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Response of human mammary epithelial cells to DNA damage induced by 4-hydroxyequilenin: lack of p53-mediated G₁ arrest

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Abstract

Long-term exposure to synthetic and endogenous estrogens has been associated with the development of cancer in several tissues. One potential mechanism of estrogen carcinogenesis involves catechol formation and these catechols are further oxidized to electrophilic/redox active *o*-quinones, which have the potential to both initiate and promote the carcinogenic process. 4-Hydroxyequilenin (4-OHEN), a major phase I metabolite of several estrogens present in Premarin®, is considerably more cytotoxic, carcinogenic, and mutagenic as compared to the catechol estrogen metabolites of endogenous estrogens. Previously, we showed that 4-OHEN autoxidized to an *o*-quinone and caused a variety of damage to DNA. Allowing more time between the induction of DNA damage and the entry of a damaged cell into the DNA synthetic phase of the cell cycle protects that cell from mutagenesis. Central to this response is the establishment of a G₁ checkpoint. This checkpoint is mediated by the cyclin-dependent kinase inhibitor p21^{WAF1}, a direct downstream target for transcriptional activation by p53. In this study, we investigated this signaling pathway. Surprisingly, exposure of the human MCF-10A immortalized nontransformed mammary epithelial cell line to 4-OHEN did not induce a p53-induced G₁ arrest. A 24 h treatment with 4-OHEN significantly induced p53 and p21^{WAF1} protein expression at 10 and 20 μM, as well as significantly induced the transactivation of a p53-luciferase reporter gene at 20 μM. Significant decreases in cell proliferation were also observed with concentrations of 5 μM and higher of 4-OHEN. However, 4-OHEN did not induce a G₁ checkpoint and cells with damaged DNA accumulated in the S phase. This S phase delay could be beneficial for the survival of the damaged cells which could contribute to the carcinogenic process.

Keywords

estrogen; quinone; MCF-10A; p53; cell cycle

1. Introduction

Long time exposure to estrogens increases the risk of developing breast or endometrial cancer in women [1–8]. Even though the mechanisms of estrogen carcinogenesis are controversial, it has been shown that hormonal potency cannot be directly correlated with the carcinogenic activity of estrogens [9]. Evidence suggest that the metabolism of estrogens to catechols and further oxidation to highly reactive *o*-quinones could play a major role in induction of DNA

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damage, leading to initiation of the carcinogenic process [10]. *o*-Quinones are Michael acceptors which cause damage in cells through alkylation of DNA, lipids, and proteins [11].

Premarin (Wyeth-Ayerst) is the most widely prescribed estrogen replacement formulation and includes the endogenous estrogens, estrone and estradiol, and the equine estrogens, equiline and equilinenin. Previously, we showed that the major phase I metabolite of equine estrogens is 4-hydroxyequilenin (4-OHEN) which autoxidizes to a potent cytotoxic *o*-quinone and causes a variety of DNA lesions [12–15]. Our recent data suggested that 4-OHEN has the potential to be a much more effective tumor promoter and complete carcinogen *in vitro* in comparison to similar experiments with the endogenous catechol estrogen, 4-hydroxyestrone [10]. In addition, 4-OHEN induced four different types of DNA lesions, including single strand breaks and oxidized bases, as shown in the mammary tissue isolated from female Sprague-Dawley rats treated with this compound [16].

The tumor suppressor protein 53 (TP53) has been shown to play a critical role in the cellular response pathway to DNA damage. When the DNA has lesions, nucleotide excision repair is of key importance in preventing toxicity and lowering induced mutation frequencies [17]. However, this process is not instantaneous, and for typical mammalian cells treated with a genotoxic chemical carcinogen only 40–60% of the lesions are removed from the genome as a whole in 24 h [17]. Indeed, allowing more time between the induction of DNA damage and the entry of a damaged cell into the DNA synthetic phase of the cell cycle protects that cell from mutagenesis [17]. Central to this response is the establishment of a G₁ checkpoint. This checkpoint is mediated by the cyclin-dependent kinase inhibitor p21^{WAF1}, a direct downstream target for transcriptional activation by p53 [18]. Additionally, a G₂/M checkpoint is often established to prevent attempted division of cells with damaged chromosomes. Much of the work that has established these p53 damage response pathways as a paradigm has utilized ultraviolet radiation, ionizing radiation, or oxidative stress as the DNA damaging agent. These forms of DNA damage are recognized by the cell as yet undetermined mechanisms, and result in post-translational modifications of p53 that result in the stabilization of the protein and its accumulation in the nuclei of damaged cells [19]. Bulky chemical adducts induce stabilization and nuclear accumulation of p53 [20]. However, recent work with direct acting metabolites of polycyclic aromatic hydrocarbons (PAH) has failed to demonstrate the establishment of a G₁ arrest in response to this form of DNA damage [21,22]. Thus, alternative DNA damage response strategies may be utilized for different kinds of damage.

The non-tumorigenic MCF-10A cell line was originally derived from a patient with proliferative breast disease [23]. This spontaneously immortalized cell line represents a provocative model for normal human breast epithelial cells in culture. Moreover, it is one of a few established cell lines that contain wild type p53. The main objective of the present study was to assess the cellular response of MCF-10A to 4-OHEN exposure. We assessed multiple endpoints such as cell proliferation, p53 and p21^{WAF1} protein expression by western blotting, p53 activation by reporter gene assay and cell cycle distribution by flow cytometry. The data suggest that the lack of p53-mediated G₁ arrest after DNA damage induced by 4-OHEN might contribute to its carcinogenicity.

2. Materials and Methods

2.1. Reagents

Caution—*The catechol estrogens were handled in accordance with NIH guidelines for the Laboratory Use of Chemical Carcinogens* [24]. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), or Fisher Scientific (Itasca, IL) unless stated otherwise. 4-OHEN was synthesized by treating equilin with Fremy's salt as described previously [25,26] with minor modifications [13]. Cholera toxin was obtained from List Biological (Campbell, CA). Culture

media, epidermal growth factor, penicillin-streptomycin, and glutamine were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA).

2.2. Cell culture conditions

Human MCF-10A mammary epithelial cells were obtained from ATCC (Manassas, VA) and they were grown in D-MEM/F-12 medium supplemented with 100 ng/ml cholera toxin, 10 µg/ml insulin, 0.5 µg/ml hydrocortisol, 20 ng/ml epidermal growth factor, 1% 10,000 U penicillin G, 10 mg/ml streptomycin, and 5% heat-inactivated FBS. 4-OHEN was freshly dissolved in DMSO and the final DMSO concentration was 0.01%.

2.3. Cell proliferation

Cells were plated (1×10^4 cells/well) in 96 well plates. The following day, cells were treated with the compound for 0, 3, 6, 12 and 24 h. After the incubation period, cells were fixed to the plastic substratum by the addition of cold 20% aqueous trichloroacetic acid. The plates were incubated at 4 °C for 1 h, washed with H₂O, and air-dried. The trichloroacetic acid-fixed cells were stained by the addition of 0.4% sulforhodamine B (w/v), dissolved in 1% acetic acid for 30 min. Free sulforhodamine B solution was removed by washing with 1% aqueous acetic acid. The plates were air-dried, and the bound dye was solubilized by the addition of 10 mM unbuffered Tris base, pH 10. The plates were placed on a shaker for 5 min, and the absorption was determined at 515 nm. Finally, the absorbance obtained with each of the treatment procedures was averaged and was expressed as a percentage, relative to the 0 h control.

2.4. Immunoblot analyses

The expression of p53, phospho Ser¹⁵ p53 and p21^{WAF1} protein was assessed by immunoblots. In brief, cells (2×10^6) were treated with various concentrations of 4-OHEN and harvested after 24 h. Whole-cell pellets were lysed with detergent lysis buffer (1 ml/ 10^7 cells, 50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 1% Nonidet[®] P40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 2 µg/ml leupeptin and 100 µM sodium vanadate) to obtain protein lysates, and protein concentrations were quantified using the Bradford method. Total protein (30 µg) was separated by 10–12.5% SDS-PAGE, electroblotted to PVDF membranes, and blocked with 5% non-fat dry milk for 1 h at room temperature. The membrane was incubated with anti-p53 antibody (1:1000, Oncogene, Cambridge, MA), anti-phospho Ser¹⁵ p53 antibody (1:1000, Cell Signaling, Beverly, MA) or anti-p21^{WAF1} antibody (1:5000, Cell Signaling), prepared in 1% blocking solution, ON at 4°C, washed three-times for 5 min with PBS-T (PBS with 0.1% v/v, Tween 20), and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed three-times for 5 min each in PBS-T and developed by enhanced chemiluminescence (Cell Signaling). Membranes were exposed to Kodak Biomax film and the resulting film analyzed using Kodak (Rochester, NY) 1D Image Analysis Software. Membranes were then stripped and reprobed for β-actin (Sigma, St. Louis, MO).

2.5. p53 transcriptional activity

The Dual-Luciferase Reporter Assay System from Promega (Madison, WI) was used to evaluate the functional formation of p53 and luciferase protein expression. Briefly, cells (1.5×10^5) were grown overnight in six well dishes. The following day, cells were cotransfected using Effectene (Qiagen, Valencia, CA) according to the company's protocol with p53 plasmid (Stratagene, La Jolla, CA, 0.5 µg), pRL-TK plasmid (0.3 µg), and the respective ratios of reagents. Following a 24 h recovery period, the cells were washed with PBS and treated with various concentrations of 4-OHEN for an additional 24 h. Cell lysates (20 µL) were placed in

96 well plates. Luciferase Assay Reagent II (100 μ L) was injected followed by a 12 s read by a FLUOstar OPTIMA (BMG LabTech, Offenburg, Germany). Stop & Glo (100 μ L) was added followed by a 12 s read, thus allowing the termination of the firefly luciferase expression and the activation of the renilla expression. The sample results were normalized to pRL-TK, to account for transfection efficiency, by dividing the sum of the luciferase activity by the sum of the renilla activity. Samples were converted into fold induction such that MCF-10A cells treated with DMSO fold induction was equal to 1.

2.6. Cell cycle analysis

Cells (10^6) were treated with various concentrations of 4-OHEN for 24 h, then trypsinized and washed with PBS. Cells were resuspended in 1 ml PBS + 9 ml ice-cold 70% EtOH and stored at -20°C . Just before analysis, samples were centrifuged to pellet the cells and resuspended in 200 μ l citrate buffer (250 mM sucrose, 40 mM trisodium citrate, 0.05% DMSO, pH 7.6).

Solution A [1.8 ml total volume of 3 mg trypsin in 100 ml stock buffer (3.4 mM trisodium citrate, 0.1% nonidet P40, 1.5 mM spermine tetrahydrochloride, 0.5 mM trizma, pH 7.6)] was added and the tube was inverted to mix the contents gently. After 10 min at room temperature, during which the tube was inverted five or six times, 1.5 ml solution B (25 mg trypsin inhibitor + 10 mg ribonuclease A in 100 ml stock buffer) was added. The solutions were again mixed by inversion of the tube and after 10 min at room temperature, 1.5 ml ice-cold solution C (41.6 mg propidium iodide + 116 mg spermine tetrahydrochloride in 100 ml stock buffer) was added. The solutions were mixed and samples were filtered through a 30- μ m nylon mesh into tubes wrapped in tinfoil for light protection of the propidium iodide and analyzed by flow cytometry.

Alternatively, cells were exposed to 5-bromo-2-deoxyuridine (BrdU) for 30 min prior to trypsinization to specifically label S-phase cells. After fixation, cells were stained with fluorescein-conjugated antibody to BrdU and counter stained with propidium iodide following the manufacturer's protocol (Phoenix Flow Systems, San Diego, CA). Cell suspensions were analyzed by flow cytometry, and data were collected using appropriate electronic gating to remove background debris and aggregates.

2.7. Statistical analysis

Data were expressed as means $\pm$ SD and analyzed through one-way analysis of variance (ANOVA), followed by pairwise comparisons made with Dunnett's test, using the SAS statistical package (SAS Institute, Cary, NC). All of the tests were two-sided, and a *p* value of less than 0.05 was considered to be significant.

3. Results

3.1. Effect of 4-OHEN on proliferation in MCF-10A cells

Previously, we have shown that estrogen receptor positive cell lines were more sensitive to induction of cell death by 4-OHEN than the estrogen receptor negative cell line in the trypan blue dye exclusion assay [27]. In this study, we used the protein-binding dye sulforhodamine B (SRB) assay which assesses the ability of compounds to inhibit the growth of cells [28]. Cells were treated for 3, 6, 12 and 24 h with various concentrations of 4-OHEN (2.5–20 μ M). The data suggest there is a concentration-dependent decrease in cell proliferation (Figure 1). Concentrations of 5–20 μ M 4-OHEN showed statistically significant inhibition of cell proliferation compared to the control.

3.2. Alterations of p53 and p21^{WAF1} after 4-OHEN treatment in MCF-10A cells

As 4-OHEN has previously been shown to induce DNA damage [29] and changes in p53 have been implicated in the cellular response to DNA damage, we studied changes in both the overall

level of p53 and the extent of phosphorylation of particular serine residues using specific antibodies. Western blots showed that the overall cellular level of p53 (Figure 2) and phosphorylation of p53 at Ser²⁰ (data not shown), a residue involved in mdm2 interactions and stabilization of p53 [30], was detected at comparable levels in both treated and control cells. However, a significant increase in phosphorylation at Ser¹⁵ was seen with 10 and 20 μ M 4-OHEN treatment (Figure 2). There was a parallel increase in p21^{WAF1}. Equal loading was confirmed by stripping and reprobing the membranes shown in Figure 2 with β -actin.

3.3. p53 activation induced by 4-OHEN in MCF-10A cells

Phosphorylation of p53 contributes to the protein stabilization and to acquisition by p53 of an active conformation capable of specifically binding the p53-responsive elements present in the regulatory regions of target genes such as *WAF-1*. MCF-10A cells that had been transiently cotransfected with the p53-luciferase and the pRL-TK control plasmid were used to measure p53 transcriptional activity in response to treatment with 4-OHEN. Samples were normalized to the control transfection and then expressed as a fold induction as compared with DMSO-treated MCF-10A cells (Figure 3). Consistent with the results obtained with p21^{WAF1} protein expression, 4-OHEN significantly induced the transactivation of a p53-luciferase reporter gene at 20 μ M.

3.4. Effects of 4-OHEN on cell cycle in MCF-10A cells

It has been demonstrated that wild-type p53 prevents replication of DNA on a damaged template by arresting cells in the G₁ phase of the cell cycle [31]. The effects of a 24 h treatment with various concentrations of 4-OHEN on cell cycle parameters were determined by flow cytometry in MCF-10A cells. Representative histograms of DNA content are shown in Figure 4 A–E, and data from three independent experiments are collected in Table I. Exposure to 10 and 20 μ M 4-OHEN induced a significant accumulation of cells in the S phase and a reduction of cells in the G₁ phase. The percentage of cells in G₂/M was equal in the treated cells relative to the control. The percentage of cells in subG₁ was insignificant. The increase in level of S phase cells in 4-OHEN-treated cultures in the face of no changes in the fraction of G₂/M cells clearly implies continued entry of G₁ cells into S phase.

To firmly establish this point, control and 4-OHEN-treated cells were allowed to incorporate BrdU for 30 minutes prior to harvest, and the level of incorporation due to new DNA synthesis was determined by staining with fluorescently labeled antibody to BrdU prior to analysis by flow cytometry. In Figure 4 F–J, total DNA content is presented along the x-axis and BrdU staining along the y-axis. Despite the reduction in number of labeled cells in the S phase compartment caused by 4-OHEN, cells did continue to enter the early stages of S phase indicating that 4-OHEN does not induce an absolute G₁ arrest.

4. Discussion

The relationship between DNA damage and carcinogenesis is very complex [32]. Previously, we showed that equine catechol estrogens can cause a variety of DNA lesions, including formation of bulky stable adducts, apurinic sites, and oxidation of the phosphate-sugar backbone and purine/pyrimidine bases *in vitro* and *in vivo* [6,16,29]. The human tumor suppressor p53 has been shown to play a critical role in the cellular response pathway to DNA damage induced by physical and chemical agents [33–35]. Recent studies have dealt with the role of p53 and/or p21^{WAF1} proteins in the cellular response to DNA damage caused by some parent PAHs or their DNA-reactive metabolites [20,36,37] and they produced contradictory results. To our knowledge, no previous studies have been done using catechol estrogens. The principal aim of this study was to investigate simultaneously the induction of p53 and p21^{WAF1} proteins, p53 activation and the cell cycle distribution in human mammary epithelial

cells after exposure to 4-OHEN. MCF-10A cells were used in this study to explore the possible mechanism(s) involved in the carcinogenic action of equine estrogen metabolites. The measure of cell proliferation (Figure 1), suggested there is a concentration-dependent decrease in cell growth. Results from the western blot analysis and the p53 reporter gene assay indicated that p53 may be stabilized by phosphorylation and its transcriptional activity was increased by 4-OHEN.

Our results of concentration-dependent increases of p53 and p21^{WAF1} protein levels after 4-OHEN treatment of mammary epithelial cells are similar to the results obtained in studies using some PAHs [34,38] in which the authors suggest that a p53-dependent transduction pathway might result in a cell cycle arrest at G₁ or G₂/M checkpoints allowing cells to repair DNA damage prior to continuing into the S phase or to undergoing mitosis. However, neither of these studies investigated the effect of exposure on the cell cycle. In contrast, Khan et al. [35] did not observe any induction of p21^{WAF1} protein in human mammary carcinoma MCF-7 cells following exposure to a PAH. In their study, they simultaneously measured p53 and p21^{WAF1} protein levels and cell cycle distribution, while the levels of DNA damage were not determined. They showed that cell treatment with this carcinogen led to an increase in p53 levels without any induction of p21^{WAF1}, and they demonstrated a lack of G₁ arrest but instead a delay of cells in the S phase. Therefore, they suggested that a lack of G₁ arrest is consistent with the absence of p21^{WAF1} cyclin-dependent kinase inhibitor induction.

In our study, despite a significant increase of p53 phosphorylated at Ser¹⁵ and p21^{WAF1} protein levels after treatment of MCF-10A cells with 4-OHEN, we did not observe a G₁ arrest but instead a delay of cells in the S phase. After treatment, there was an increase in the proportion of cells in S phase and equal percentages of cells in G₂/M phase, along with a decrease of cells in G₁ phase. Similarly, an S phase delay was observed by Binkova et al. [36] after exposure of normal human diploid lung fibroblast cells to PAHs. They also showed an increase in p53 and p21^{WAF1} protein levels. Shenberger et al. [39] observed an S phase growth arrest with increased expression of p53 and p21^{WAF1} proteins induced by oxidative DNA damage in human bronchial smooth muscle cells. They suggested that activation of these genes may prevent replication without inducing apoptosis to allow for the repair of oxidative damage. Black et al. [40] detected an S phase block in human lymphoblasts exposed to PAHs. They found that the perturbation of the cell cycle was preceded by a reduction of cell viability and was associated with an inhibition of population growth. However, their study did not investigate the effect of exposure on p53 and p21^{WAF1} expression. In the present study, 4-OHEN induced S phase arrest and, at high concentrations, also cell death. It is becoming clear that p53 activation does not always induce G₁ arrest and that the absence of p53 induction does not necessarily mean the absence of DNA damage and of G₁ arrest. It seems that data on DNA damage, as well as cell cycle regulation and alterations are necessary for a better understanding of the effect and consequences of genotoxic agents. Moreover, differences in the measure of defects in cell cycle progression by using static flow cytometric DNA histograms versus BrdU labeling can lead to confusion and potential misinterpretation of results [41]. The vast majority of molecular biology studies, in which the role of p53 is investigated, use only static methods to assess cell cycle progression delays. In these cases, it remains unclear whether cells in general show a G₁ arrest following DNA damage and, if so, whether such a G₁ arrest is dependent of p53 status. Thus, the need to use dynamic methods, such as BrdU labeling, to measure cell cycle progression is important. Studies that failed to find a G₁/S arrest utilized epithelial cells or tumor cells of epithelial origin, whereas the ones that established the p53 paradigm utilized fibroblasts or lymphoid cells. Thus, it is not clear whether the altered responses noted in this study are a result of a difference in cell type or in DNA damaging agent.

In conclusion, the present study clearly demonstrated that exposure of MCF-10A cells to 4-OHEN resulted in increased levels of p53 and p21^{WAF1} proteins, and p53 activation.

Surprisingly, there was no G₁ arrest but instead a delay of cells in the S phase. This S phase delay could be beneficial for the survival of the damaged cells which could contribute to the carcinogenic process. To the best of our knowledge, the concentration of 4-OHEN in the breast or endometrium is not known. However, on the basis of estimates for the concentrations of endogenous estrogens, we would predict they would be in the nanomolar range. As a result, it is quite possible that at physiologically relevant concentrations, 4-OHEN is capable of causing DNA damage, mutations, and carcinogenesis in these target tissues. The mechanism behind the cell cycle perturbation is unclear and requires further study.

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Abbreviations

4-OHEN	4-hydroxyequilenin
BrdU	5-bromo-2-deoxyuridine
ER	estrogen receptor
FBS	fetal bovine serum
PAH	polycyclic aromatic hydrocarbon
ROS	reactive oxygen species

Fig. 1. Proliferation of MCF-10A cells. The cells were incubated with various concentrations of 4-OHEN (expressed in μM) for 3, 6, 12 and 24 h as described in Materials and Methods. The data represent the means of three determinations $\pm$ SD and are expressed as a percentage, relative to the 0 h control.

* Significantly different from control values ($p < 0.01$).

Fig. 2.

Changes in p53, phosphorylation of p53 at Ser¹⁵ and p21^{WAF1} protein levels following a 24 h treatment with 4-OHEN. MCF-10A cells were treated with various concentrations of 4-OHEN (expressed in μ M) and whole cell extracts were prepared as described in Materials and Methods. Aliquots were analyzed by western blot using antibodies specific for total p53, p53 phosphorylated at Ser¹⁵ and p21^{WAF1}. Densitometric analyses results are shown as fold induction of protein expression relative to levels observed in cells treated with solvent after normalization for β -actin. Results are the means of three experiments $\pm$ SD.

* Significantly different from control values ($p < 0.01$).

Fig. 3.

p53-luciferase induction in MCF-10A cells by 4-OHEN. After transfection, cells were treated with 4-OHEN for 24 h and then analyzed for chemiluminescence as described in Materials and Methods. Results were normalized for transfection efficiency, and they are shown as a fold induction relative to the level observed in MCF-10A cells treated with solvent only. The positive control plasmid expressed a kinase from the constitutive CMV promoter that is ultimately responsible for activating p53 from the enhancer element-TATA box region. Results are the means of three determinations $\pm$ SD.

* Significantly different from control values ($p < 0.01$).

Fig. 4.

Lack of establishment of a G₁ arrest. MCF-10A cells were treated with 0 (A, F), 2.5 (B, G), 5 (C, H), 10 (D, I), or 20 μ M (E, J) of 4-OHEN, and harvested for cell cycle analysis 24 h later. For 30 min prior to harvest, cells synthesizing DNA were allowed to incorporate BrdU. Cells synthesizing DNA during this period were then labeled using fluorescein-modified antibody to BrdU and were analyzed by flow cytometry. Histograms of propidium iodide fluorescence corresponding to DNA content is shown in A–E. A dot plot of DNA content against BrdU labeling is shown in F–J.

Table IChanges in cell cycle parameters^a

Concentration [μ M]	G ₁	S	G ₂
0	73 $\pm$ 9	11 $\pm$ 4	16 $\pm$ 6
2.5	68 $\pm$ 13	15 $\pm$ 6	17 $\pm$ 8
5	63 $\pm$ 3	18 $\pm$ 3	19 $\pm$ 5
10	57 $\pm$ 5	23 $\pm$ 4 [*]	20 $\pm$ 7
20	59 $\pm$ 2	25 $\pm$ 2 [*]	16 $\pm$ 4

^aCell cycle changes in MCF-10A cells following a 24 h treatment with 4-OHEN. Cells were harvested, stained with propidium iodide, and analyzed for DNA content by flow cytometry. Values are expressed as percentage of total cells and represent the mean $\pm$ SD of three determinations. Experimental details are described in Material and Methods.

* Significantly different from control values ($p < 0.05$).