Published in final edited form as:
Free Radic Biol Med. 2010 August 1; 49(3): 392–400. doi:10.1016/j.freeradbiomed.2010.04.028.

N-Acetylcysteine blocks formation of cancer-initiating estrogen-DNA adducts in cells

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Abstract

Catechol estrogens, especially 4-hydroxylated metabolites of 17β-estradiol (E_2), are responsible for estrogen-induced carcinogenesis. 4-Hydroxyestradiol (4-OHE_2), a major metabolite of E_2 formed preferentially by cytochrome P-450 1B1, is oxidized to E_2-3,4-quinone, which can react with DNA to yield the depurinating adducts 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua. The apurinic sites generated by the loss of these depurinating adducts induce mutations that could lead to cancer initiation. In the present study, we have evaluated the effects of *N*-acetycysteine (NAcCys) on the metabolism of two cell lines, MCF-10F (a normal human breast epithelial cell line) and E6 (a normal mouse mammary epithelial cell line), treated with 4-OHE_2 or its reactive metabolite, E_2-3,4-quinone. Extensive HPLC with electrochemical detection and UPLC-MS/MS analyses of the cell media demonstrated that the presence of NAcCys very efficiently shifted the estrogen metabolism towards protective methoxylation and conjugation pathways in multiple ways, while formation of depurinating DNA adducts was inhibited. Protection by NAcCys appears to be similar in both cell lines irrespective of their origin (human or mouse) or the presence of estrogen receptor-alpha. This finding suggests that NAcCys, a common dietary supplement, could be used as a potential chemopreventive agent to block the initial step in the genotoxicity caused by catechol estrogen quinones.

Keywords

catechol estrogen quinones; depurinating estrogen-DNA adducts; cancer prevention, *N*-acetylcysteine; homeostasis of estrogen metabolism; breast epithelial cells

Introduction

Breast cancer is the most commonly occurring neoplasm and the second most frequent cause of death in women in the United States [1]. Present evidence shows the involvement of estrogens in processes leading to cancers, including breast, endometrial and ovarian [2–5]. A

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substantial amount of evidence from experiments on estrogen metabolism, formation of DNA adducts, mutagenicity, cell transformation and carcinogenicity [6–24] provides the basis for the paradigm that certain estrogen metabolites, catechol estrogen-3,4-quinones, react with DNA to form predominantly depurinating adducts 4-hydroxyestrone(estradiol)-1-N3Ade [4-$OHE_1(E_2)$-1-N3Ade] and 4-$OHE_1(E_2)$-1-N7Gua (Fig. 1) [10–13]. Apurinic sites resulting from formation of these adducts can undergo error-prone repair to generate critical mutations that initiate cancer [14–16]. The paradigm of cancer initiation by natural or synthetic estrogens is based on estrogen metabolism that generates a disrupted homeostatic balance between activating (leading to DNA adducts) and deactivating (leading to methoxylation and conjugation) pathways. In support of this, we have recently shown that in healthy women, the level of estrogen-DNA adducts in urine is low and the levels of methoxylated estrogen metabolites and conjugates are high (Fig. 1) [9,25]. In contrast, higher levels of DNA adducts and lower levels of methoxylated estrogen metabolites and conjugates are present in the urine of breast cancer patients and women at high risk for the disease [9,25].

The oxidation of catechol estrogens to quinones can be reduced by the actions of Phase II enzymes. Specifically, catechol-*O*-methyltransferase (COMT) catalyzes the methylation of catechol estrogens, preventing their conversion to estrogen semiquinones and quinones (Fig. 1) [26]. We have documented that low COMT activity is a contributory factor in the increased formation of estrogen-DNA adducts [27,28]. In turn, the estrogen quinones are expected to undergo conjugation with glutathione (GSH), either nonenzymatically or more efficiently, via the catalytic action of glutathione-*S*-transferases, a superfamily of multifunctional phase II enzymes that play a key role in cellular detoxification [29–31]. Other phase II enzymes, NADPH quinone oxidoreductase 1 and 2 (NQO1 and NQO2) are also involved in detoxification of the quinones by their reduction back to catechol estrogens [32–34].

The activating and deactivating pathways leading to DNA adducts or methoxylation and conjugation can be modulated by using antioxidant compounds. These agents, in mechanism-based action, either prevent the formation of estrogen quinones and their reaction with DNA or covalently bind with the quinones to form nontoxic conjugates. Towards this goal, in a preliminary *in vitro* study, we have tested several antioxidants, NAcCys, GSH, cysteine (Cys), melatonin, resveratrol and reduced lipoic acid [35]. NAcCys was found to be one of the best inhibitors of the formation of depurinating adducts in reaction mixtures containing E_2-3,4-Q or enzyme-activated 4-OHE_2 and DNA.

NAcCys is an aminothiol, and its hydrolytic product, Cys, is a precursor of intracellular GSH [36]. Changes in GSH homeostasis have been implicated in the etiology and progression of a variety of human diseases, including cancer [37]. NAcCys was initially used as an antidote for paracetamol poisoning [38] and as a mucolytic drug [39]. Later it was shown to possess antioxidant, anticoagulant and platelet-inhibiting properties with low systemic toxicity [40–44]. In light of our recent findings on the mechanism of initiation of breast cancer and *in vitro* studies, it was of interest to investigate further the effect of NAcCys on estrogen metabolism in breast epithelial cells.

In the present study using the human breast epithelial cell line MCF-10F (estrogen receptor negative and aryl hydrocarbon receptor positive), a well-developed cell culture model for studying the carcinogenesis of non-estrogen receptor pathways, and E6 (a mouse mammary epithelial cell line), we have demonstrated that NAcCys modulates estrogen metabolism in multiple ways. The data presented in this article suggest that NAcCys could be used as a chemopreventive agent against the initiation of breast cancer.

Materials and Methods

Chemicals and Reagents

4-OHE_2 and all standards were synthesized in our laboratory, as previously described [10,11, 45,46]. For quinone treatments, E_2-3,4-Q was freshly synthesized as previously described [12]. NAcCys and all other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture and treatment

MCF-10F cells were obtained from the ATCC (Rockville, MD), and cultured in DMEM and Ham's F12 media (Mediatech, Inc.) with 20 ng/ml epidermal growth factor, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, 5% horse serum and 100 μg/ml penicillin/streptomycin mixture. Estrogen-free medium was prepared in phenol red-free DME/F12 medium with charcoal-stripped FBS (HyClone, Logan, UT).

Mouse mammary epithelial cells (E6) were a generous gift from Dr. K.H. Cowan (University of Nebraska Medical Center, Omaha, NE) [47]. These cells were originally isolated from the mammary gland of a $Brca1^{fl/fl}$ mouse (loxP sites flanking exon 11 of the Brca1 gene). They were immortalized by infection 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. Cell viability of both cell lines was determined by the MTT [3,(4,5-dimethylthiazole-2-yl)-2,5-diphenyltrazolium bromide] assay [48].

The cells (0.75×10^5 cells) were seeded for 48 h in estrogen-containing medium. The medium was changed to estrogen-free medium and the cells were grown for another 72 h. After changing the medium, cells were treated with E_2-3,4-Q or 4-OHE_2 (1, 10 or 30 μM) for 24 h. To see the effect of NAcCys on the metabolic profile of each cell line, the cells were first pre-incubated for 2 h with a 1:1 or 1:0.5 ratio of NAcCys to estrogen and then treated with E_2-3,4-Q or 4-OHE_2 (1, 10 or 30 μM) for 24 h. Only MCF-10F were used to determine whether NAcCys can prevent the formation of estrogen-DNA adducts in a dose dependent manner. The cells were pre-incubated with 1, 3, 10 or 30 μM NAcCys for 2 h and then treated with a fixed moderate dose of 4-OHE_2 (10 μM) for 24 h. To keep the concentration of organic solvents (0.001%) the same in all experiments, different stock solutions of E_2-3,4-Q and 4-OHE_2 (1–30 mM) were prepared. Similar media from five flasks, either treated with an equal amount of organic solvent or untreated, were used as controls.

After collecting the medium from five flasks, 1 ml of trypsin was added to each flask and incubated for 10 min. Once the cells had detached, the cells from all five flasks were combined and 10 ml of double serum medium was added to neutralize the trypsin. Cell numbers were estimated by using a Coulter counter (Beckman Coulter Inc., USA).

Analysis of estrogen-DNA adducts

Following the treatments, media were harvested, supplemented with 2 mM ascorbic acid (to prevent possible decomposition of the compounds) and processed immediately. Sample preparation and analysis by HPLC-ECD and UPLC-MS/MS were carried out as follows:

i. *Sample Preparation*: Culture media pooled from five flasks (50 mL) were processed by passage through C8 Certify II cartridges (Varian, Harbor City, CA), which were equilibrated by sequentially passing 1 mL of methanol, distilled water, and potassium phosphate buffer (100 mM, pH 8) through them. The harvested media (50 mL) were adjusted to pH 8.0 and passed through these cartridges. The retained analytes in the cartridges were washed with the above phosphate buffer, eluted with

methanol:acetonitrile:water:trifluoroacetic acid (8:1:1:0.1), and processed as described previously [20,49].

ii. *HPLC-ECD and UPLC-MS/MS*: Analyses of all samples were conducted on an HPLC system equipped with dual ESA Model 580 solvent delivery modules, an ESA Model 540 auto-sampler and a 12-channel CoulArray electrochemical detector (ESA, Chelmsford, MA) [20,49] (Fig. 2). 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 comparison with known amounts of standards. The % recovery of each standard was used to normalize our data. To determine the % recovery for each experiment, a complete set of analytes was spiked into medium (same batch as used with the cells), processed as the other samples and analyzed. The % recovery for each analyte was calculated from this set of standards for the samples analyzed in this experiment. The metabolites and adducts showed better recovery (85–93%) than the quinone conjugates (56–78%).

The statistical significance of data was determined by using the t-test in which the value obtained from cells treated in the presence of NAcCys was compared individually with the control in which NAcCys was not added. The identity of the DNA adducts was further confirmed on an Acquity UPLC connected to a MicroMass QuattroMicro triple quadrupole mass spectrometer (Waters, Milford, MA), as described previously [9].

Immunoblotting analysis

After treatment for 24 h with NAcCys (0.1, 1, 10 or 30 μM), whole cell lysate was prepared by suspending the MCF-10F cells in RIPA buffer with protease inhibitor and lysing by three freeze-thaw cycles. Nuclei and unlysed cells were removed by centrifugation. The protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology, Inc, Rockford, IL). Protein samples (20 μg) were solubilized with SDS-polyacrylamide gel electrophoresis sample loading buffer and electrophoresed on 12% SDS-polyacrylamide gel at 200 V for 30 min. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane at 100 V for 60 min. The blots were blocked for 60 min at room temperature in fresh blocking buffer (0.1% Tween-20 in Tris-buffered saline, pH 7.4, 5% nonfat dried milk, PBST). Dilutions of the primary antibodies [COMT (1:2000, Chemicon International, Temecula, CA); NQO1 (1:2000, Abcam, Temecula, CA), CYP1B1 (1:1000, Genetest, Bedford, MA) and β-actin (1:10000, Genetest, Bedford, MA)] were made in blocking solution. The blots were incubated overnight with primary antibody at 4 °C. Following three washes with PBST for 10 min each, the blots were incubated with appropriate secondary antibody (horseradish peroxidase-conjugated) for 45 min at room temperature. The blots were washed again three times in PBST, incubated with Super Signal West Pico Chemiluminescent kit (Pierce Biotechnology, Inc., Rockford, IL) for 2 min, and visualized with radiographic film. The intensities of the bands were quantified by Alpha DigiDoc 1201 (Alpha Innotech, San Leandro, CA).

Results

The effects of NAcCys on estrogen metabolism were studied in MCF-10F (human) and E6 (mouse) cells, which were treated with E_2-3,4-Q or 4-OHE_2 alone or in the presence of NAcCys (Tables 1–4) for 24 h. The cell culture media from all the treatment groups were analyzed by HPLC-ECD. The presence of DNA adducts was confirmed by UPLC-MS/MS. MCF-10F and E6 cell treatments with either E_2-3,4-Q or 4-OHE_2 were divided into three groups. First, control group (A) was treated with 1, 10 or 30 μM E_2-3,4-Q or 4-OHE_2 alone. In the second group (B), cells were treated with NAcCys at half the molar concentration of E_2-3,4-Q or 4-OHE_2.

In the third group (C), cells were treated with equal molar ratios of NAcCys and E_2-3,4-Q or 4-OHE_2.

Effect of NAcCys on E_2-3,4-Q metabolism and DNA adduct formation in MCF-10F and E6 cells treated with E_2-3,4-Q

The control group, in which cells were treated with 1, 10 or 30 μM E_2-3,4-Q, showed the formation of hydroxycatechols, methoxycatechols, conjugates and depurinating DNA adducts in a dose-dependent manner (Table 1). The methoxycatechol and depurinating DNA adduct levels are also shown in Figure 3. NAcCys treatment of MCF-10F cells resulted in a decrease in 4-hydroxycatechol and 4-methoxycatechol levels (Table 1, Fig. 3A), whereas there were significant increases in 4-OHE_2-2-SG, 4-OHE_2-2-Cys and 4-OHE_2-2-NAcCys (Table 1). 4-OHE_2-1-N3Ade adduct levels were decreased up to 31% when NAcCys was present at half the concentration of E_2-3,4-Q and up to 69% when NAcCys and E_2-3,4-Q were equimolar (Table 1, Fig. 3B). Similarly, 4-OHE_2-1-N7Gua levels were reduced up 29% with NAcCys at half the molar level of E_2-3,4-Q and up to 69% with equimolar NAcCys and E_2-3,4-Q (Table 1, Fig. 3B).

Similar effects of NAcCys were observed in E6 cells treated with E_2-3,4-Q (Table 2). The levels of metabolites, conjugates and DNA adducts in the E6 cells were similar to or somewhat higher than those observed in the MCF-10F cells (Table 1). The levels of 4-hydroxycatechols, 4-methoxycatechols and depurinating DNA adducts were decreased in the E6 cells, whereas there were significant increases in quinone conjugates (Table 2).

Effect of NAcCys on 4-OHE_2 metabolism and formation of DNA adducts in MCF-10F and E6 cells treated with 4-OHE_2

Analysis of the culture media from MCF-10F cells treated with 1, 10 or 30 μM 4-OHE_2 alone showed an increase of 4-methoxycatechols, quinone conjugates and depurinating DNA adducts in a dose-dependent manner (Table 3, Fig. 4). In the presence of NAcCys, there was an increase in 4-methoxylation (Fig. 4A) and in quinone conjugates (4-OHE_2-2-SG, 4-OHE_2-2-Cys and 4-OHE_2-2-NAcCys) (Table 3). The depurinating adduct, 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua, levels were reduced 46% and 37%, respectively, with NAcCys present at half the concentration of 4-OHE_2. Their levels were reduced 86% and 85%, respectively, with equimolar NAcCys and 4-OHE_2 (Table 3, Fig. 4B).

The levels of 4-methoxycatechols, quinone conjugates and depurinating DNA adducts were similarly altered when E6 cells were exposed to 4-OHE_2 in the presence of half or equimolar ratios of NAcCys (Table 4). The levels of metabolites, conjugates and DNA adducts were similar to those detected in the MCF-10F cells (Table 3), except that higher levels of DNA adducts were observed in the E6 cells. In fact, when the cells were treated with 30 μM 4-OHE_2, about 3–4-fold higher levels of DNA adducts were seen in the E6 cells compared to MCF-10F cells, with or without NAcCys present.

Dose-response of NAcCys

To observe the effect of NAcCys on estrogen metabolism, we decided to use a moderate dose of 4-OHE_2 in human breast epithelial cells (MCF-10F). The MCF-10F cells were treated with 10 μM 4-OHE_2 and varying ratios (0, 0.1, 0.3, 1 and 3) of NAcCys (Table 5). The metabolic profile of cell media revealed a dose-dependent response in the elevation of 4-methoxycatechol and quinone conjugate levels (Table 5). Significant inhibition of 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua adducts was observed with increased NAcCys concentrations (Table 5). Complete inhibition of both depurinating adducts was observed when cells were treated with a 1:3 ratio of 4-OHE_2 to NAcCys (Table 5).

Effect of NAcCys on enzyme (COMT, NQO1 and CYP1B1) expression in MCF-10F cells

To see the effect of NAcCys on estrogen activating (CYP1B1) or protective (COMT and NQO1) enzymes (Fig. 1), MCF-10F cells were treated with various concentrations of NAcCys (1, 10 and 30 μM) for 24 h. These enzymes are of particular interest because breast tissue from women with breast cancer was found to contain higher levels of the activating enzyme and lower levels of the protective enzymes than breast tissue from control women [50]. Immunoblotting of the samples showed that the COMT, NQO1 and CYP1B1 protein expression remained unaffected (Fig. 5). Similar results were observed when E6 cells were treated with NAcCys (data not shown).

Discussion

To determine the antioxidant effect of NAcCys on estrogen metabolism, two different cell cultures were used: the MCF-10F human breast epithelial cell line (estrogen receptor-α negative and aryl hydrocarbon receptor positive) and the E6 mouse mammary epithelial cell line. In the treatment of MCF-10F cells with E_2-3,4-Q alone, increased levels of the quinone led to increased levels of 4-OHE_2, 4-OCH_3E_2, GSH conjugates and their derivatives, as well as increased levels of both depurinating DNA adducts (Table 1). When the cells were incubated with E_2-3,4-Q plus NAcCys at two dose levels, decreased amounts of the catechol, the methoxycatechol and the depurinating DNA adducts were observed, due to the reaction of E_2-3,4-Q with NAcCys [35,46]. This led to less formation of catechol from the reduction of E_2-3,4-Q by NQO1 (Fig. 1). Thus, less methylation of 4-OHE_2 by COMT to form 4-OCH_3E_2 could occur (Table 1, Fig. 3A). Reduced formation of the DNA adducts was also due to reaction of the quinone with NAcCys instead of DNA (Table 1, Fig. 3B).

The increased levels of GSH, Cys and NAcCys conjugates arose from the added NAcCys, its hydrolysis to Cys, and further formation of GSH. Similar results were obtained by incubation of the E6 mouse mammary cells with E_2-3,4-Q alone or in the presence of NAcCys (Table 2).

When MCF-10F cells were treated with 4-OHE_2 alone, a dose-response was observed for all of the analytes (Table 3). The catechol was detected almost entirely as the methoxycatechol. Most of the remaining catechol was converted to the quinone, which reacted with GSH, Cys and endogenous NAcCys or DNA. In the presence of NAcCys at half or equimolar concentrations, the levels of GSH conjugates and their derivatives increased, as expected (Table 3). The levels of 4-OHE_2 decreased, but 4-OCH_3E_2 increased (Table 3, Fig. 4A). The 4-OCH_3E_2 arises partly from methylation of the added 4-OHE_2 by COMT. The oxidation of 4-OHE_2 to E_2-3,4-Q proceeds through the E_2-3,4-semiquinone. When NAcCys is present, some of the E_2-3,4-semiquinone is efficiently reduced back to 4-OHE_2 by NAcCys [51]. This results in greater formation of 4-OCH_3E_2 from the newly formed 4-OHE_2 (Fig. 4A). The decreased levels of the 4-OHE_2-1-N3Ade and 4-OHE_2-1-N7Gua adducts (Fig. 4B) are due to two factors: first, the reduced amount of E_2-3,4-Q formed and, second, its reaction with NAcCys [35,46].

The E6 cells treated with 4-OHE_2 produced higher levels of the depurinating adducts (Table 4) than the MCF-10F cells (Table 3). The effects of NAcCys on the metabolism of 4-OHE_2 in the E6 cells (Table 4) were, however, strikingly similar to those observed in the MCF-10F cells (Table 3). Use of the E6 cells enabled us to investigate the metabolism of 4-OHE_2 and formation of estrogen-DNA adducts in cells that have the estrogen receptor, as well as MCF-10F cells, which lack the receptor. These results indicate that the presence of the estrogen receptor has no effect on how 4-OHE_2 is metabolized and estrogen-DNA adducts are formed in mammary epithelial cells.

The effects of NAcCys on the metabolism of 4-OHE_2 in MCF-10F cells was further studied over a dose range of 0 to 30 μM NAcCys with a constant 10 μM concentration of 4-OHE_2 (Table 5). Over this expanded ratio of NAcCys to 4-OHE_2 (0 to 3-fold), the level of 4-OHE_2 decreased more than 90%, while the amount of 4-OCH_3E_2 more than doubled. The GSH and derivative conjugates also increased. The levels of the depurinating DNA adducts decreased with increasing amounts of NAcCys and were undetectable when the ratio of NAcCys to 4-OHE_2 was 3:1 (Table 5).

To gain further insight into the various mechanisms by which NAcCys affects estrogen metabolism, the expression of three key enzymes, COMT, NQO1 and CYP1B1 (Fig. 1) [50], was determined in MCF-10F cells incubated with 0 to 30 μM NAcCys (Fig. 5). The presence of NAcCys had no effect on the expression of any of these three enzymes. These results are consistent with the known effects of NAcCys, i.e., reduction of catechol estrogen semiquinones to catechol estrogens [51] and reaction of NAcCys with catechol estrogen quinones [8,35, 46].

Conclusions

NAcCys exerts its action through multiple protective mechanisms, including nucleophilicity, antioxidant activity and inhibition of DNA adduct formation. Its hydrolysis to Cys, the precursor to GSH, guarantees replenishment of this critical tripeptide in the cell. GSH has similar chemical properties to Cys and NAcCys. NAcCys reacts with E_2-3,4-Q to prevent their reaction with DNA [35]. NAcCys, like Cys, also operates as an antioxidant in reducing catechol estrogen semiquinones to catechol estrogens [51]. All of these properties have been observed in human breast epithelial cells and mouse mammary epithelial cells, as reported here. In addition, NAcCys has been shown to prevent malignant transformation of E6 mouse mammary epithelial cells treated with 4-OHE_2 or E_2-3,4-Q [20]. These properties of NAcCys suggest that this compound can help keep estrogen metabolism balanced and help rebalance it if it becomes out of balance. Therefore, by blocking formation of estrogen-DNA adducts, NAcCys is a potential agent to prevent the initiation of cancer by estrogens.

Abbreviations

CE	catechol estrogen(s)
CE-Q	catechol quinone(s)
COMT	catechol-*O*-methytransferase
Cys	cysteine
E_2-3,4-Q	estradiol-3,4-quinone
GSH	glutathione
GST	glutathione-*S*-transferase
HPLC-ECD	high performance liquid chromatography with electrochemical detection
NAcCys	*N*-acetylcysteine
NQO1	NAD(P)H quinone oxidoreductase 1
4-OCH_3E_2	4-methoxyestradiol
4-OHE_2	4-hydroxyestradiol
-SG	glutathione moiety
UPLC-MS/MS	ultraperformance liquid chromatography-tandem mass spectrometry

Acknowledgments

The authors wish to thank Dr. Fang Lu and Dr. Nilesh W. Gaikwad for their contribution to these experiments. This research was supported by Prevention, LLC. Core support at the Eppley Institute was supported by Grant CA P30 36727 from the National Cancer Institute.

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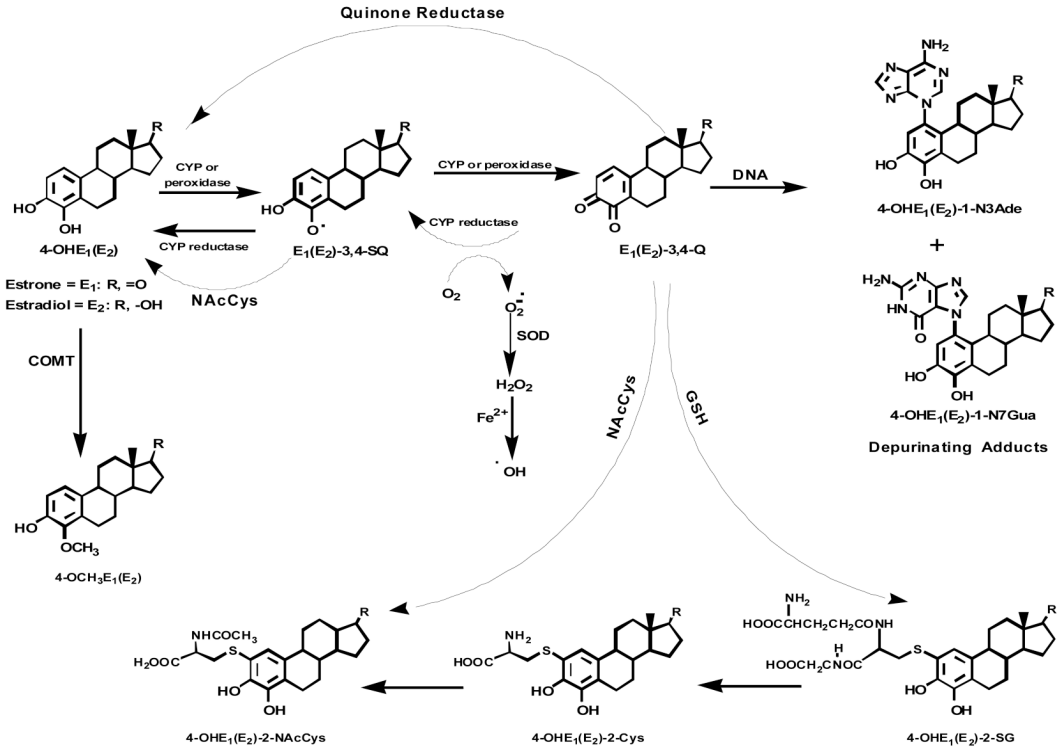

Figure 1.
Metabolic pathway for 4-$OHE_1(E_2)$ in the absence or presence of NAcCys.

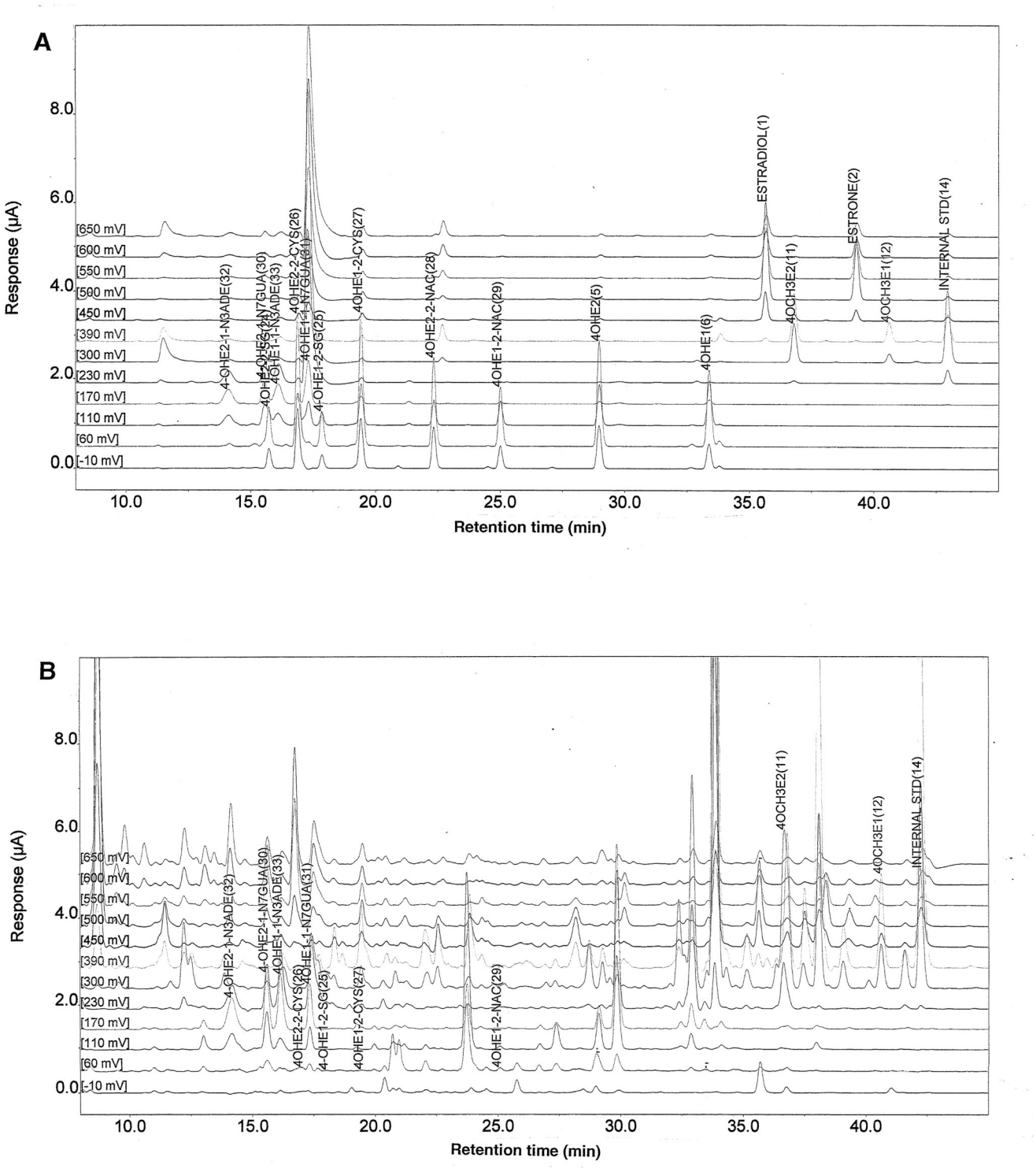

Figure 2.

HPLC-ECD of (A) a 95 µl aliquot of the standard compounds at a concentration of 1.0 µg/µl each, and (B) a representative chromatogram of culture medium following 24 h of treatment of MCF-10F cells with 10 µM 4-OHE_2.

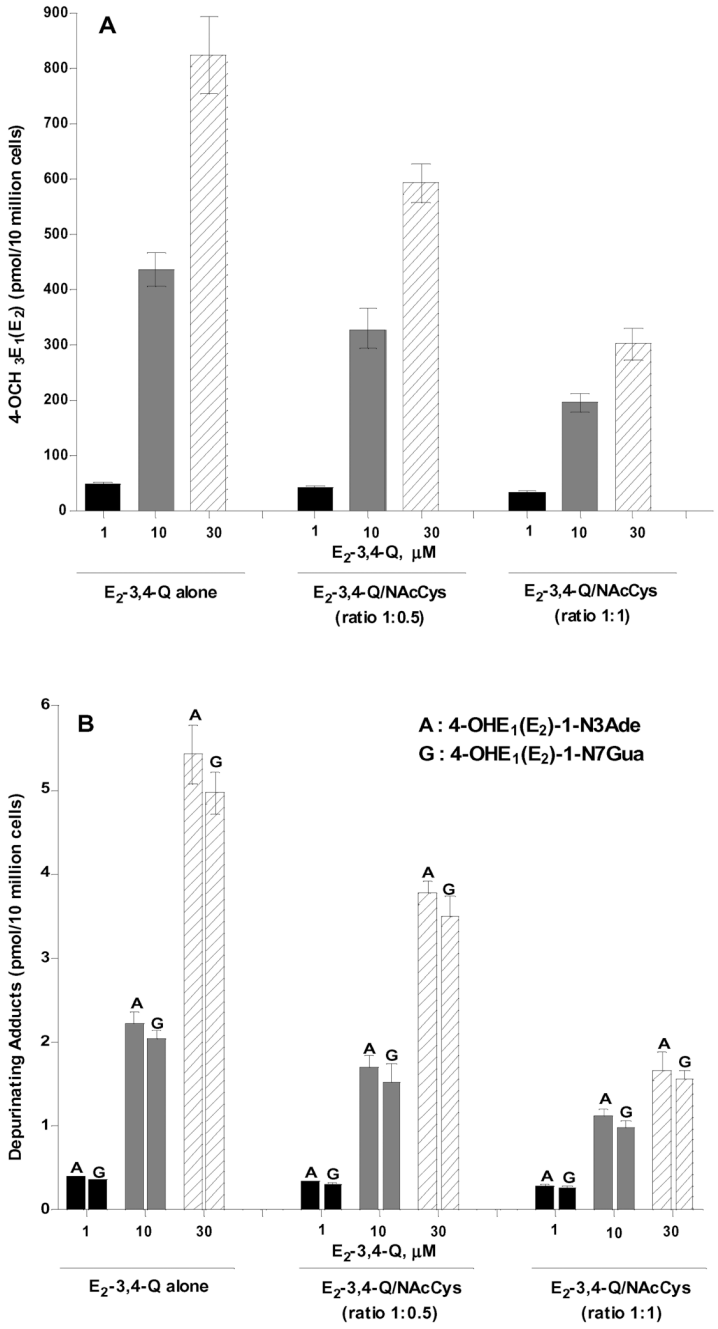

Figure 3.

Effects of NAcCys on the formation of (A) 4-$OCH_3E_1(E_2)$ and (B) estrogen-DNA adducts in MCF-10F cells treated with E_2-3,4-Q.

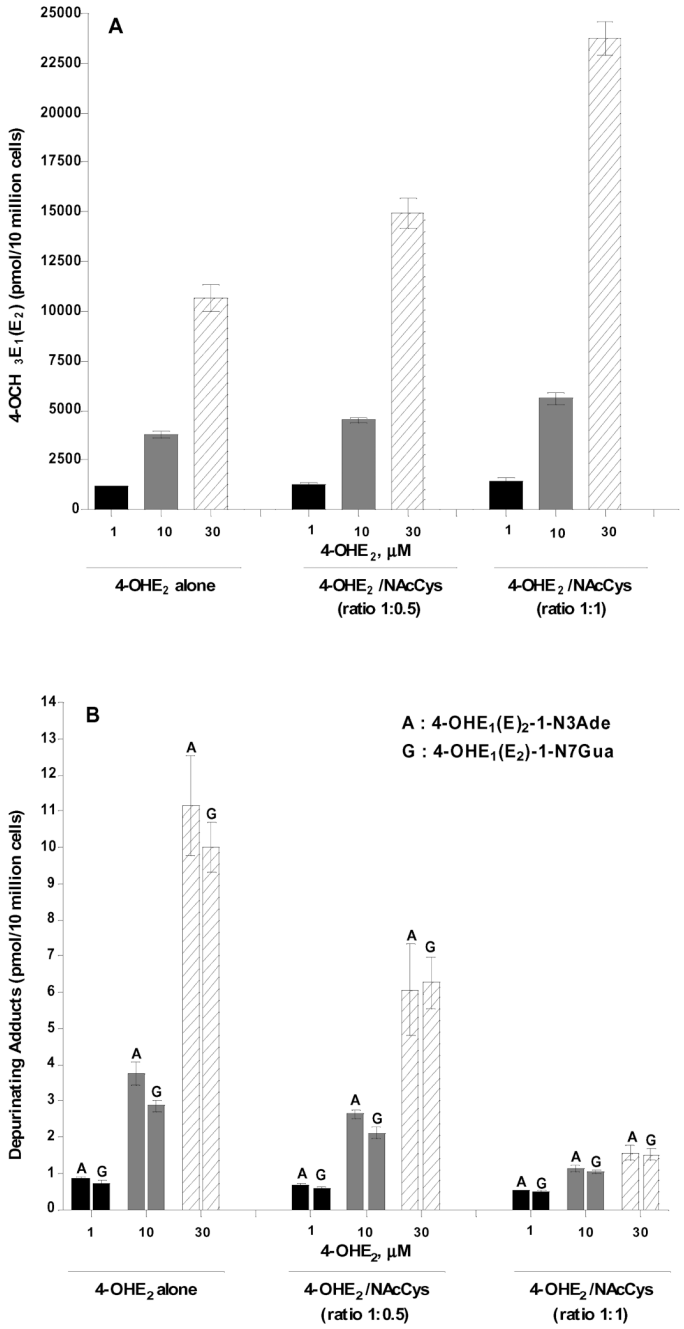

Figure 4.
Effects of NAcCys on the formation of (A) 4-$OCH_3E_1(E_2)$ and (B) estrogen-DNA adducts in MCF-10F cells treated with 4-OHE_2.

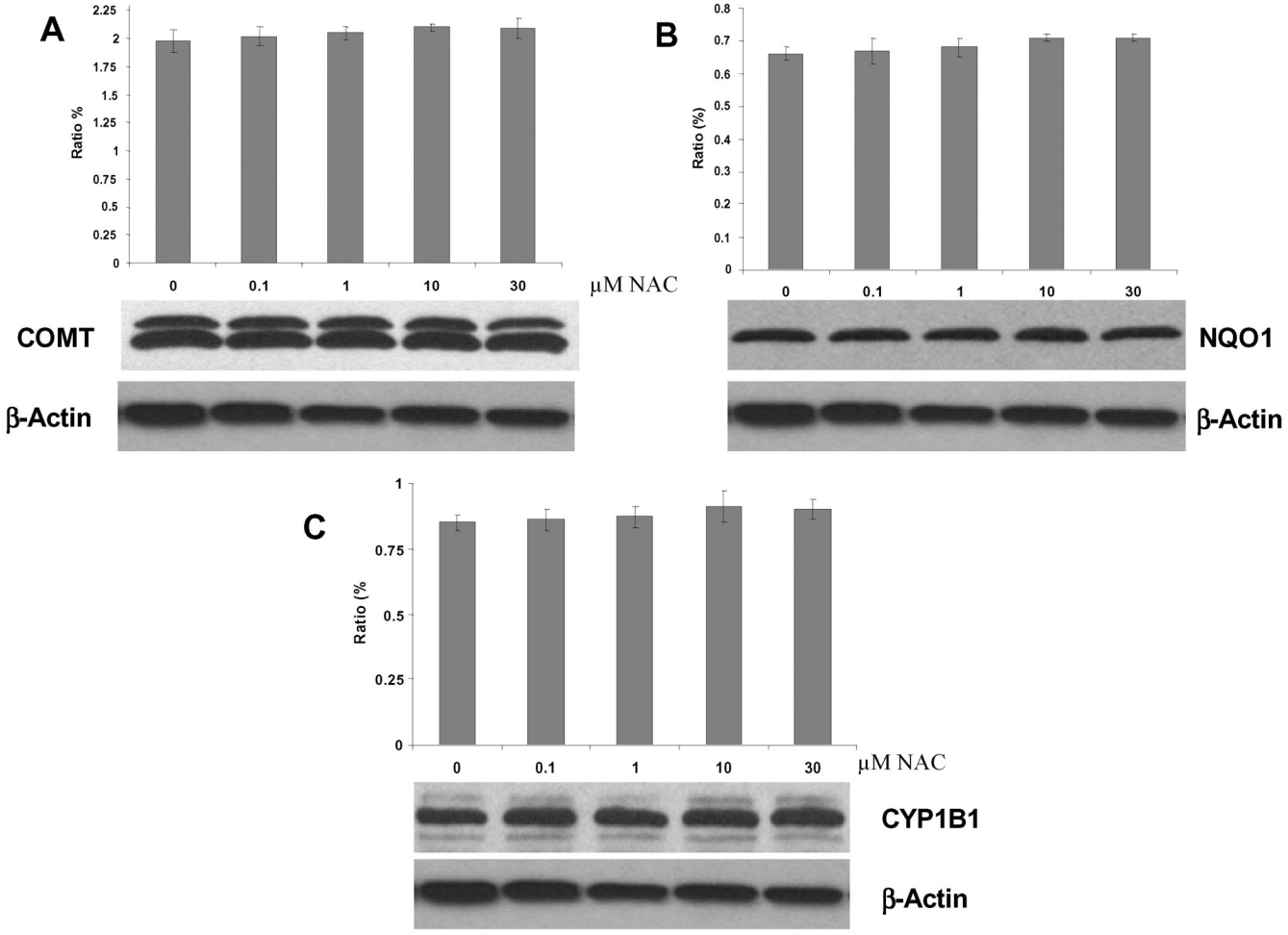

Figure 5.
Protein levels (COMT, NQO1, and CYP1B1) in MCF-10F cells after NAcCys treatment.

Table 1

Incubation of MCF-10F cells with E_2-3,4-Q alone or in the presence of NAcCys[1]

Detected Compounds[2]	E_2-3,4-Q alone			E_2-3,4-Q: NAcCys (1:0.5)			$E_2$3,4-Q: NAcCys (1:1)		
	1	10	30	1	10	30	1	10	30
	pmol/10 million cells								
4-OHE$_1$(E_2)	4.05 ± 0.32	7.79 ± 0.34	14.2 ± 1.41	2.96 ± 0.24	5.79 ± 0.51	9.57 ± 1.15	1.17 ± 0.26	3.07 ± 0.32	5.11 ± 0.47
4-OCH$_3$E$_1$(E_2)	49.7 ± 4.27	437 ± 50.9	824 ± 120	41.9* ± 4.06	329* ± 62.3	593* ± 59.6	32.3* ± 5.48	196* ± 28.9	302* ± 51.3
4-OHE$_1$(E_2)-2-SG	0.58 ± 0.05	3.13 ± 0.38	6.39 ± 0.77	0.65 ± 0.06	3.75 ± 0.59	8.10 ± 1.54	0.74* ± 0.06	4.36* ± 0.31	10.2* ± 1.71
4-OHE$_1$(E_2)-2-Cys	3.95 ± 0.71	8.78 ± 0.56	28.1 ± 2.40	4.42 ± 0.46	10.6* ± 0.72	34.7* ± 2.49	5.02 ± 0.70	12.2* ± 1.28	42.1* ± 2.84
4-OHE$_1$(E_2)-2-NAcCys	1.36 ± 0.28	6.37 ± 0.81	15.9 ± 2.82	1.63 ± 0.29	8.15* ± 1.04	21.6* ± 1.61	1.98* ± 0.26	10.3* ± 1.53	28.0* ± 3.41
4-OHE$_1$(E_2)-1-N3Ade	0.39 ± 0.03	2.22 ± 0.24	5.42 ± 0.60	0.34* ± 0.01	1.70* ± 0.24	3.76* ± 0.26	0.28* ± 0.02	1.12* ± 0.14	1.67* ± 0.35
4-OHE$_1$(E_2)-1-N7Gua	0.36 ± 0.02	2.08 ± 0.18	4.97 ± 0.44	0.30 ± 0.04	1.52 ± 0.39	3.50* ± 0.40	0.26* ± 0.03	0.99* ± 0.13	1.55* ± 0.19

[1] MCF-10F cells were incubated at 37 °C for 24 h with E_2-3,4-Q (1, 10 or 30 μM) alone or in the presence of different ratios of NAcCys.

[2] The compounds were identified and quantified by HPLC-ECD and the adducts were confirmed by UPLC-MS/MS; values are an average of three replicates from three independent pools of medium each from a set of five flasks; ± standard deviation.

* The value is significantly different from that in the cell s not treated with NAcCys ($p<0.05$) as determined by the t-test.

Table 2

Incubation of E6 cells with E_2-3,4-Q alone or in the presence of NAcCys[1]

Detected Compounds[2]	E_2-3,4-Q alone			E_2-3,4-Q: NAcCys (1:0.5)			E_2-3,4-Q: NAcCys (1:1)		
	1	10	30	1	10	30	1	10	30
	pmol/10 million cells								
4-OHE$_1$(E_2)	0.41 ± 0.07	1.34 ± 0.16	2.35 ± 0.30	0.27 ± 0.06	0.90 ± 0.05	1.50 ± 0.18	0.14 ± 0.03	0.65 ± 0.06	0.92 ± 0.09
4-OCH$_3$E$_1$(E_2)	60.2 ± 5.45	555 ± 29.4	1142 ± 74.8	48.9* ± 5.89	414* ± 29.9	761* ± 85.7	37.5* ± 2.47	264* ± 65.8	345* ± 81.3
4-OHE$_1$(E_2)-2-SG	0.76 ± 0.05	6.38 ± 1.0	14.9 ± 2.36	0.86* ± 0.06	7.58 ± 2.26	18.5* ± 1.83	1.0* ± 0.09	8.98* ± 1.48	21.8* ± 2.41
4-OHE$_1$(E_2)-2-Cys	4.52 ± 0.48	11.4 ± 0.95	32.7 ± 3.43	5.18 ± 0.48	14.1* ± 1.09	41.0* ± 3.23	6.27* ± 0.67	16.8* ± 1.35	48.2* ± 3.17
4-OHE$_1$(E_2)-2-NAcCys	1.80 ± 0.37	8.53 ± 0.96	18.6 ± 3.26	2.30 ± 0.46	11.6 ± 2.74	28.5* ± 3.28	2.85* ± 0.40	15.2* ± 2.29	42.6* ± 2.08
4-OHE$_1$(E_2)-1-N3Ade	0.49 ± 0.06	2.92 ± 0.27	6.22 ± 0.59	0.41 ± 0.04	2.36 ± 0.58	4.37* ± 0.32	0.32* ± 0.03	1.58* ± 0.16	1.86* ± 0.35
4-OHE$_1$(E_2)-1-N7Gua	0.47 ± 0.08	2.76 ± 0.15	5.82 ± 0.54	0.39 ± 0.02	2.07* ± 0.36	3.94* ± 0.74	0.33* ± 0.02	1.42* ± 0.25	1.79* ± 0.21

[1] E6 cells were incubated at 37 °C for 24 h with E_2-3,4-Q (1, 10, or 30 μM) alone or in the presence of different ratios of NAcCys.

[2] The compounds were identified and quantified by HPLC-ECD and the adducts were confirmed by UPLC-MS/MS; values are an average of three replicates from three independent pools of medium each from a set of five flasks; ± standard deviation.

* The value is significantly different from th at in the cell s not treated with NAcCys (p<0.05) as determined by the t-test.

Table 3

Incubation of MCF-10F cells with 4-OHE_2 alone or in the presence of NAcCys[1]

Detected Compounds[2]	4-OHE_2 alone			4-OHE_2: NAcCys (1:0.5)			4-OHE_2: NAcCys (1:1)		
	1	10	30	1	10	30	1	10	30
	pmol/10 million cells								
4-OHE_1(E_2)	2.20 ± 0.45	9.49 ± 2.04	29.5 ± 3.45	1.11 ± 0.33	5.47 ± 0.71	14.2 ± 2.17	0.27 ± 0.07	2.42 ± 0.45	7.37 ± 1.4
4-OCH_3E_1(E_2)	1139 ± 106	3788 ± 353	10648 ± 1211	1282 ± 124	4500* ± 259	14944* ± 1316	1461* ± 200	5598* ± 500	23736* ± 1467
4-OHE_1(E_2)-2-SG	1.10 ± 0.21	7.48 ± 0.77	12.2 ± 3.34	1.19 ± 0.16	8.57 ± 0.57	14.4 ± 2.09	1.32* ± 0.15	9.87 ± 2.19	17.3 ± 2.94
4-OHE_1(E_2)-2-Cys	5.16 ± 0.55	9.98 ± 0.24	37.3 ± 1.26	5.74 ± 0.53	11.7* ± 0.72	46.2* ± 4.29	6.76* ± 1.08	14.2* ± 2.29	58.6* ± 3.30
4-OHE_1(E_2)-2-NAcCys	5.0 ± 0.65	11.5 ± 2.0	31.0 ± 3.86	5.57 ± 1.10	13.2 ± 2.26	38.6* ± 3.95	6.84* ± 0.60	16.6* ± 3.31	51.2* ± 3.63
4-OHE_1(E_2)-1-N3Ade	0.84 ± 0.09	3.76 ± 0.53	11.2 ± 2.38	0.68* ± 0.07	2.63* ± 0.20	6.07* ± 2.20	0.53* ± 0.03	1.14* ± 0.19	1.57* ± 0.34
4-OHE_1(E_2)-1-N7Gua	0.70 ± 0.19	2.86 ± 0.28	9.99 ± 1.20	0.59 ± 0.05	2.11* ± 0.28	6.26* ± 1.24	0.51* ± 0.04	1.05* ± 0.08	1.52* ± 0.29

[1] MCF-10F cells were incubated at 37 °C for 24 h with 4-OHE_2 (1, 10 or 30 μM) alone or in the presence of different ratios of NAcCys.

[2] The compounds were identified and quantified by HPLC-ECD and the adducts were confirmed by UPLC-MS/MS; values are an average of three replicates from three independent pools of medium each from a set of five flasks; ± standard deviation.

* The value is significantly different from th at in the cells not treated with NAcCys ($p<0.05$) as determined by the t-test.

Table 4

Incubation of E6 cells with 4 -OHE_2 alone or in presence of NAcCys[1]

Detected Compounds[2]	4-OHE_2 alone			4-OHE_2: NAcCys (1:0.5)			4-OHE_2: NAcCys (1:1)		
	1	10	30	1	10	30	1	10	30
	pmol/10 million cells								
4-OHE_1(E_2)	1.29 ± 0.11	4.46 ± 1.01	20.1 ± 1.76	0.78 ± 0.09	2.86 ± 0.86	10.5 ± 2.40	0.21 ± 0.07	0.59 ± 0.10	3.56 ± 1.13
4-OCH_3E_1(E_2)	2077 ± 327	4669 ± 440	15112 ± 1652	2377 ± 338	5718* ± 342	19804* ± 1325	2785* ± 335	7599* ± 963	36372* ± 3355
4-OHE_1(E_2)-2-SG	2.38 ± 0.47	13.9 ± 2.02	33.0 ± 4.19	2.67 ± 0.55	16.4 ± 0.57	40.8* ± 1.70	3.07 ± 0.70	18.6* ± 2.32	50.4* ± 3.23
4-OHE_1(E_2)-2-Cys	6.19 ± 1.29	18.12 ± 2.53	38.5 ± 7.06	7.25 ± 1.16	22.3* ± 2.27	50.4 ± 7.53	8.80 ± 1.81	28.4* ± 2.92	64.2* ± 7.61
4-OHE_1(E_2)-2-NAcCys	6.22 ± 0.66	17.9 ± 1.53	40.5 ± 6.46	7.23 ± 0.96	22.1* ± 1.92	54.3* ± 6.82	8.78* ± 1.07	26.8* ± 2.04	70.8* ± 11.9
4-OHE_1(E_2)-1-N3Ade	1.33 ± 0.28	4.18 ± 0.87	41.9 ± 4.97	1.07 ± 0.05	2.81* ± 0.33	25.3* ± 1.88	0.53* ± 0.03	1.63* ± 0.26	4.00* ± 0.73
4-OHE_1(E_2)-1-N7Gua	0.97 ± 0.11	3.97 ± 0.59	39.6 ± 4.65	0.79* ± 0.09	2.70* ± 0.51	24.3* ± 1.48	0.51* ± 0.04	1.58* ± 0.25	4.59* ± 0.83

[1] E6 cells were incubated at 37 °C for 24 h with 4-OHE_2 (1, 10 or 30 μM) alone or in the presence of different ratios of NAcCys.

[2] The compounds were identified and quantified by HPLC-ECD and the adducts were confirmed by UPLC-MS/MS; values are an average of three replicates from three independent pools of medium each from a set of five flasks; ± standard deviation.

* The value is significantly different from th at in the cell s not treated with NAcCys ($p<0.05$) as determined by the t-test.

Free Radic Biol Med. Author manuscript; available in PMC 2011 August 1.

Table 5

Incubation of MCF-10F cells with 10 μM 4-OHE_2 alone or in the presence of NAcCys[1]

Treatment ratio: 4-OHE_2: NAcCys					
Detected Compounds [2]	**1:0**	**1:0.1**	**1:0.3**	**1:1**	**1:3**
	pmol/10 million cells				
4-OHE_1(E_2)	11.5 ± 2.73	9.38 ± 2.35	5.73 ± 0.92	3.03 ± 0.59	0.70 ± 0.09
4-OCH_3E_1(E_2)	3684 ± 346	3931 ± 427	4515 ± 275	5780* ± 386	8143* ± 942
4-OHE_1(E_2)-2-SG	8.35 ± 0.75	8.52 ± 0.92	9.24 ± 1.12	10.4* ± 0.95	12.2* ± 1.78
4-OHE_1(E_2)-2-Cys	11.9 ± 1.71	12.2 ± 1.06	14.3 ± 1.77	17.2* ± 1.96	20.8* ± 3.99
4-OHE_1(E_2)-2-NAcCys	13.7 ± 2.07	14.2 ± 1.72	16.7 ± 1.72	20.2* ± 1.30	25.1* ± 2.30
4-OHE_1(E_2)-1-N3Ade	4.43 ± 0.84	4.31 ± 0.45	3.23 ± 0.25	1.29* ± 0.45	nd[3]
4-OHE_1(E_2)-1-N7Gua	3.56 ± 0.72	3.43 ± 0.51	2.62 ± 0.39	1.13* ± 0.27	nd

[1] MCF-10F cells were incubated at 37 °C for 24 h with 4-OHE_2 (10 μM) alone or in the presence of different ratios of NAcCys.

[2] The compounds were identified and quantified by HPLC-ECD and the adducts were confirmed by UPLC-MS/MS; values are an average of three replicates from three independent pools o f medium each from a set of five flasks; ± standard deviation.

[3] not detected.

* The value is significantly different from th at in the cells not treated with NAcCys ($p<0.05$) as determined by the t-test.