated that cancerous human breast tissue can have 30-fold more estrogen-induced depurinating adducts than normal human breast [8], and another study indicated that the urine of breast cancer patients as well as high risk women contain ~2-fold more of these adducts than normal women (Gaikwad et al, submitted). The depurinating adducts spontaneously dissociate from DNA, forming abasic sites. In the breast, these abasic sites induce the mutations that may initiate breast cancer. These ideas are supported by the observation that 4-catecholestrogens and estrogen-3,4-quinones account for the majority of the mutagenic [9-12], transforming [3,13] and carcinogenic activities of estrogens [14-17].

To establish the initiating role of estrogen-3,4-quinones in breast cancer, it is necessary to demonstrate a direct link between oxidative estrogen metabolism and an early event of breast cancer. In a preliminary study, four antioxidants (*N*-acetylcysteine, resveratrol, melatonin and reduced lipoic acid) were examined for their ability to inhibit estrogen-DNA adduct formation *in vitro* [18]. These antioxidants, when added in equimolar doses to 4-catecholestrogens, showed 33-77% inhibition of DNA adduct formation [18]. In the present study, we have examined whether *N*-acetylcysteine can be used to explore the role of oxidative metabolites of estrogen in breast cancer initiation. Specifically, we examined whether *N*-acetylcysteine can reduce estrogen-DNA adduct formation and inhibit estrogen-induced transformation of murine breast epithelial cells. A demonstration that targeting estrogen metabolism can prevent the transformation of murine breast epithelial cells may lead to new approaches to breast cancer prevention.

N-aceylcysteine is an aminothiol antioxidant and a precursor of cysteine and glutathione [19]. It was initially used as a topical mucolytic drug [20] and as an antidote for paracetamol poisoning [21]. Later, its antioxidant properties were found useful for preventing the progression of chronic obstructive pulmonary disease (COPD) [22]. Other studies have demonstrated that *N*-acetylcysteine has very low systemic toxicity and can cross the blood-brain barrier [23]. Its long-term use has been evaluated at 1-3 × 600 mg/d in clinical trials for the prevention of chronic bronchitis [24,25] and renal dysfunction [26]. Thus, *N*-acetylcysteine has desirable properties for long-term use. The discovery that *N*-acetylcysteine can prevent mutagenesis by oxidative DNA damage [27-29] and decrease the incidence of pre-neoplastic and neoplastic lesions by various smoke-related chemical carcinogens in rodents [23,30-32] pointed to its chemopreventive potential. Unfortunately, clinical trials did not indicate *N*-acetylcysteine to be effective in preventing lung cancer in smokers [33].

Materials and Methods

Chemicals and reagents

Estradiol (E_2) was oxidized to 4-OHE_2 and E_2-3,4-Q as described previously [34]. E_2-3,4-Q was reacted with Ade and dG to generate the depurinating adduct standards as described previously [34]. *N*-acetylcysteine was purchased from Sigma (St. Louis, MO).

Cell lines and culture conditions

Mouse mammary epithelial cells (E6) were a gift from Dr. K.H. Cowan (Univ of Nebraska Medical Center, Omaha, NE) [35]. These cells were originally isolated from the mammary

gland of the Brca1$^{fl/fl}$ mouse (loxP sites flanking exon 11 of the Brca1 gene). They were immortalized by infecting with HPV-16E6 (Neo^{+}) retrovirus to inhibit p53. The E6 cells express a full-length Brca1 protein and are considered "normal" cells. E6 cells were cultured in 1:1 Dulbecco's minimal essential medium: Ham's F-12 (DMEM:F-12, Mediatech, Herndon, VA) supplemented with 10% bovine growth serum (BGS, Hyclone, Logan, UT) in a 5% CO_2 incubator at 37 °C.

Estrogen cytotoxicity

Exponentially-growing cells were seeded at a density of 5,000 cells/well in 96-well plates. After a day (Day 0), cells in one 96-well plate were counted by the MTT assay (see below), while other cells were treated with 4-OHE$_2$ (5-100 μM) or E$_2$-3,4-Q (5-100 μM) and incubated for 24 h. The estrogen solutions were made in acetonitrile (final concentration 0.007%). Following this incubation, cells were rinsed with PBS (Invitrogen, Carlsbad, CA), fresh media was added, and the cells were cultured for another three days. Cell numbers at days 1-3 were determined by the MTT assay. The effect of *N*-acetylcysteine on the growth of estrogen-treated cells was similarly determined (used in 1:1 molar ratio).

In the MTT [3-(4, 5-dimethyl-thiazolyl-2)-2,5-diphenyltetrazolium bromide, Sigma] assay, the media in the 96-well plate cultures were replaced with 100 μl fresh medium containing 25 μl of MTT (5 mg/ml in PBS), and incubated for 2 h at 37°C to allow the reduction of MTT by metabolically-active cells to form a purple formazan precipitate. The precipitate was then solubilized by adding 20% SDS in 1:1 DMF:H_2O, pH 4.7 (100 μL) and incubating overnight at 37 °C. The purple color was read (at 570 nm) in a μQuant microplate spectrophotometer (Bio-Tek Instruments) and analyzed by the KCjunior (version 1.41) software. The absorbance values were converted into cell numbers using a standard curve constructed by plotting MTT assay absorbance against cell counts. Linear interpolation was used to estimate the IC50. Survival estimates are presented using means and 95% confidence intervals.

Analysis of estrogen-DNA adducts

Cell culture and treatment—E6 cells were cultured for 72 h in estrogen-free medium (phenol red-free DMEM-F12 with charcoal stripped FBS), and treated (20 × 10^6 cells) either with *N*-acetylcysteine (30 μM, 30 min pre-treatment) and 4-OHE$_2$ (30 μM) or with *N*-acetylcysteine (50 μM, 30 min pre-treatment) and E$_2$-3,4-Q (50 μM) for 24 h. Following the treatments, the media were harvested and supplemented with 2 mM ascorbic acid (to prevent possible decomposition of the compounds) and processed immediately. Media from E6 cells treated with 10 μl acetonitrile (solvent) were used as controls.

Sample preparation for analyzing estrogen metabolites and DNA adducts—Varian C8 Certify II cartridges (Varian, Harbor City, CA) were equilibrated by sequentially passing 1 mL of methanol, distilled water, and potassium phosphate buffer (100 mM, pH 8) through them. The harvested media (40 mL) were adjusted to pH 8.0 with 1 mL of 1 M potassium phosphate buffer, and passed through these cartridges. The retained analytes in the cartridges were washed with the above phosphate buffer, eluted with 8:1:1:0.1 of methanol:acetonitrile:water:trifluoroacetic acid, and processed as described previously [34].

Analysis of estrogen metabolites and adducts—The eluted samples were analyzed in an HPLC apparatus equipped with a multi-channel electrochemical detector (Model 580 solvent delivery modules, Model 540 auto-sampler fitted with a 12-channel CoulArray electrochemical detector, Environmental Sciences Association, Chelmsford, MA). The analytes and adducts were separated with solvent gradients generated by Solvent A [15:5:10:70 of acetonitrile:methanol:CAA buffer (5.25% citric acid, 3.85% ammonium acetate, 11.5% acetic acid): water] and solvent B [50:20:10:20 of acetonitrile:methanol:CAA buffer:water].

The samples were injected into a Phenomenex Luna-2 C-18 column (250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA), and eluted isocratically (90% solvent A:10% solvent B) for 10 min, then by a linear gradient (up to 90% solvent B) in the next 35 min, at a flow rate of 1 ml/min. The 12 coulometric electrodes were set at potentials of −35, 10, 70, 140, 210, 280, 350, 420, 490, 550, 620 and 690 mV. The analyte and adduct peaks were identified by their retention times and peak height ratios between the dominant peak and the peaks in the two adjacent channels. The data were quantified by comparing with known amounts of standards. The results were compared between groups using a Mann-Whitney test.

The results were confirmed by a MicroMass QuattroMicro triple stage quadrupole mass spectrometer attached to a Waters Acquity UPLC (Waters, Milford, MA) as described previously [34].

Soft agar colony formation assay

Cells (1.17×10^6) were cultured for 24 h, and then treated with 4-OHE_2 (3 or 30 μM) or E_2-3,4-Q (5 or 50 μM) with or without equimolar amounts of *N*-acetylcysteine for another 24 h. Next, the cells were harvested, counted (Coulter Z-series particle count and size analyzer, Beckman, Fullerton, CA) and seeded in 6-well plates for the soft agar assay. Briefly, 5000 cells were suspended in 2 mL of 0.4 % Noble agar (Sigma, St Louis, MO) in 10% BGS (Hyclone) and poured over a 2-mL underlayer of 0.6 % Noble agar (in the culture medium) in each well. After 3 weeks of incubation at 37 °C in a 5% CO_2 incubator, colonies were counted under a phase-contrast microscope. The results were compared between groups using a Mann-Whitney test.

Analysis of H-*ras* mutations

The procedure for mutation analysis has been described previously [9,36,37]. Briefly, the exon 1-2 region of the mouse H-*ras* gene was PCR amplified, the product cloned in pUC18, the recombinant plasmids transformed in *E.coli*, and using the X-gal/IPTG color assay, bacterial colonies harvested, cultured for the extraction of plasmids and the sequence of H-*ras* DNA analyzed. The results were statistically evaluated by Fisher's Exact test.

Results

4-OHE_2 and E_2-3,4-Q toxicity in E6 mammary epithelial cells

Survival plots for examining estrogen cytotoxicity were constructed by converting the MTT absorbance values to cell numbers using a standard curve. The ratios of cell numbers at days 0-3 with respect to those in the control wells at day 0 were plotted as a time course (Fig. 1). These experiments were conducted under exponential growth conditions, as can be seen from the growth of the untreated control cells.

Both 4-OHE_2 and E_2-3,4-Q showed a remarkably narrow spectrum of cytotoxicity in E6 breast epithelial cells. For 4-OHE_2, a dose-dependent response was observed between 10-50 μM, and for E_2-3,4-Q this spectrum was between 25-70 μM. The duration of decline in cell numbers in the 4-OHE_2- or E_2-3,4-Q-treated cells was dose-dependent. For example, at the high doses (≥ 50 μM for 4-OHE_2 and ≥ 60 μM for E_2-3,4-Q), cell numbers declined for all three days and there was no regrowth beyond this period (not shown). At the low and intermediate doses (3-40 μM 4-OHE_2 and 25-40 μM E_2-3,4-Q), cell numbers declined initially (compared to control) and then increased again (not shown). The short cytotoxic period followed by regrowth suggests that these treatments of the estrogen metabolites induce acute cytotoxicity. Although these results suggest 4-OHE_2 to be more cytotoxic than E_2-3,4-Q, it may be an experimental artifact, as the extreme reactivity of the quinone with various cellular macromolecules may minimize its availability for mechanisms that elicit the cytotoxic response.

To determine the 50% killing dose (IC50), we chose estrogen doses within the linear decline periods. For both 4-OHE_2 and E_2-3,4-Q, the cytotoxic doses showed linear declines up to 1 d. Therefore, the IC50 values were calculated at 1 d. The IC50 for 4-OHE_2 is estimated to be 34 μM and for E_2-3,4-Q it is estimated to be 48 μM.

N-*acetylcysteine* inhibits estrogen cytotoxicity

N-acetylcysteine is known to rescue cells from the cytotoxic effects of various chemicals. Several mechanisms of rescue have been indicated, including *N*-acetylcysteine blocking oxidative DNA damage [38-43] by reacting with reactive oxygen species [44]. Metabolism of estrogens and similar compounds is known to induce oxidative DNA damage as a byproduct of redox cycling between semiquinones and quinones [45-47]. A previous study showed that *N*-acetylcysteine can inhibit cytotoxicity by the catechol estrogen 2-OHE_2 by minimizing the formation of oxidative DNA damage by 2-OHE_2 [48]. However, whether 2-OHE_2-induced oxidative DNA damage is the target of the protective activity of *N*-acetylcysteine is unclear.

Although *ortho*-semiquinones are thought to have poor ability to reduce oxygen to superoxide [41,49], both 2-OHE_2 and 4-OHE_2 are known to induce oxidative stress [48,50]. They have similar redox potentials [51,52], and form comparable levels of oxidative DNA damage [53-55]. Despite these similarities, 4-OHE_2 is a much stronger carcinogen than 2-OHE_2 [17, 56,57] and it is detected in greater amounts in cancerous breast compared to normal human breast [5,58]. These results support the hypothesis that 4-OHE_2 is the precursor of the quinone that initiates breast cancer.

We treated E6 cells with 1:1 mixtures of *N*-acetylcysteine and either 4-OHE_2 or E_2-3,4-Q for 24 h and examined cell survival at 48 h (Fig. 2). The results indicate that *N*-acetylcysteine can protect cells from the cytotoxic effects of these estrogen metabolites. For example, the addition of *N*-acetylcysteine improved the mean survival of E6 cells treated with 30 μM 4-OHE_2 from 0% to 43.9% (i.e., a mean survival of 43.9%, 95% confidence interval, range 35.6% to 52.2%). Similarly, *N*-acetylcysteine improved the mean survival of E6 cells treated with 50 μM E_2-3,4-Q from 0.7% to 64.5% (i.e., a mean survival of 63.8%, 95% confidence interval, range 41.8% to 85.8%). This level of protection is similar to that reported when MCF-10A human breast epithelial cells were treated with 2-OHE_2 (10-20 μM) and *N*-acetylcysteine (10 mM) [48]. The similarity of *N*-acetylcysteine rescue of E6 cells from cytotoxicity by 4-OHE_2 or E_2-3,4-Q suggests that the quinone may play a critical role in cytotoxicity.

E_2-3,4-Q has a relatively short half-life ($t_{1/2} \approx 45$ min at pH 7.0) (unpublished results); it reacts rapidly with DNA [59] or self-polymerizes to form inert compounds [60]. Although E_2-3,4-Q can be reduced by NAD(P)H alone [61] or by NAD(P)H-quinone oxidoreductase 1 (NQO1) *in vitro* [62], previous studies suggest that the reduction of E_2-3,4-Q is inefficient in cells [47,63]. Therefore, the cytotoxic effect of E_2-3,4-Q may be related mainly to the chemical reactions of the quinone.

A previous study also implicated E_2-3,4-Q in 4-OHE_2 cytotoxicity [41]. It was found that hypoxic or aerobic culture conditions did not alter 4-OHE_2 cytotoxicity in human breast carcinoma cells (MCF-7), but the addition of ascorbic acid or cysteine protected the cells, whereas a nitroxide (Tempol) increased the cytotoxicity. It was proposed that ascorbic acid and cysteine act by minimizing oxidation of 4-OHE_2 to E_2-3,4-Q, whereas Tempol acts by favoring quinone formation [41].

N-acetylcysteine may act as a quencher of E_2-3,4-Q to minimize estrogen cytotoxicity. It conjugates with E_2-3,4-Q to form 4-OHE_2-2-NAcCys (Fig. 3A), thereby decreasing the quinone pool for the DNA adduct-forming reaction [18,64]. In addition, *N*-acetylcysteine, through the mercapturic acid biosynthesis pathway, can be transformed to cysteine, which is

involved in the formation of glutathione that can also quench E_2-3,4-Q. Furthermore, the intracellular glutathione level itself is related to cell survival [41].

N-acetylcysteine inhibits estrogen oxidation and DNA adduct formation in E6 cells

To address the above issues, we examined the effects of *N*-acetylcysteine on estrogen metabolism and DNA adduct formation (Fig. 3B & C). Treatment of the cells with 4-OHE_2 or E_2-3,4-Q produces three major conjugates (methoxy, glutathione and *N*-acetylcysteine conjugates) and two major DNA adducts (adenine- and guanine-depurinating adducts). Under these conditions, relatively little of the 4-OHE_2 remains free, a major portion is converted by the abundant catechol-*O*-methyltransferase (COMT) to form the methoxy conjugates (4-$OCH_3E_{1/2}$)[5,6,65-67] and the remaining 4-OHE_2 appears to be oxidized to form the E_2-3,4-Q. The quinones have three major fates. Some are reduced by NAD(P)H:quinone oxidoreductases back to the catechols [7,62], which are then methylated by COMT. Second, the quinones react with endogenous glutathione (GSH) to form glutathione conjugates (4-$OHE_{1/2}$-2-SG), a part of which are catabolized by the mercapturic acid biosynthesis pathway, forming *N*-acetylcysteine conjugates (4-$OHE_{1/2}$-2-NAcCys) [68]. Third, the quinones react with DNA and form adducts. E_2-3,4-Q forms primarily (99.99%) depurinating DNA adducts (roughly equal amounts of N3Ade and N7Gua adducts) and 0.001% stable DNA adducts [59] Treatment of E6 cells with 4-OHE_2 or E_2-3,4-Q for 24 h showed the expected three conjugates and the two DNA adducts (Fig. 3B &C).

We used moderately cytotoxic doses of 4-OHE_2 and E_2-3,4-Q for these experiments for efficient analysis of the estrogen analytes. As can be seen from Fig. 1, the 30 μM dose of 4-OHE_2 kills ~22 % of the cells and the 50 μM dose of E_2-3,4-Q kills ~59% of the cells at 24 h. Under these conditions, the 4-OHE_2 -treated cells generated greater quantities of the three conjugates and the two DNA adducts than the E_2-3,4-Q-treated cells (Fig. 3 B&C, note the scales on the Y-axes).

The addition of equimolar amounts of *N*-acetylcysteine measurably altered the levels of the estrogen conjugates. Specifically, in the 4-OHE_2-treated cells (Fig. 3B), *N*-acetylcysteine altered the methoxy conjugate levels from 19,192 ± 952 (SEM) to 45,600 ± 3,308 (SEM) pmol (138% increase, p=0.05), the glutathione conjugate levels from 36.7 ± 4.1 to 79.3 ± 6.4 (116% increase, p=0.05) and *N*-acetylcysteine-conjugate levels from 66.6 ± 6.4 to 112.7 ± 4.4 pmol / 20 million cells (69% increase, p=0.05). In E_2-3,4-Q-treated cells (Fig. 3C), *N*-acetylcysteine altered the methoxy conjugate levels from 3079.7 ± 132.9 to 1260.7 ± 42.4 pmol (59% decrease, p=0.05), the glutathione conjugate levels from 36.7 ± 0.7 to 32.7 ± 3.3 pmol (no significant change, p=0.2), and *N*-acetylcysteine-conjugate levels from 36 ± 3.1 to 78 ± 3.1/20 million cells (117% increase, p=0.05). *N*-acetylcysteine can react directly with E_2-3,4-Q to form the 4-OHE_2-2-NAcCys conjugate [64] (Fig. 3A). As expected, the addition of *N*-acetylcysteine increased the levels of these conjugates in both 4-OHE_2 and E_2-3,4-Q-treated cultures. Similarly, *N*-acetylcysteine is a precursor of glutathione [19], which can react with E_2-3,4-Q to produce a conjugate (Fig. 3A). *N*-acetylcysteine increased glutathione conjugate levels in 4-OHE_2-treated cultures. However, this was not observed in the E_2-3,4-Q-treated cultures. This difference in result could be due to rapid depletion of E_2-3,4-Q by reaction with various nucleophilic groups present in the cell and the medium, before sufficient glutathione is produced from *N*-acetylcysteine.

The most remarkable result was that in 4-OHE_2-treated cells, *N*-acetylcysteine caused a great increase in the levels of the 4-methoxy conjugates (Fig. 3B). This result may be explained with the observation that the semiquinone produced by oxidation of 4-OHE_2 in these cells, can be reduced back to 4-OHE_2 by the thiol group of cysteine [41]. This process can increase the pool size of 4-OHE_2 that is methylated by catechol-*O*-methyltransferase (Fig. 3A). In contrast, *N*-acetylcysteine caused a decrease in the levels of 4-methoxy conjugates in the E_2-3,4-Q-treated

cells (Fig. 3C). In this case, the 4-OHE_2 can only be obtained by reduction of the quinone, and *N*-acetylcysteine apparently interferes with this process. We are conducting further studies to understand the contributions of *N*-acetylcysteine towards conjugate formation.

Thus, *N*-acetylcysteine can interfere with estrogen oxidation and minimize quinone levels. Since the quinones are needed for reaction with DNA to form adducts, *N*-acetylcysteine can be expected to reduce DNA adduct formation. A drastic reduction in the depurinating DNA adduct levels was observed. In 4-OHE_2-treated cells, *N*-acetylcysteine reduced the N3Ade adducts from 52 ± 2.0 to 5.3 ± 0.7 pmol (90 % reduction, p = 0.04) and N7Gua adducts from 43.3 ± 1.8 to 7.3 ± 0.7 pmol / 20 million cells (83% reduction, p=0.05). In E_2-3,4-Q-treated cells, *N*-acetylcysteine reduced the N3Ade adducts from 18 ± 1.2 to 5.3 ± 0.7 pmol (71% reduction, p=0.05) and N7Gua adducts from 18 ± 1.2 to 6.7 ± 0.7 pmol / 20 million cells (63% reduction, p=0.05).

Thus, *N*-acetylcysteine can drastically reduce depurinating estrogen-DNA adduct formation in breast epithelial cells. Since the depurinating adducts are considered to be the precursor lesions that induce the mutations leading to breast cancer [9-12], *N*-acetylcysteine may be useful for probing the link between oxidative metabolism of estrogens and the initiation of breast cancer.

N-*acetylcysteine* inhibits estrogen-induced transformation of E6 cells

Previous studies showed that estrogens can transform normal human breast epithelial cells [3]. We examined whether 4-OHE_2 or E_2-3,4-Q can similarly transform E6 cells and whether equimolar amounts of *N*-acetylcysteine can block the transformation. A typical estrogen-transformed E6 colony is shown in Fig. 4.

E6 cells showed a background frequency of transformation of 0.016 % (Table 1). Addition of *N*-acetylcysteine did not significantly alter transformation: 0.015% (p=0.99) and 0.016% (p=0.99) with 30 or 50 μM *N*-acetylcysteine, respectively.

Treatment of E6 cells with 4-OHE_2 (3 or 30 μM) or E_2-3,4-Q (5 or 50 μM) increased the number of transformed colonies [frequency = 0.06% (p<0.001) or 0.067 % (p<0.001) for 4-OHE_2, and 0.053% (p<0.001) or 0.058% (p<0.001) for E_2-3,4-Q, respectively]. The chosen estrogen doses [4-OHE_2 (3 or 30 μM) or E_2-3,4-Q (5 or 50 μM)] did not show a dose-response in transformation. The lack of a dose-response suggests that the extent of cytotoxicity induced by these doses of estrogen does not allow significant changes in the frequency of transformation.

In contrast to 4-OHE_2 and E_2-3,4-Q, treatment with 10 μM E_2-2,3-Q showed a minimal increase in transformation over background (0.025 %, p=0.99, compared with control). The poor transforming activity of E_2-2,3-Q was expected, as its precursor, 2-OHE_2, is known to have weaker transforming activity compared to 4-OHE_2 [3]. It has been suggested that the weak carcinogenic activity of 2-OHE_2/E_2-2,3-Q is related to their poor ability to form depurinating adducts [59].

Next, we examined whether the addition of equimolar amounts of *N*-acetylcysteine (30 μM to 30 μM 4-OHE_2 and 50 μM to 50 μM E_2-3,4-Q) would reduce transformation (Table 1). In both cases, *N*-acetylcysteine drastically reduced transformation of the E6 cells. *N*-acetylcysteine reduced transformation by 4-OHE_2, from 0.067% to 0.028% for 30 μM 4-OHE_2 (58% reduction, p=0.01), and by E_2-3,4-Q from 0.058% to 0.042% for 50 μM E_2-3,4-Q (28% reduction, p=0.09). *N*-acetylcysteine reduction of transformation was more effective against 4-OHE_2 (residual transformation was similar to control, p=0.50), than against E_2-3,4-Q (residual transformation higher than control, p=0.002). These effects of *N*-acetylcysteine

provide a tool to directly correlate oxidative metabolism of estrogens with transformation of breast epithelial cells, which is expected to be an early event of breast cancer.

It is possible that the background frequency of transformation could be related to intrinsic properties of the E6 cells, such as pre-existing mutations. To examine whether E6 cells contained pre-existing mutations, we PCR amplified the exon 1-2 region of the H-*ras* gene (500 bp), cloned the product in pUC18, transformed into *E.coli*, isolated single colonies using the IPTG-X-gal colony color assay, extracted the recombinant plasmids and sequenced the inserts (Fig. 5). This PCR protocol for mutation analysis generates low levels of mutations (one mutation in ~40 plasmids, mutation frequency= 5×10^{-5}, calculated by dividing the number of mutations with the total number of bases sequenced) [9,12,37]. Analysis of 31 plasmid clones from the E6 cells showed 8 mutations (mutation frequency = 51.6×10^{-5}, p=0.01, Fisher's Exact test comparison of PCR-induced vs. pre-existing mutations). Seven of these mutations were G.C to T.A and the eighth mutation was A.T to T.A. Since the E6 cells contained pre-existing mutations, a role of these mutations in spontaneous transformation of E6 cells warrants consideration.

Discussion

The E6 are normal mouse breast epithelial cells. Similar human cells exist (MCF-10A and MCF-10F). In particular, the MCF-10F cells are considered to be a good model of the human breast [13]. Unfortunately, both MCF-10A and 10F cells have low CYP1B1, and thus show poor ability to oxidize estrogens [69]. Estrogen oxidation is more efficient in normal human breast [5]. Estrogens can transform both MCF-10A and 10F cells, but four treatments are required. Results presented here show that the E6 cells are efficient estrogen oxidizers, and can be transformed with a single estrogen treatment. This study is the first to demonstrate the transforming activity of estrogen-3,4-quinones.

The purpose of this study was to examine whether the anti-oxidant *N*-acetylcysteine can be used to test the hypothesis that depurinating DNA adduct formation by estrogen-3,4-quinones is the initiating event in breast cancer. A transformation protocol that requires four treatments can model the combined effects of initiation and promotion from sustained carcinogen exposure. On the other hand, transformation by a single treatment of a carcinogen can report its initiating activity. Therefore, for this study, we think that the E6 are more suitable than the MCF-10A and 10F cells.

N-acetylcysteine prevented estrogen-DNA damage and associated death of E6 cells (Figs 2 & 3). In addition to preventing estrogen-DNA adduct formation, *N*-acetylcysteine strongly inhibited estrogen-induced transformation (Table 1). The ability of *N*-acetylcysteine in inhibiting estrogen-induced transformation should be validated in appropriate human cells. However, we think that the present results provide a proof-of-principle and warrant the exploration of *N*-acetylcysteine in the prevention of breast cancer.

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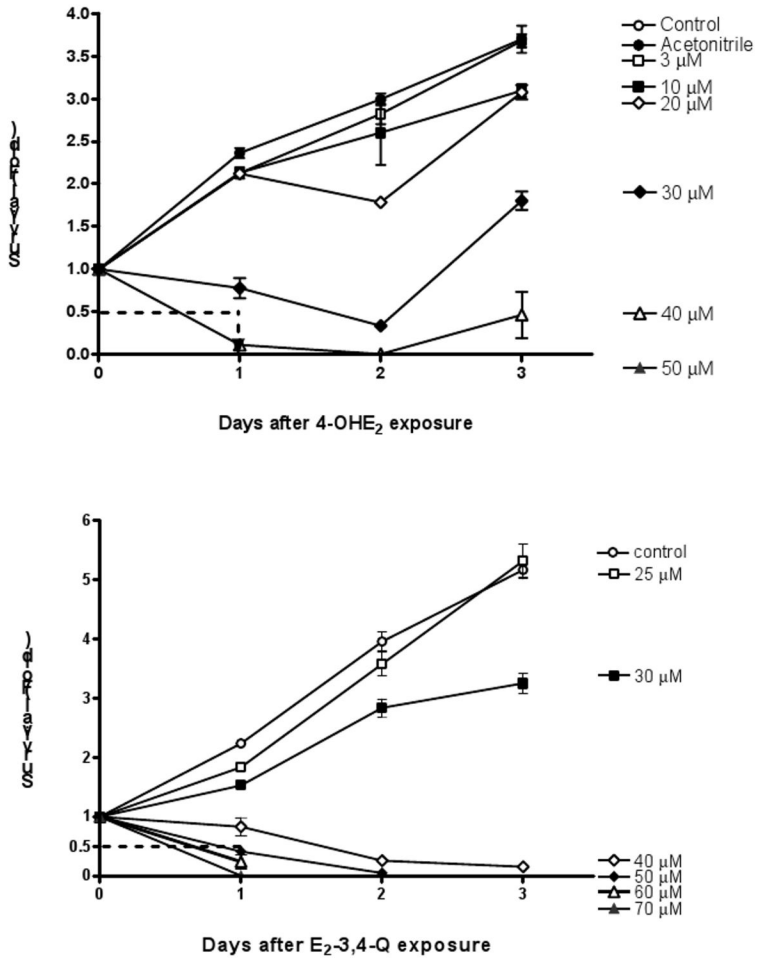

Figure 1. Cytotoxicity of 4-OHE$_2$ and E$_2$-3,4-Q in normal mouse mammary epithelial cells (E6)
Cells were treated with the estrogens at Day 0. Cell numbers were determined by the MTT assay using a standard curve.

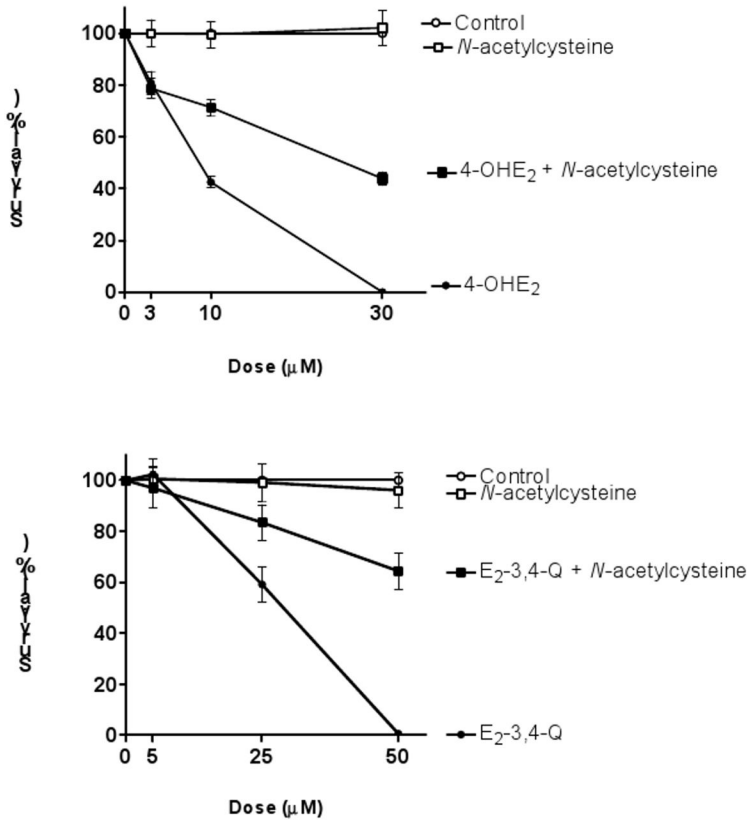

Figure 2. *N*-acetylcysteine rescues E6 cells from 4-OHE$_2$ and E$_2$-3,4-Q cytotoxicity

Equimolar amounts of *N*-acetylcysteine were added to the experiments at Day 0. Cell numbers were determined by the MTT assay using a standard curve.

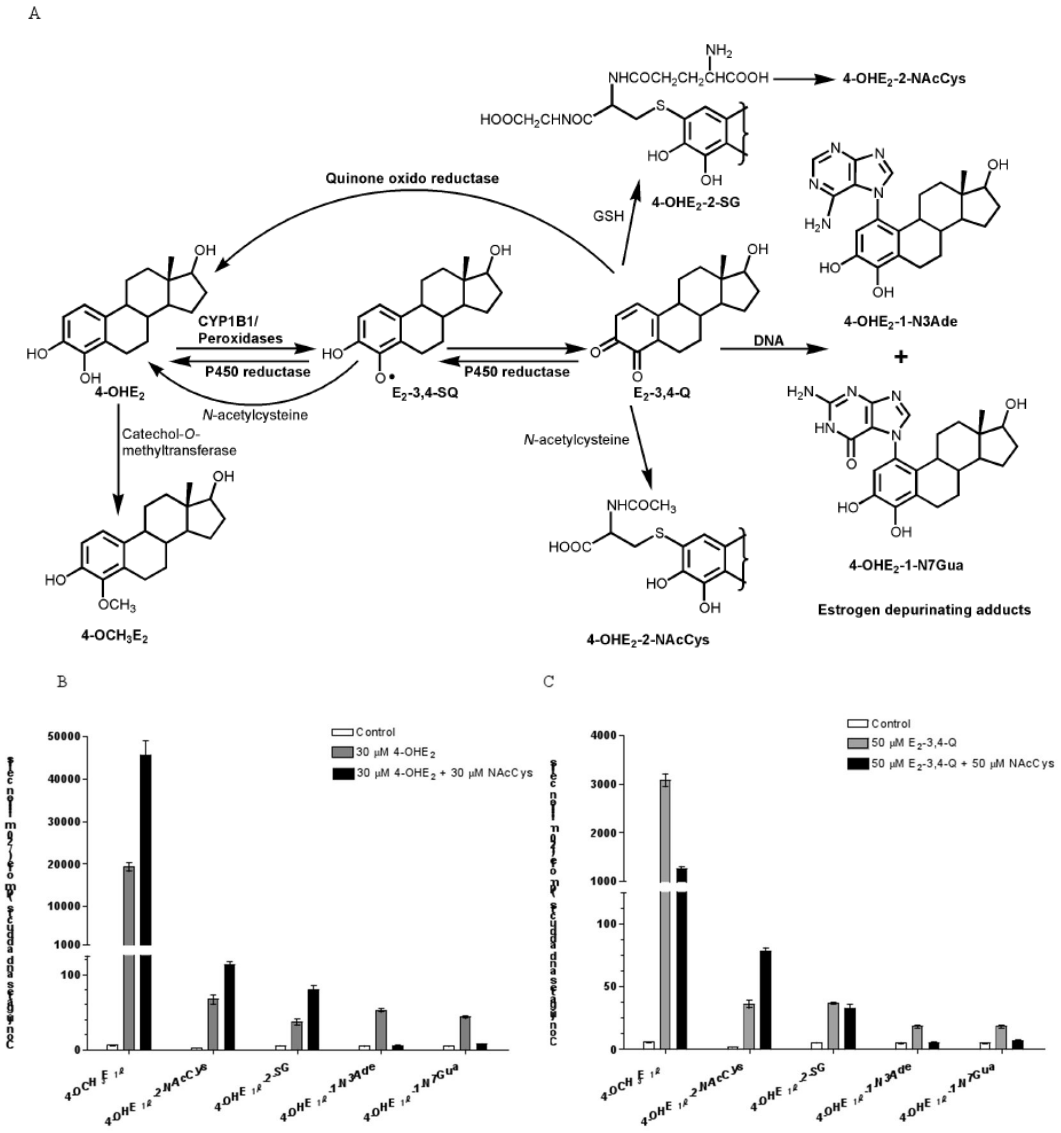

Figure 3. ***N*-acetylcysteine inhibits estrogen-DNA adduct formation in E6 cells**

E_2 and E_1 are interconverted in the cell; therefore, we combined the conjugates and adducts from E_1 and E_2 together. The quinone produced less conjugates and DNA adducts than the catechol (note the difference in the Y-axes of B & C).

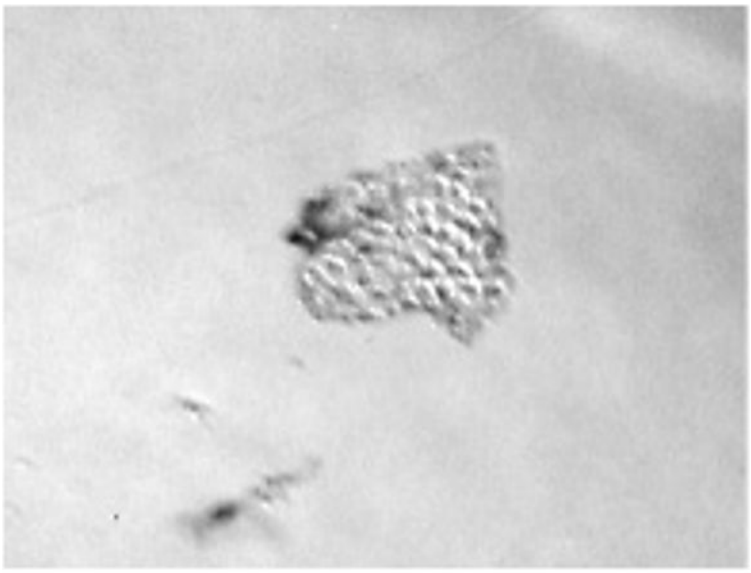

Figure 4. Colony morphology of estrogen-transformed E6 cells

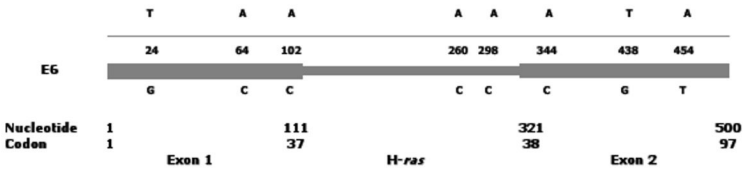

Figure 5. Spectrum of pre-existing H-*ras* mutations in E6 cells

Exon 1-2 region (GenBank Accession No. U89950) was analyzed for mutations. The mutations are shown over the top line of the cartoon, and the wild-type nucleotides are shown below.

Table 1

Treatments		Transformation Total colonies/Total wells (% cells transformed *)	p-value ** (compared with Control)
Estrogen metabolite (μM)	N-acetylcysteine (μM)		
Control	-	-	70/88 (0.016%)	-
	-	30	17/24 (0.015%)	0.99
	-	50	18/24 (0.016%)	0.99
4-OHE_2	3	-	58/19 (0.060%)	<0.001
	30	-	52/16 (0.067%)	<0.001
	30	30	21/15 (0.028%)	0.50
E_2-3,4-Q	5	-	53/20 (0.053%)	<0.001
	50	-	60/20 (0.058%)	<0.001
	50	50	42/20 (0.042%)	0.002
E_2-2,3-Q	10	-	27/24 (0.025%)	0.99

* 5000 cells/well.

** P-values are adjusted for multiple comparisons with the control using the method of Bonferroni.