

# Regulation of cell cycle and cyclins by 16 $\alpha$ -hydroxyestrone in MCF-7 breast cancer cells

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## ABSTRACT

It has been suggested that alterations in estradiol (E<sub>2</sub>) metabolism, resulting in increased production of 16 $\alpha$ -hydroxyestrone (16 $\alpha$ -OHE<sub>1</sub>), is associated with an increased risk of breast cancer. In the present study, we examined the effects of 16 $\alpha$ -OHE<sub>1</sub> on DNA synthesis, cell cycle progression, and the expression of cell cycle regulatory genes in MCF-7 breast cancer cells. G<sub>1</sub> synchronized cells were treated with 1 to 25 nM 16 $\alpha$ -OHE<sub>1</sub> for 24 and 48 h. [<sup>3</sup>H]Thymidine incorporation assay showed that 16 $\alpha$ -OHE<sub>1</sub> caused an 8-fold increase in DNA synthesis compared with that of control cells, whereas E<sub>2</sub> caused a 4-fold increase. Flow cytometric analysis of cell cycle progression also demonstrated the potency of 16 $\alpha$ -OHE<sub>1</sub> in stimulating cell growth. When G<sub>1</sub> synchronized cells were treated with 10 nM 16 $\alpha$ -OHE<sub>1</sub> for 24 h, 62  $\pm$  3% of cells were in S phase compared with 14  $\pm$  3% and 52  $\pm$  2% of cells in the control and E<sub>2</sub>-treated groups

respectively. In order to explore the role of 16 $\alpha$ -OHE<sub>1</sub> in cell cycle regulation, we examined its effects on cyclins (D1, E, A, B1), cyclin dependent kinases (Cdk4, Cdk2), and retinoblastoma protein (pRB) using Western and Northern blot analysis. Treatment of cells with 10 nM 16 $\alpha$ -OHE<sub>1</sub> resulted in 4- and 3-fold increases in cyclin D1 and cyclin A, respectively, at the protein level. There was also a significant increase in pRB phosphorylation and Cdk2 activation. In addition, transient transfection assay using an estrogen response element-driven luciferase reporter vector showed a 15-fold increase in estrogen receptor-mediated transactivation compared with control. These results show that 16 $\alpha$ -OHE<sub>1</sub> is a potent estrogen capable of accelerating cell cycle kinetics and stimulating the expression of cell cycle regulatory proteins.

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## INTRODUCTION

Estrogens are known to play an important role in the development and progression of breast cancer (McGuire *et al.* 1976, Henderson *et al.* 1988, Russo

& Russo 1998, Clemons & Goss 2001). The endogenous conversion of estradiol to its metabolites appears to contribute to the tumorigenic potential of estradiol (Schneider *et al.* 1982, Lippert *et al.* 2000). Estradiol (E<sub>2</sub>) is metabolized primarily

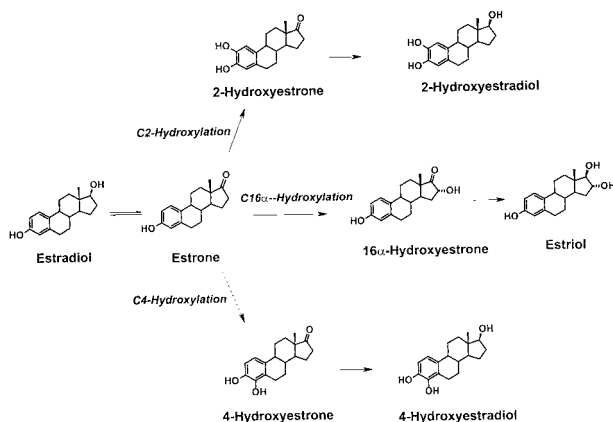


FIGURE 1. Pathways of endogenous estradiol (E<sub>2</sub>) metabolism. E<sub>2</sub> is metabolized by one of three pathways: C<sub>2</sub>-hydroxylation (major pathway), C<sub>16 $\alpha$</sub> -hydroxylation (major pathway), and C<sub>4</sub>-hydroxylation (minor pathway).

by hydroxylation at either the C-2 or the C-16 $\alpha$  position (Fig. 1). Hydroxylation at the C-2 position yields the catecholestrogens, 2-hydroxyestrone (2-OHE<sub>1</sub>) and 2-hydroxyestradiol (2-OHE<sub>2</sub>), while hydroxylation at the C-16 $\alpha$  position yields 16 $\alpha$ -hydroxyestrone (16 $\alpha$ -OHE<sub>1</sub>) and estriol (E<sub>3</sub>) (Ball & Knuppen 1980, Zhu & Conney 1998). The products of these two hydroxylation pathways exert markedly different biological properties: the 16 $\alpha$ -metabolites are active estrogens whereas the 2-hydroxy metabolites are not (Martucci & Fishman 1977, 1979, Fishman & Martucci 1980, Swaneck & Fishman 1988). Epidemiological and animal studies suggest that increased production of 2-hydroxyestrone confers a decreased risk for breast cancer, whereas elevated levels of 16 $\alpha$ -OHE<sub>1</sub> are associated with an increased risk for the disease (Schneider *et al.* 1982, Bradlow *et al.* 1985, Osborne *et al.* 1993, Meilahn *et al.* 1998).

Estradiol regulates target cell growth and differentiation through a sequence of events triggered by its binding to the intracellular estrogen receptors, ER $\alpha$  and ER $\beta$  (Greco *et al.* 1993, Tsai & O'Malley 1994, Jensen 1996, Gustafsson & Warner 2000, Katzenellenbogen *et al.* 2000). The ERs are members of the thyroid/vitamin D/retinoic acid nuclear receptor superfamily that function as ligand-activated transcription factors (Lin *et al.* 1998, Katzenellenbogen *et al.* 2000). ER $\alpha$  and ER $\beta$  have the potential to function as homo- or hetero-dimers, but their distribution varies according to tissue type; hence, the two receptors may function as homo- or hetero-dimers in different tissues (Gustafsson & Warner 2000). Following the binding of E<sub>2</sub>, ER dimerizes and binds

to specific DNA sequences called estrogen response elements (EREs) located in the upstream region of estrogen-responsive genes, regulating their transcriptional activity (Katzenellenbogen *et al.* 2000). The minimal consensus ERE sequence is a palindromic inverted repeat: 5'-GGTCAnnnTGACC-3', whereas many estrogen-regulated genes contain imperfect and non-palindromic EREs (Klein-Hitpass *et al.* 1988, Driscoll *et al.* 1998). 16 $\alpha$ -OHE<sub>1</sub> binds to ER $\alpha$  with low affinity (Fishman & Martucci 1980, Anstead *et al.* 1997); however, the effect of this binding on ER-mediated events associated with cell cycle progression has not been characterized.

Estradiol stimulates cell proliferation by increasing the expression of genes that regulate cell growth and cell cycle progression (Lippman *et al.* 1976, Altucci *et al.* 1996, Sutherland *et al.* 1998). The rate of progression of cells through the different phases of the cell cycle is controlled by a group of functionally related proteins, cyclins, cyclin-dependent kinases (CDKs) and their inhibitors (Hartwell & Kastan 1994, Hunter & Pines 1994, Pestell *et al.* 1999). The D-type cyclins (D1, D2, D3) in complex with their catalytic partners, Cdk4 and Cdk6, and cyclin E in complex with Cdk2 are the primary regulators of G<sub>1</sub> progression. Cyclin A in complex with Cdk2 is important for S phase progression and S $\rightarrow$ G<sub>2</sub> transition while cyclin B1 in complex with its catalytic subunit Cdk1/Cdc2 is essential for cellular entrance into G<sub>2</sub>/M phase and progression through mitosis. Estrogens, anti-estrogens, and other steroid hormones initiate their cell cycle effects in the G<sub>1</sub> phase (Taylor *et al.* 1983, Prall *et al.* 1998). Estradiol stimulates G<sub>1</sub> progression in MCF-7 cells via an up-regulation of cyclin D1 expression, increased activation of Cdk2/Cdk4, increased retinoblastoma protein (pRB) phosphorylation, and by decreasing the level of Cdk inhibitors such as p21 and p27 (Foster & Wimalasena 1996, Planas-Silva & Weinberg 1997, Prall *et al.* 1997).

We examined the effects of 16 $\alpha$ -OHE<sub>1</sub> on DNA synthesis, cell cycle kinetics, cyclin/CDK expression, and ER-mediated transactivation in MCF-7 breast cancer cells. Our results showed that 16 $\alpha$ -OHE<sub>1</sub> significantly enhanced DNA synthesis in MCF-7 cells and caused an accumulation of cells in the S phase of the cell cycle. The induction of cell cycle progression by 16 $\alpha$ -OHE<sub>1</sub> was associated with a significant increase in cyclin D1 and cyclin A expression (mRNA and protein), Cdk2 activation, and hyperphosphorylation of pRB. Transient transfection studies showed that 16 $\alpha$ -OHE<sub>1</sub> induced ER-mediated gene transcription, causing a 15-fold increase in the expression of an ERE-driven

luciferase gene. These results show that 16 $\alpha$ -OHE<sub>1</sub> is a potent estrogen with the ability to modulate the expression of genes with important roles in the regulation of breast cancer cell cycle progression.

## MATERIALS AND METHODS

### Chemicals, antibodies, and reagents

17 $\beta$ -Estradiol (E<sub>2</sub>) and 16 $\alpha$ -OHE<sub>1</sub> were purchased from Steraloids, Inc. (Wilton, NH, USA). Steroids were prepared in 100% ethanol (1 mM stock concentration) and stored at -20 °C. Monoclonal anti-human cyclin A (E23), anti-cyclin B1 (V152), anti-cyclin D1 (DCS-6), anti-cyclin E (HE12), anti-Cdk2 (2B6+8D4), and anti-Cdk4 (DCS-31+DCS-35) antibodies were purchased from Neomarkers (Union City, CA, USA). Monoclonal anti-pRB antibody (G3-245) was purchased from PharMingen (San Diego, CA, USA). Monoclonal anti- $\beta$ -actin antibody was purchased from Sigma Chemical Co. (St Louis, MO, USA). Horseradish peroxidase conjugated mouse anti-IgG secondary antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibiotics, trypsin, and other additives for cell culture medium were purchased from Gibco Laboratories (Grand Island, NY, USA). Dulbecco's modified Eagle's medium (DMEM), phenol red-free DMEM, isoleucine-free DMEM, and fetal bovine serum were obtained from either Gibco or Sigma. For Western blot detection, a SuperSignal Chemiluminescence reagent kit was purchased from Pierce (Rockford, IL, USA). Polyscreen PVDF Immobilon transfer membrane, multicolored protein markers, and reflection autoradiography film were purchased from Dupont/New England Nuclear (Boston, MA, USA). A protein assay kit was obtained from BIO-RAD (Richmond, CA, USA). The plasmids harboring human cyclin D1 and cyclin E were provided by Dr Steven Reed of the Scripps Research Institute (La Jolla, CA, USA) and cyclin A and cyclin B1 were gifts from Drs Jonathan Pines and Tony Hunter of the Salk Institute (La Jolla, CA, USA) (Lew *et al.* 1991, Thomas & Thomas 1994).

### Cell culture

MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in DMEM supplemented with 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 40  $\mu$ g/ml gentamicin, 2  $\mu$ g/ml insulin, 0.5 mM sodium pyruvate, 50 mM nonessential amino acids, 2 mM L-glutamine, and 10% fetal

bovine serum. Two weeks prior to each experiment, MCF-7 cells were grown in phenol red-free DMEM containing serum treated with dextran-coated charcoal (DCC) to remove estrogenic compounds (Berthois *et al.* 1986, Thomas *et al.* 1989, Shah *et al.* 1999).

### Thymidine incorporation assay and cell counting

MCF-7 cells ( $0.5 \times 10^6$ ) were seeded in 60-mm culture dishes in phenol red-free DMEM supplemented with 10% DCC-treated serum and additives. After 24 h of plating, cells were synchronized in G<sub>1</sub> phase by isoleucine starvation for 40 h. Cells were then allowed to progress through the cell cycle by a change of medium to that containing isoleucine. For each desired time point (24 and 48 h), triplicate plates were treated with 1, 4, 10, or 25 nM E<sub>2</sub> or 16 $\alpha$ -OHE<sub>1</sub>. Control cells received ethanol vehicle, which was maintained at less than 0.1%. DNA synthesis was measured by adding 4  $\mu$ Ci/ml [<sup>3</sup>H]thymidine to cells 1 h prior to the specified time points. After 1-h incubation at 37 °C, cells were washed twice with ice-cold PBS and ice-cold 5% trichloroacetic acid. The cell layer was then solubilized in 1 M NaOH and neutralized with 1 M HCl. The radioactive thymidine incorporated in cellular DNA was quantified by liquid scintillation counting (Hong *et al.* 1998).

For cell counting experiments, cells ( $0.25 \times 10^6$ ) were seeded in 100-mm culture dishes and after 24 h, cells were treated with 10 nM 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub>. Cells were then harvested in 1  $\times$  PBS on days 2, 4, 6, 8, and 10 and counted in a hemocytometer using the trypan blue exclusion.

### Cell cycle analysis/flow cytometry

MCF-7 cells ( $2 \times 10^6$ ) were seeded in 100 mm culture dishes and after 24 h of plating, cells were synchronized by isoleucine deprivation for 40 h. Cell cycle progression was induced by fresh medium and the addition of 10 nM E<sub>2</sub> or 16 $\alpha$ -OHE<sub>1</sub>. At the desired time points (0, 2, 4, 6, 8, 12, 16, 24, 30, 36, 48 h), triplicate plates from each treatment group were washed with PBS and covered with a buffer containing 40 mM sodium citrate, 250 mM sucrose, and 5% dimethylsulfoxide (DMSO) and stored at -70 °C. On the day of DNA analysis, cells were thawed and the citrate buffer removed. Cells were trypsinized for 10 min and then treated with a solution containing trypsin inhibitor and RNase (Sigma Chemical Co.) for 10 min. Cells were then stained by adding propidium iodide solution in sodium citrate buffer and

analyzed by a Coulter flow cytometer. The distribution of cells in the different phases of the cell cycle was calculated using cytologic software.

### Western blot analysis

Cell lysates were prepared according to procedures previously described (Thomas & Thomas 1994). Briefly, monolayers of MCF-7 cells ( $2 \times 10^6$ ) were washed twice with ice-cold PBS and lysed by addition of ice-cold lysis buffer (150 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 50 mM sodium fluoride, 0.2% SDS, 100 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride). After 30–60 min on ice, lysates were cleared of cellular debris by centrifugation (15 000 g) for 20 min at 4 °C and the supernatants were frozen at  $-20$  °C in aliquots. Thirty micrograms protein (determined by the Bradford protein assay) were diluted in 2X SDS-PAGE sample buffer (150 mM Tris base (pH 6.8), 30% glycerol, 4% SDS, 7.5 mM dithiothreitol (DTT), 0.01% bromophenol blue) and separated on 10% SDS-polyacrylamide gel. After electrophoresis, separated proteins were transferred to PVDF Polyscreen membranes. Membranes were incubated in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h to saturate the nonspecific binding sites. Membranes were then incubated with a 1:200 dilution of one of the following primary antibodies: cyclin D1, cyclin E, cyclin A, cyclin B1, RB, Cdk2 or Cdk4 overnight at 4 °C. Protein bands were visualized using horseradish peroxidase-conjugated secondary antibody with a chemiluminescence-based detection system. For molecular weight determinations, we used multicolored protein markers (NEN Life Science Products, Boston, MA, USA). To verify equal protein loading, membranes were stripped and reprobed with anti- $\beta$ -actin monoclonal antibody (1:5000). Intensity of protein bands was quantified using a Scanjet 4C flatbed scanner (Hewlett-Packard) with NIH Image v1.52 software. Lightly exposed films were used for quantification.

### Cdk2 kinase activity assay

For Cdk2 and cyclin E-Cdk2 kinase activity measurements, lysates were prepared as previously described (Prall *et al.* 1997). Briefly, cell monolayers were washed twice with ice-cold PBS and lysed by the addition of ice-cold lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 200  $\mu$ M sodium

orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF, and 1 mM DTT). After 15 min on ice, cellular debris was cleared by centrifugation at 20 000 g for 5 min at 4 °C. Equal amounts of lysates were precleared with protein A-Sepharose (1 h, 4 °C) and immunoprecipitated with an anti-Cdk2 monoclonal antibody (Neomarkers, Union City, CA, USA) or an anti-cyclin E monoclonal antibody (Neomarkers) overnight at 4 °C. After incubation with protein-A-Sepharose, the immunoprecipitates were washed three times with ice-cold lysis buffer and twice with ice-cold 50 mM HEPES (pH 7.5), 1 mM DTT. For the kinase reaction, the immunoprecipitates were suspended in 30  $\mu$ l kinase buffer (50 mM HEPES (pH 7.5), 1 mM DTT, 2.5 mM EGTA, 10 mM MgCl<sub>2</sub>, 20  $\mu$ M ATP, 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP, 0.1 mM orthovanadate, 1 mM NaF, 10 mM  $\beta$ -glycerophosphate) supplemented with either 3  $\mu$ g histone H1 (Cdk2 immunoprecipitates) or 10  $\mu$ g histone H1 (cyclin E immunoprecipitates) as a substrate for 30 min at room temperature. The reaction was terminated with the addition of 20  $\mu$ l of 3X SDS sample buffer (187 mM Tris-HCl (pH 6.8), 30% (v/v) glycerol, 6% SDS, 15% (v/v)  $\beta$ -mercaptoethanol). The samples were then boiled for 5 min and the reaction products separated using 12% SDS-PAGE, and the dried gel exposed to X-ray film. All data presented are representative of at least three separate experiments and the relative band intensities were quantified by densitometric analysis.

### Northern blot analysis

Total cellular RNA was extracted using TRIZOL according to the manufacturer's protocol (Gibco Laboratories, Long Island, NY, USA). G<sub>1</sub> synchronized cells ( $2 \times 10^6$ ) were treated with 10 nM E<sub>2</sub>, 16 $\alpha$ -OHE<sub>1</sub>, or ethanol vehicle (<0.1%) for specific time periods. The RNA solution was extracted with a 4:1 mixture of chloroform and isopropanol, precipitated with absolute ethanol, and the pellet was resuspended in diethyl pyrocarbonate-treated water. Total RNA (20  $\mu$ g/lane) was separated by 1% agarose gel electrophoresis under denaturing conditions and transferred onto a Sure Blot Hybridization membrane (Oncor, Gaithersburg, MD, USA) using the MilliBlot-V Transfer system according to the manufacturer's instructions (Sigma). The plasmids harboring human cyclin and cyclin A were cleaved by EcoRI, while those harboring cyclin D1 and cyclin B1 were cleaved by Not1 and BamH1 respectively. The inserts were purified from agarose gel by a freeze-thaw procedure. The DNA was recovered by ethanol precipitation and resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) at a concentration of 0.2  $\mu$ g/ $\mu$ l. The DNA



was labeled with [ $\alpha$ - $^{32}$ P]dCTP (3000 Ci/mmol; NEN, Boston, MA, USA) using a Prime-a-Gene labeling kit from Promega (Madison, WI, USA) and the labeled probes were purified using the STE MIDI SELECT-D G-50 Microcentrifuge Spin Columns from Eppendorf-5 Prime, Inc. (Boulder, CO, USA).

The RNA blots were hybridized with the following cDNA probes: human cyclin D1, cyclin E, cyclin A, and cyclin B1. The specific activities of the cDNA probes were in the range of  $1 \times 10^8$  c.p.m./ $\mu$ g DNA. Prehybridization, hybridization, and washing solutions were obtained from Molecular Research Center (Cincinnati, OH, USA). Prehybridization was for 2 h using Pre-Hyb solution. Hybridization was conducted in the High Efficiency Hybridization solution containing 50% formamide at 42 °C for 24 h, according to the manufacturer's protocol. Following hybridization, membranes were washed 3 times with washing solution at room temperature for 15 min and twice at 50 °C for 30 min (Thomas *et al.* 1989, Thomas & Thomas 1994). After washing, membranes were exposed to Kodak Biomax film for 24–48 h at –70 °C before development. To verify equal RNA loading, membranes were stripped and rehybridized with a  $^{32}$ P-labeled glyceraldehyde-3 phosphate dehydrogenase (GAPDH) probe from Clontech (La Jolla, CA, USA).

### Transient transfection assay

MCF-7 cells were grown in phenol red-free DMEM for 2 weeks prior to transfection experiments. Cells ( $5 \times 10^4$ ) were plated in 24-well culture dishes and allowed to adhere for 48 h. A 10:1 ratio of the pGL3–4(EREc38)-luciferase vector and pRLtk control vector were cotransfected using the calcium phosphate mammalian transfection kit, as recommended by the manufacturer (Promega). The pGL3–4(EREc38) vector contains the firefly luciferase reporter gene with 4 tandem copies of EREc38 sequence (Klinge *et al.* 1997) that induces transcription of the luciferase reporter upon ER binding. The pRL-TK control vector contains the *Renilla* luciferase gene under the control of thymidilate kinase promoter, and serves as a control for normalization of transfection efficiencies. For transfection, 4  $\mu$ g of the ERE plasmid were used per well. Twenty-four hours after transfection, cells were treated in triplicate with the indicated concentrations of  $E_2$  or 16 $\alpha$ -OHE $_1$ , and assayed for luciferase activity at 8 h using the dual luciferase reporter assay system, as recommended by the manufacturer (Promega). Luciferase activity was detected using a tube luminometer TD-20/20

(Turner Designs, Sunnyvale, CA, USA). Light signals were recorded as 10 s integrals. Reporter activity was normalized for each sample using the following equation:

$$\text{normalized luciferase activity} = \frac{\text{observed firefly luciferase activity}}{\text{Renilla luciferase activity}}$$

### Statistical analysis

Statistical significance of difference between control and treated samples was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test (GraphPad Prism Software program, San Diego, CA, USA).

## RESULTS

### Effects of 16 $\alpha$ -OHE $_1$ on DNA synthesis and growth of ER-positive MCF-7 cells

In the first set of experiments, we examined the effects of 16 $\alpha$ -OHE $_1$  and  $E_2$  on DNA synthesis in MCF-7 breast cancer cells using the [ $^3$ H]thymidine incorporation assay.  $G_1$  synchronized cells were released from cell cycle arrest by a change of medium and then treated with 1, 4, 10, and 25 nM of either 16 $\alpha$ -OHE $_1$  or  $E_2$  for 24 and 48 h, as described in the Materials and Methods section. As can be seen in Fig. 2A, treatment of MCF-7 cells with 16 $\alpha$ -OHE $_1$  resulted in a significant ( $P < 0.001$ ) concentration-dependent increase in DNA synthesis with a maximum 8-fold increase (above control) at 24 h and a 6-fold increase at 48 h. Similar experiments performed with  $E_2$  also showed a dose-dependent increase of DNA synthesis in MCF-7 cells; however,  $E_2$  was slightly less potent than 16 $\alpha$ -OHE $_1$  in stimulating DNA synthesis, causing a maximum 4.5-fold increase at these time points (Fig. 2B).

We also performed the [ $^3$ H]thymidine incorporation assay on an ER-negative breast cancer cell line, MDA-MB-468, to test whether the stimulatory effect of 16 $\alpha$ -OHE $_1$  was mediated by the ER. Our results showed that 16 $\alpha$ -OHE $_1$  and  $E_2$  did not enhance DNA synthesis in MDA-MB-468 cells at any of the concentrations examined (data not shown). This result indicates that the presence of endogenous ER is necessary for the growth stimulatory effect of 16 $\alpha$ -OHE $_1$  in MCF-7 cells.

We also examined the effects of  $E_2$  and 16 $\alpha$ -OHE $_1$  on cell number. MCF-7 cells ( $0.25 \times 10^6$ ) were treated with 10 nM  $E_2$  or 16 $\alpha$ -OHE $_1$ , and on days 2, 4, 6, 8, and 10. Cells were harvested and counted using a hemocytometer. Cells that were stained by trypan blue dye (dead cells) were excluded from the count. 16 $\alpha$ -OHE $_1$  caused a significant ( $P < 0.001$ )

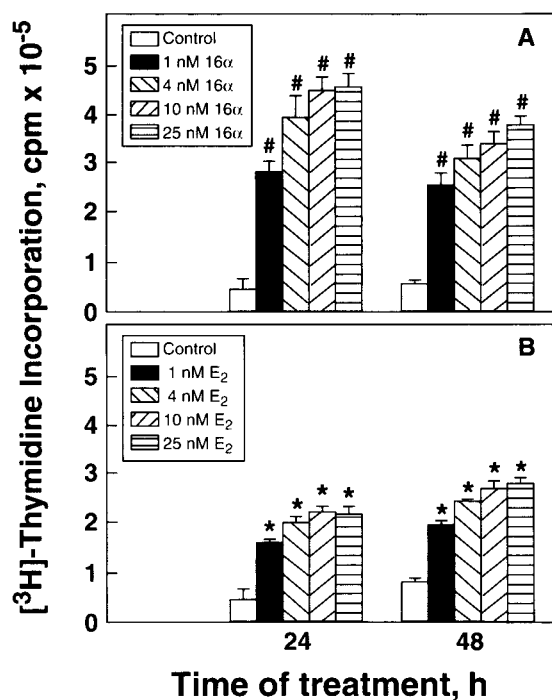


FIGURE 2. Thymidine incorporation assay of MCF-7 breast cancer cells in the presence of 16 $\alpha$ -OHE<sub>1</sub> and E<sub>2</sub>. G<sub>1</sub> synchronized MCF-7 cells ( $0.5 \times 10^6$ ) were treated with 1, 4, 10 or 25 nM 16 $\alpha$ -OHE<sub>1</sub> (A) or E<sub>2</sub> (B) for 24 and 48 h. One hour prior to the 24 and 48 h time points, cells were treated with 4  $\mu$ Ci [<sup>3</sup>H]thymidine and then processed according to procedures set out in the Materials and Methods section. Results shown are the means  $\pm$  s.e. based on three separate experiments, and the *P* values were determined using ANOVA followed by Dunnett's test. Values that are significantly different from control, \**P*<0.01 and #*P*<0.001, are indicated.

increase in cell numbers on days 6, 8, and 10 with the most dramatic increases observed on days 8 and 10 (Table 1). With 16 $\alpha$ -OHE<sub>1</sub> treatment, cell numbers increased  $\sim 6.3$ -fold above control by day 8 and 5.1-fold by day 10. In comparison, E<sub>2</sub> treatment caused a 5.1-fold increase by day 8 and a 4.1-fold increase by day 10. Thus, the effects of 16 $\alpha$ -OHE<sub>1</sub> and E<sub>2</sub> on DNA synthesis are reflected in cell growth.

#### Effects of 16 $\alpha$ -OHE<sub>1</sub> on cell cycle progression in MCF-7 cells

We next examined the effect of 16 $\alpha$ -OHE<sub>1</sub> on cell cycle progression in MCF-7 cells. G<sub>1</sub> synchronized cells were treated with 10 nM 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub>, and harvested at various time points (0 to 48 h). Cells were then stained with propidium iodide and

analyzed by flow cytometry. The time-course of changes in cell cycle phase distribution of cells treated with 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub> is shown in Fig. 3. Treatment of G<sub>1</sub> synchronized cells with 16 $\alpha$ -OHE<sub>1</sub> caused a significant decline (from 80 to 23%) in the proportion of cells in the G<sub>1</sub> phase and a concomitant 5-fold increase in the amount of S phase cells. At 24 h,  $62 \pm 3\%$  of 16 $\alpha$ -OHE<sub>1</sub>-treated cells were in the S phase compared with  $14 \pm 3\%$  of untreated cells (Fig. 3A and B). A significant increase (2-fold) in G<sub>2</sub>/M cells was also observed following 16 $\alpha$ -OHE<sub>1</sub> treatment (30–36 h) as compared with untreated cells. Similarly, treatment of cells with E<sub>2</sub> caused a 4-fold increase in the proportion of cells in S phase – from  $14 \pm 3\%$  to  $52 \pm 2\%$  at 24 h (Fig. 3C). E<sub>2</sub> treatment also caused an increase in the percentage of cells in G<sub>2</sub>/M phase (1.3-fold). This result shows that 16 $\alpha$ -OHE<sub>1</sub> is an effective inducer of G<sub>1</sub> to S phase transition in MCF-7 cells and suggests that the potent growth stimulatory effects of this compound may be due to its ability to modulate cell cycle progression.

#### Effects of 16 $\alpha$ -OHE<sub>1</sub> on cyclin and CDK expression

To investigate the possible mechanism by which 16 $\alpha$ -OHE<sub>1</sub> enhances G<sub>1</sub> to S phase progression, we examined its effect on cyclin D1, an important regulator of early G<sub>1</sub> progression. G<sub>1</sub> synchronized MCF-7 cells were treated with 10 nM 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub> and cells were harvested at various time points. Cell lysate was separated by 10% SDS-polyacrylamide gel, proteins were transferred to PVDF membranes, and the membranes were immunoblotted with anti-cyclin D1 monoclonal antibody.

Figure 4A shows a representative Western blot of cyclin D1 levels in MCF-7 cells following treatment with 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub> for 2, 4, 6, and 8 h. Treatment of cells with 10 nM 16 $\alpha$ -OHE<sub>1</sub> caused a significant time-dependent increase in cyclin D1 protein, with maximum induction ( $\sim 4$ -fold above control) observed between 4 and 8 h after the initiation of the cell cycle. E<sub>2</sub> treatment also produced a marked increase in cyclin D1 levels, with maximum induction (4-fold) observed at 6 and 8 h (Fig. 4A). Cyclin D1 levels remained significantly (*P*<0.01) elevated above control up to the 12-h time point after which there was a decline (data not shown). We also tested whether the effects of 16 $\alpha$ -OHE<sub>1</sub> on cyclin D1 protein expression could be blocked by the pure antiestrogen ICI 182,780. The addition of 100 nM ICI 182,780 slightly reduced 16 $\alpha$ -OHE<sub>1</sub>-mediated induction of cyclin D1 at 4 and 6 h (data not shown) but completely inhibited the induction at 8, 12, and 24 h (Fig. 4C), indicating

TABLE 1. Effects of E<sub>2</sub> and 16 $\alpha$ -OHE<sub>1</sub> on MCF-7 cell number. Data shown are the means  $\pm$  standard deviation from three separate experiments. Fold induction (shown in parentheses) was determined by dividing the total number of treated cells by the total number of untreated cells

Days of treatment	Cell number ( $\times 10^{-5}$ )		
	Control	E <sub>2</sub>	16 $\alpha$ -OHE <sub>1</sub>
2	5.3 $\pm$ 0.13	8.1 $\pm$ 0.14 (1.5) NS	7.3 $\pm$ 0.17 (1.4) NS
4	9.4 $\pm$ 0.19	24.1 $\pm$ 0.22 (2.6)*	22.8 $\pm$ 0.52 (2.3)*
6	11.3 $\pm$ 0.30	46.4 $\pm$ 0.53 (4.1)**	53.1 $\pm$ 0.54 (4.7)**
8	24.6 $\pm$ 0.29	126.7 $\pm$ 0.44 (5.1)**	156.1 $\pm$ 0.67 (6.3)** <sup>†</sup>
10	45.0 $\pm$ 0.39	168.0 $\pm$ 0.60 (4.1)**	244.0 $\pm$ 0.84 (5.0)** <sup>†</sup>

Cells were treated with 10 nM E<sub>2</sub> or 16 $\alpha$ -OHE<sub>1</sub> and at the indicated time points cells were harvested and counted in a hemocytometer. Control cells were treated with 0.1% ethanol vehicle. NS, not significantly different from control; \* $P < 0.05$ , \*\* $P < 0.001$  compared with control; <sup>†</sup> $P < 0.001$  compared with E<sub>2</sub>-treated samples.  $P$  values were determined by ANOVA followed by Dunnett's test.

a time lag in the suppressive effects of ICI 182,780 on 16 $\alpha$ -OHE<sub>1</sub>. However, we did not observe any change in cyclin E protein levels following 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub> treatment (data not shown).

Next, we examined the effects of 16 $\alpha$ -OHE<sub>1</sub> on cyclin A and cyclin B1, important regulators of S and G<sub>2</sub>/M phase progression. We found that treatment of cells with 10 nM 16 $\alpha$ -OHE<sub>1</sub> resulted in a time-dependent increase in cyclin A protein level, with a maximum 3-fold induction observed at 24 h (Fig. 4D). This increase in cyclin A correlated with the increase in DNA synthesis (Fig. 2A) and the accumulation of cells in S phase of the cell cycle as determined by flow cytometry (Fig. 3B). Interestingly, when similar experiments were performed with E<sub>2</sub>, a less than 25% increase in cyclin A was observed at comparable time points (Fig. 4D). 16 $\alpha$ -OHE<sub>1</sub> treatment caused a 1.4-fold increase in cyclin B1 protein at 16 h and a  $\sim$ 2-fold increase at 24 and 36 h (Fig. 5), a finding consistent with its established role as a regulator of G<sub>2</sub>/M progression. In contrast, E<sub>2</sub> treatment caused only a 20% increase at 16 h and a 30–40% increase at 24 h (Fig. 5).

The effects of 16 $\alpha$ -OHE<sub>1</sub> on expression of Cdk2, Cdk4, and Cdk1 were also investigated by Western blot analysis (data not shown). There were no major changes in the levels of Cdk2, Cdk4, or Cdk1 at any of the time points examined. We did, however, observe the presence of a fast migrating form of Cdk2 in 16 $\alpha$ -OHE<sub>1</sub>-treated cells at 12, 16, and 24 h of treatment (data not shown). This fast migrating form of Cdk2 has been described previously (Gu *et al.* 1992, Foster & Wimalasena 1996) and may represent the phosphorylated, active form of Cdk2.

### Effects of 16 $\alpha$ -OHE<sub>1</sub> on pRB phosphorylation

Retinoblastoma (pRB) is a tumor suppressor protein that inhibits progression through the G<sub>1</sub> phase of the cell cycle in its hypophosphorylated state by sequestering transcription factors, such as members of the E2F family (Harbour & Dean 2000). pRB phosphorylation is a critical step leading to S-phase commitment at the G<sub>1</sub> checkpoint of the cell cycle. We investigated the effects of 16 $\alpha$ -OHE<sub>1</sub> on pRB phosphorylation in G<sub>1</sub> arrested MCF-7 cells using Western blot analysis. In G<sub>1</sub> arrested cells (0 h time point), pRB was found primarily in the hypophosphorylated state. In cells allowed to progress in the cell cycle in the absence of 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub>, pRB remained primarily in the hypophosphorylated state up to 24 h (Fig. 6A). Treatment of cells with 16 $\alpha$ -OHE<sub>1</sub> resulted in a significant time-dependent increase in pRB phosphorylation, with initial induction observed at 2 h (Fig. 6C). E<sub>2</sub> treatment also resulted in a time-dependent increase in pRB phosphorylation with initial induction observed at 4 h and maximum induction at 16 to 24 h (Fig. 6B). These results show that 16 $\alpha$ -OHE<sub>1</sub> is capable of inducing pRB phosphorylation, and that this induction follows a time-course that is slightly different from that of E<sub>2</sub>.

### Effects of 16 $\alpha$ -OHE<sub>1</sub> on Cdk2 activity

Although E<sub>2</sub> and 16 $\alpha$ -OHE<sub>1</sub> did not change cyclin E protein levels, E<sub>2</sub> has been reported to alter Cdk2 activity. Therefore, we examined the effect of 16 $\alpha$ -OHE<sub>1</sub> on Cdk2 activity in MCF-7 cells. Cdk2 activity was measured by immune-complex kinase assays using histone H1 as the substrate (Fig. 7).

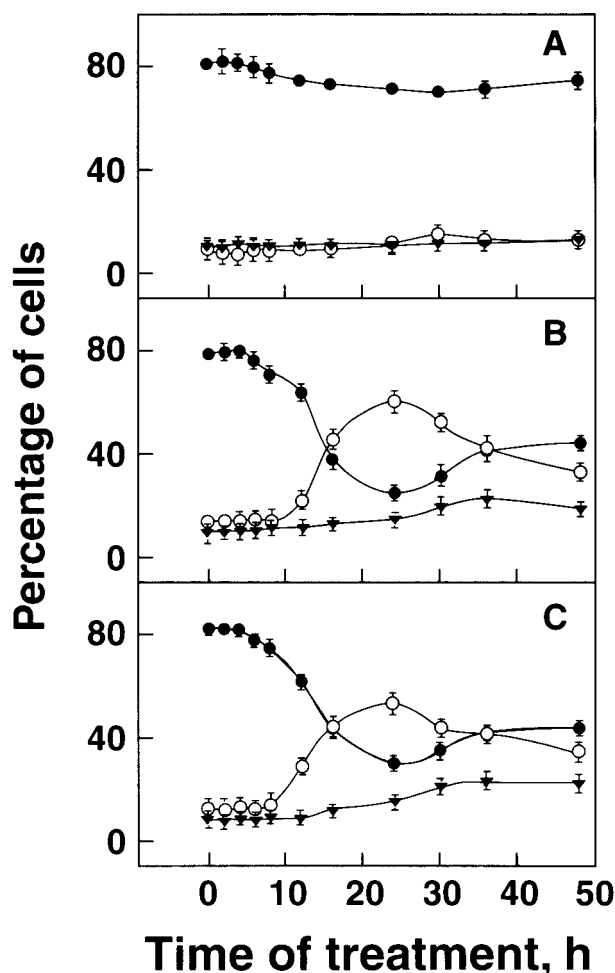


FIGURE 3. The effect of 16 $\alpha$ -OHE<sub>1</sub> and E<sub>2</sub> on cell cycle progression in MCF-7 cells. G<sub>1</sub> synchronized cells ( $2 \times 10^6$ ) were treated with <0.1% ethanol vehicle (A), 10 nM 16 $\alpha$ -OHE<sub>1</sub> (B), or 10 nM E<sub>2</sub> (C). At the indicated time intervals, cells were harvested and stained with propidium iodide for DNA content, and the percentage distribution of cells in G<sub>1</sub> (●), S (○), and G<sub>2</sub>/M (▼) phases of the cell cycle were determined using the cytologic software program. Values are the means  $\pm$  S.E. for three separate experiments performed in triplicate.

Treatment with 16 $\alpha$ -OHE<sub>1</sub> caused a significant time-dependent increase in Cdk2-associated kinase activity in MCF-7 cells, with maximum induction (20-fold above control) observed at 24 h (Fig. 7). This increase in total Cdk2 activity was  $\sim$ 1.5-fold higher than that observed with E<sub>2</sub> (data not shown) and it coincided with the increase in S phase (Fig. 3C) and cyclin A protein expression (Fig. 4D). To determine whether the effects of 16 $\alpha$ -OHE<sub>1</sub> on Cdk2 activity are mediated by the ER, cells were treated with 16 $\alpha$ -OHE<sub>1</sub> plus 100 nM ICI 182,780

and total Cdk2 kinase activity was measured at various time points (0, 2, 4, 6, 8, 12, and 24 h). ICI 182,780, when added together with 16 $\alpha$ -OHE<sub>1</sub>, significantly suppressed ( $P < 0.0001$ ) Cdk2 activity at all time points tested, with maximum inhibition (15-fold) observed at 12 and 24 h. (Fig. 7). This finding is consistent with an ER-mediated pathway for the activation of Cdk2 by 16 $\alpha$ -OHE<sub>1</sub>.

#### Effects of 16 $\alpha$ -OHE<sub>1</sub> and E<sub>2</sub> on cyclin D1, cyclin A, and cyclin B1 mRNA expression

Since the earliest and largest effect on G<sub>1</sub> cyclin protein expression following 16 $\alpha$ -OHE<sub>1</sub> treatment was that of cyclin D1, we further examined the effect of 16 $\alpha$ -OHE<sub>1</sub> on cyclin D1 mRNA level. In the presence of 16 $\alpha$ -OHE<sub>1</sub>, cyclin D1 mRNA level increased 3-fold at 6 h and 4-fold at 8 h (Fig. 8A) compared with controls. Similarly, E<sub>2</sub> treatment caused a 1.8-fold increase in cyclin D1 mRNA levels at 4 h and a maximal 4-fold increase at 8 h (Fig. 8A). To verify equal RNA loading, membranes were stripped and rehybridized with GAPDH cDNA probe. There was no significant difference in GAPDH mRNA level between untreated (control) and treated samples.

We also examined the effects of 16 $\alpha$ -OHE<sub>1</sub> on cyclin A and cyclin B1 mRNA levels (Fig. 8B and C). 16 $\alpha$ -OHE<sub>1</sub> stimulated cyclin A mRNA expression at 12, 16, 24, and 36 h. Densitometric scanning and normalization with GAPDH indicated  $\sim$ 2- to 3-fold increase (above control) in the intensity of the cyclin A mRNA band between 12 and 24 h of treatment. Treatment with E<sub>2</sub> also stimulated cyclin A mRNA with a maximum 2-fold increase at 24 h. Cyclin B1 mRNA levels remained relatively unchanged at the 12, 16, and 24 h time points; however, 16 $\alpha$ -OHE<sub>1</sub> caused a 40% increase in cyclin B1 mRNA levels at 36 h and E<sub>2</sub> caused a 20% increase (Fig. 8C). We did not observe any significant change in cyclin E mRNA levels at any of the time points examined (data not shown).

#### Effect of 16 $\alpha$ -OHE<sub>1</sub> on ERE-mediated gene transactivation

The transcription of a number of genes is under the control of EREs in the genome. Previous studies have shown that 16 $\alpha$ -OHE<sub>1</sub> binds the ER with lower affinity than that of E<sub>2</sub> (Swaneck & Fishman 1988). Since our studies suggested that the action of 16 $\alpha$ -OHE<sub>1</sub> on the molecular mechanisms controlling the cell cycle was mediated through the ER, we examined the ability of 16 $\alpha$ -OHE<sub>1</sub> to stimulate transcription of genes controlled by the ERE. We performed transient transfection experiments in



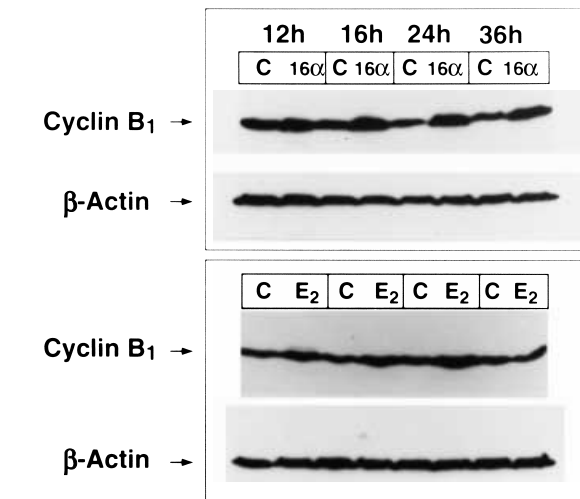
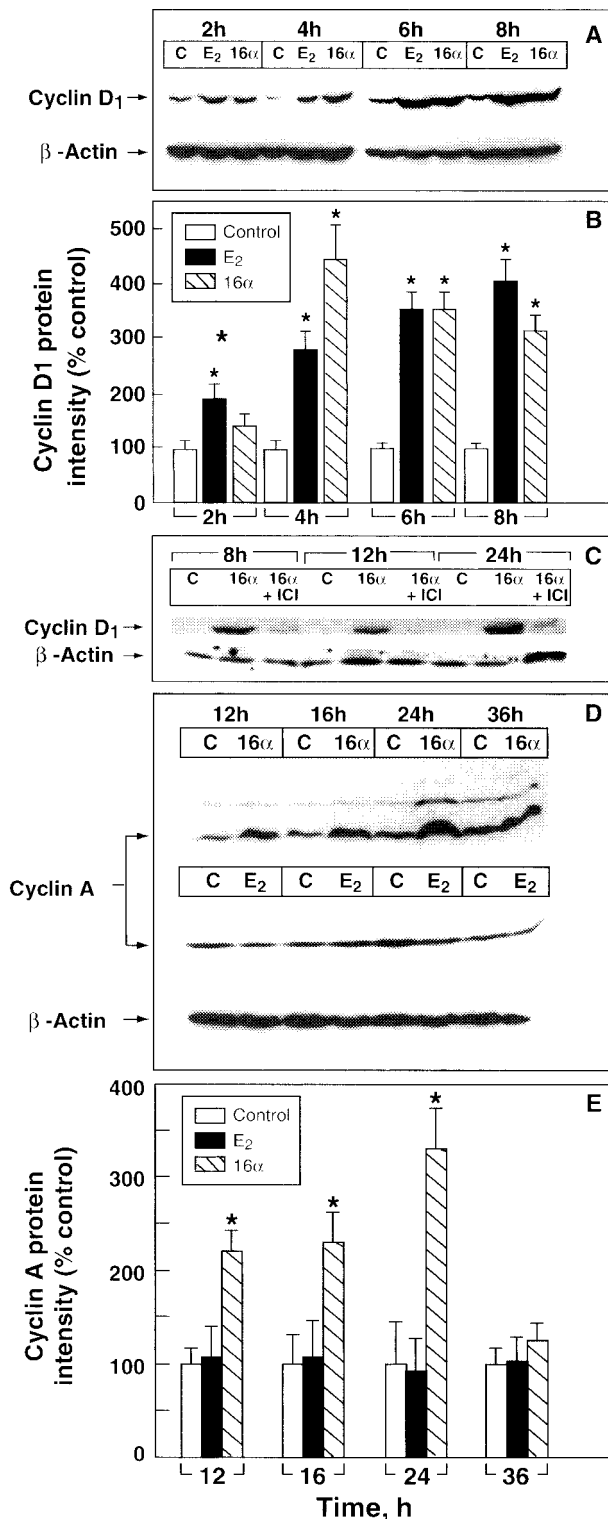


FIGURE 5. Effects of 16 $\alpha$ -OHE<sub>1</sub> on cyclin B1 protein expression in MCF-7 cells. G<sub>1</sub> synchronized cells were treated with <0.1% ethanol (control, C), 10 nM 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub> and at the indicated time points (12, 16, 24, and 36 h) cells were harvested and analyzed as described in Fig. 4. To verify equal protein loading, membranes were stripped and reprobbed with anti- $\beta$ -actin antibody. Results shown are representative of two separate experiments producing identical results.

MCF-7 cells using an ERE-driven luciferase reporter plasmid. A *Renilla* luciferase control plasmid was used to normalize for transfection efficiencies. As shown in Fig. 9A, treatment of cells with 1, 4, and 10 nM 16 $\alpha$ -OHE<sub>1</sub> resulted in a 10-, 15-, and 14-fold induction, respectively, in ER-mediated gene transcription at 6 h as compared with control. Treatment of cells with E<sub>2</sub> (at similar concentrations) caused a 10-, 13-, and 14-fold increase respectively. To determine the specificity of 16 $\alpha$ -OHE<sub>1</sub>/E<sub>2</sub> in activating ER-mediated gene

FIGURE 4. Effects of 16 $\alpha$ -OHE<sub>1</sub> and E<sub>2</sub> on cyclin D1 and cyclin A protein expression. G<sub>1</sub> synchronized MCF-7 cells were treated with <0.1% ethanol (control, C), 10 nM 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub>. Representative Western blots are shown for cyclin D1 (A) and cyclin A (D). To verify equal protein loading, membranes were stripped and reprobbed with anti- $\beta$ -actin antibody, and cyclin D1 and cyclin A protein bands were normalized to the density of the  $\beta$ -actin band. Quantification of Western blot intensity for cyclin D1 and cyclin A protein signals is shown in (B) and (E). (C) The effect of ICI 182,780 on cyclin D1 protein expression. Quantification of band intensities are based on the mean  $\pm$  s.e. for three separate experiments and the *P*-values were determined using ANOVA followed by Dunnet's test. \*Significantly different from control (*P*<0.01).

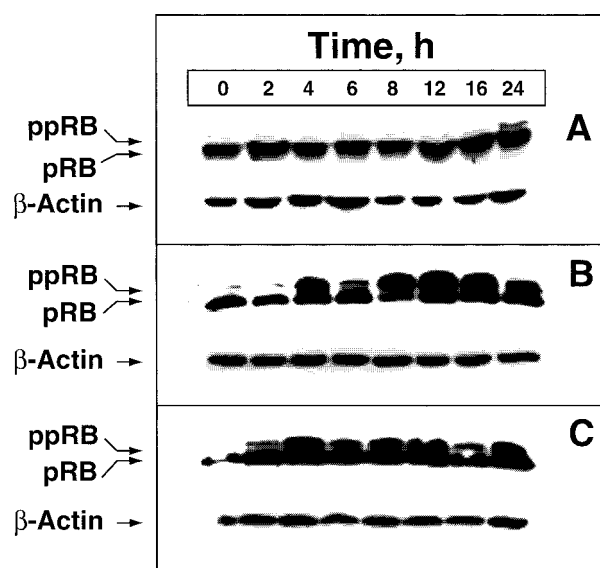


FIGURE 6. Effects of 16 $\alpha$ -OHE<sub>1</sub> and E<sub>2</sub> on pRB phosphorylation in MCF-7 cells. G<sub>1</sub> synchronized MCF-7 cells were treated with 10 nM 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub> and at the indicated time points (0, 2, 4, 6, 8, 12, 16 and 24 h) whole cell lysates were prepared, separated by 7.5% SDS-PAGE, transferred to PVDF membranes, and analyzed by Western blotting using anti-pRB monoclonal antibody. A representative Western blot is shown for pRB following treatment with (A) <0.1% ethanol vehicle (control), (B) E<sub>2</sub>, or (C) 16 $\alpha$ -OHE<sub>1</sub>. The upper band represents the hyperphosphorylated form of RB (ppRB) and the lower band represents the hypophosphorylated form (pRB). To verify equal protein loading, membranes were stripped and reprobed with anti- $\beta$ -actin antibody. Results shown are representative of three separate experiments.

transcription, we tested whether the pure antiestrogen ICI 182,780 could block this response. The addition of ICI 182,780 (100 nM) simultaneously with 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub>, completely blocked the estrogen-induced stimulation (Fig. 9B). These data indicate that ER is required for the effects of 16 $\alpha$ -OHE<sub>1</sub> on transcriptional activation, and thus support the hypothesis that the estrogenic action of this compound is mediated through ER.

## DISCUSSION

This study describes the effects of 16 $\alpha$ -hydroxyestrone on cell cycle regulation of estrogen-responsive MCF-7 breast cancer cells. Our results show that 16 $\alpha$ -OHE<sub>1</sub> causes a significant increase in DNA synthesis in MCF-7 cells and stimulates the progression of cells from G<sub>1</sub> to S phase of the cell

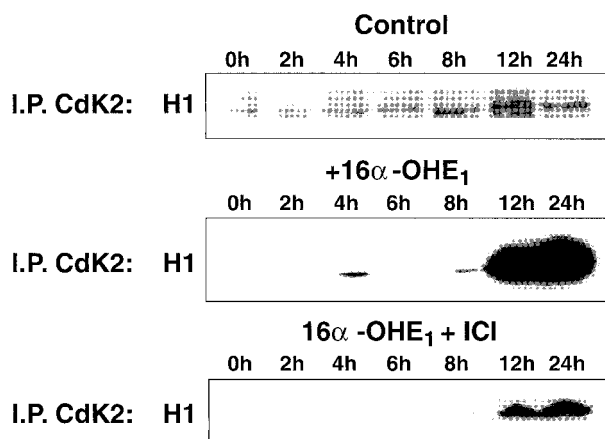


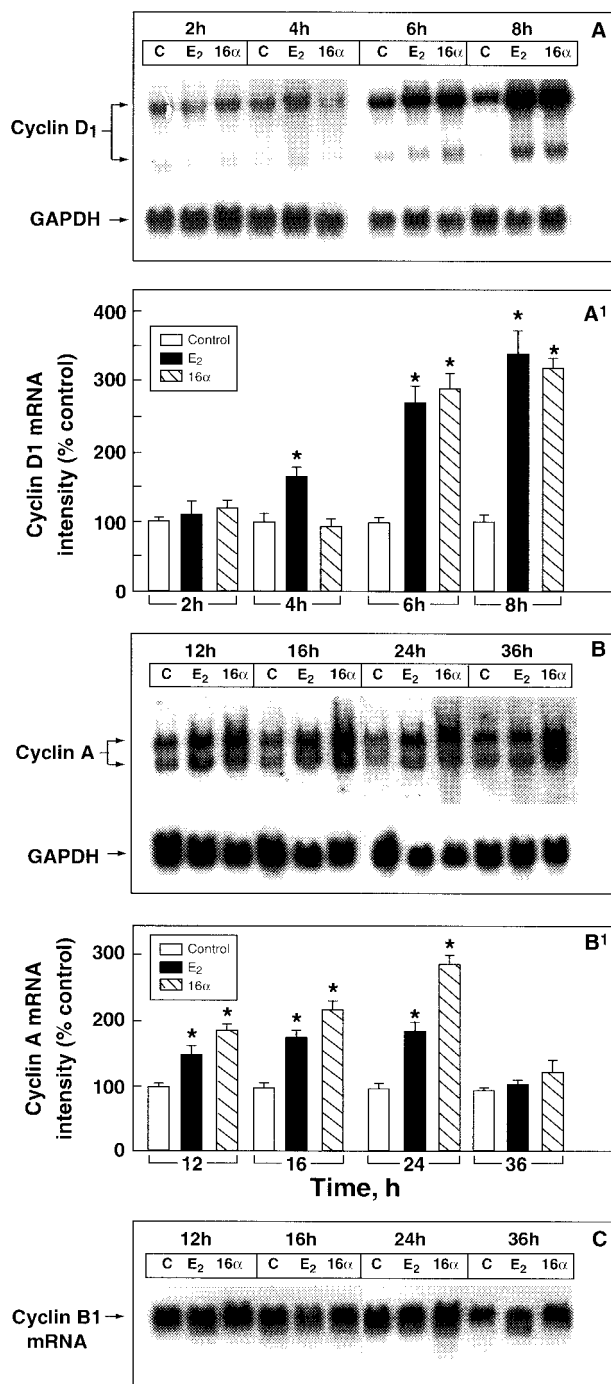
FIGURE 7. Effects of 16 $\alpha$ -OHE<sub>1</sub> on Cdk2 kinase activation in MCF-7 cells. Growth arrested cells were treated with 10 nM 16 $\alpha$ -OHE<sub>1</sub> or 10 nM 16 $\alpha$ -OHE<sub>1</sub> plus 100 nM ICI 182,780 at time 0, and at intervals thereafter whole cell lysates were prepared and analyzed for Cdk2-associated histone kinase activity as described in the Materials and Methods section. The top panel shows a representative time-course of Cdk2 activation in untreated cells (control). The middle panel shows Cdk2 activation following 16 $\alpha$ -OHE<sub>1</sub> treatment. The bottom panel shows Cdk2 activation following 16 $\alpha$ -OHE<sub>1</sub> and 100 nM ICI 182,780 (ICI) treatment. Phosphorylated histone H1 was separated by SDS-PAGE and detected by autoradiography. Results shown are representative of three separate experiments. I.P., immunoprecipitation.

cycle. Further analysis shows that the acceleration of G<sub>1</sub> to S phase transition is associated with increased expression of cyclin D1, cyclin A, Cdk2 activation, and pRB phosphorylation. Transient transfection using an ERE-driven luciferase vector shows that the mechanism of 16 $\alpha$ -OHE<sub>1</sub> action involves the estrogen receptor. To the best of our knowledge, this is the first study demonstrating growth stimulation and altered expression of cyclins by 16 $\alpha$ -OHE<sub>1</sub>.

16 $\alpha$ -Hydroxyestrone is found in the plasma in pg/ml levels and in bile and urine in  $\mu$ g/ml levels (Ikegawa *et al.* 1983, Naganuma *et al.* 1989). Several studies have shown that the 16 $\alpha$ -hydroxylation pathway (yielding 16 $\alpha$ -OHE<sub>1</sub> and estriol) is significantly elevated in mammary tissues of subjects at risk, and in patients with identifiable breast cancer (Schneider *et al.* 1982, Fishman *et al.* 1984). While the exact physiological function(s) of 16 $\alpha$ -OHE<sub>1</sub> is not known, this metabolite exhibits estrogenicity comparable to that of E<sub>2</sub> (as measured by stimulation of uterine growth). 16 $\alpha$ -OHE<sub>1</sub> is known to have low binding affinity to sex hormone-binding globulin (Fishman & Martucci 1980) and it binds

irreversibly to the estrogen receptor and up-regulates c-myc oncogene expression (Swanek & Fishman 1988).

It is well known that basal and mitogen-induced cell growth is regulated by multiple proteins that control cell cycle progression. The effects of E<sub>2</sub> on



cell cycle kinetics in ER-positive breast cancer cell lines have been studied by several investigators (Sutherland *et al.* 1983, Foster & Wimalasena 1996, Planas-Silva & Weinberg 1997, Prall *et al.* 1997, Foster *et al.* 2001). Rapid induction of cyclin D1 protein is one of the early events associated with E<sub>2</sub>-induced G<sub>1</sub> progression. Foster & Wimalasena (1996) reported that the synthesis of cyclin D1 protein increased within 3 h after E<sub>2</sub> treatment of growth arrested MCF-7 cells and peaked at 6 h, while Cdk4-associated RB kinase activity was evident within 6 h after E<sub>2</sub> treatment. In our study, cyclin D1 protein level increased within the first 2 to 4 h of E<sub>2</sub> treatment and peaked at 6 h which is consistent with previous findings. We found that 16 $\alpha$ -OHE<sub>1</sub> emulated (to some extent) the effects of E<sub>2</sub> on cyclin D1 protein expression with initial induction occurring at 4 h. The effects of 16 $\alpha$ -OHE<sub>1</sub> on cyclin D1 were inhibited by the pure antiestrogen ICI 182,780. Although cyclin D1 induction was detected at both mRNA and protein levels, data on 16 $\alpha$ -OHE<sub>1</sub> showed protein levels to be induced at earlier time points than the mRNA (Figs 4A and 8A). The reason for this disparity is not known at present; however, in a previous study by Muise-Helmericks *et al.* (1998), cyclin D1 mRNA levels were shown to increase several hours after the increase in cyclin D1 protein expression. This phenomenon, demonstrated in MCF10A cells, was serum induced and involved a phosphatidylinositol 3-kinase mediated increase in the rate of cyclin D1 protein synthesis. In MCF-7 cells, serum factors

FIGURE 8. Effects of 16 $\alpha$ -OHE<sub>1</sub> and E<sub>2</sub> on cyclin D1, cyclin A, and cyclin B1 mRNA expression in MCF-7 cells. G<sub>1</sub> synchronized cells were treated with 10 nM 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub> and at the indicated time points (2, 4, 6, and 8 h) total cellular RNA was harvested in TRI-Reagent. Fifteen micrograms of total RNA were resolved by formaldehyde gel electrophoresis, transferred to a nylon membrane, and hybridized with cyclin D1, cyclin A, or cyclin B1 cDNA probes, as described in the Materials and Methods section. Representative Northern blots are shown for cyclin D1 (panel A), cyclin A (panel B), and cyclin B1 mRNA (panel C) from 16 $\alpha$ -OHE<sub>1</sub> and E<sub>2</sub>-treated cells. The control cells (C) were treated with <0.1% ethanol vehicle. To verify equal RNA loading, membranes were stripped and rehybridized with the GAPDH probe and the corresponding blot is also shown. Cyclin D1, cyclin A, and cyclin B1 band densities were normalized to the density of the GAPDH band. Quantification of cyclin D1 and cyclin A mRNA signals is shown in panels A<sup>1</sup> and B<sup>1</sup>. Error bars represent S.E. for three separate experiments. \*Significantly different from control ( $P < 0.01$ ).

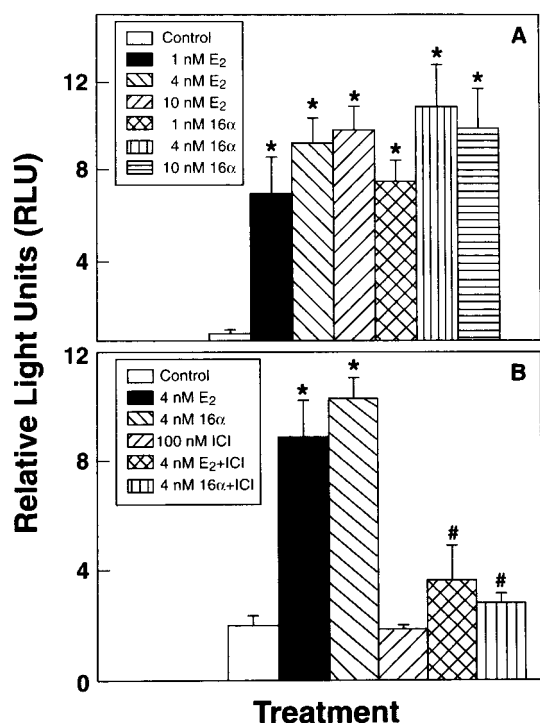


FIGURE 9. (A) Effects of 16 $\alpha$ -OHE<sub>1</sub> and E<sub>2</sub> on ER-mediated transcriptional activation of luciferase reporter gene. Plasmids, pGL3-4(ERE38) and pRL-TK control vector, were cotransfected in MCF-7 cells using the calcium phosphate precipitation procedure as described in the Materials and Methods section. Following transfection, cells were allowed to grow for 48 h and then treated with (A) 1, 4, and 10 nM 16 $\alpha$ -OHE<sub>1</sub> (16 $\alpha$ ) or E<sub>2</sub>, or (B) 100 nM ICI 182,780 (ICI) alone or in combination with 4 nM 16 $\alpha$ -OHE<sub>1</sub> or E<sub>2</sub>. After 8 h, cells were harvested and luciferase activities were measured. Luciferase activity is expressed as relative light units (RLU) and was normalized for each sample by dividing the firefly luciferase activity by the *Renilla* luciferase activity. Error bars represent average  $\pm$  s.e. from three separate experiments performed in triplicate. Statistical significance was evaluated by ANOVA followed by Dunnet's test. All 16 $\alpha$ -OHE<sub>1</sub> and E<sub>2</sub> doses produced significantly more RLU than controls (\* $P$ <0.002). Treatment with ICI 182,780 significantly reduced 16 $\alpha$ -OHE<sub>1</sub>- and E<sub>2</sub>-induced luciferase gene activity from their corresponding values (# $P$ <0.01).

such as insulin-like growth factor-I (IGF-I) and E<sub>2</sub> are known to have a synergistic growth stimulatory effect (Lee *et al.* 1999). Thus, it is possible that under the conditions of our experiments, a combination of transcriptional and translational regulation of cyclin D1 might be occurring. The lag-time in reducing cyclin D1 levels by ICI 182,780 may also be related to multiple pathways for the regulation of this protein.

In current models of G<sub>1</sub> control, the expression of activated Cdk4/cyclin D1 complex is thought to be critical for the early increases in pRB phosphorylation while activated Cdk2/cyclin E/A complex is required for late stage pRB phosphorylation and G<sub>1</sub> to S transition (Morgan 1997, Dictor *et al.* 1999, Harbour & Dean 2000). In the present study, a significant increase in hyperphosphorylated pRB was detected as early as 4 h after 16 $\alpha$ -OHE<sub>1</sub> treatment, with maximum phosphorylation occurring at 24 h (Fig. 6C). It is possible that the accumulation of cyclin D1 protein observed 4 h after treatment may have led to an increase in cyclin D1/Cdk4 complex formation and thus activation of Cdk4 kinase activity. While Cdk4 activity was not measured directly in the present study, we did measure Cdk2 and cyclin E-associated Cdk2 activities. 16 $\alpha$ -OHE<sub>1</sub> caused a significant time-dependent increase in Cdk2 activity with maximum induction observed during the entry of cells to the S phase (Fig. 7). A significant increase in cyclin E/Cdk2 activity was also observed following 16 $\alpha$ -OHE<sub>1</sub> and E<sub>2</sub> treatment (data not shown). Cdk2 is known to be activated by its association with both cyclin E and cyclin A (Prall *et al.* 1997, Foster *et al.* 2001). While we did not detect any significant changes in cyclin E protein expression following 16 $\alpha$ -OHE<sub>1</sub> treatment, we detected a significant increase (3-fold above control) in cyclin A protein at 12, 16, and 24 h (Fig. 4D), coinciding with the increase in Cdk2 activity and the accumulation of cells in S phase of the cell cycle. In addition, a phosphorylated fast migrating form of Cdk2 was observed following 16 $\alpha$ -OHE<sub>1</sub> treatment. Phosphorylation of Cdk2 on Thr-160, a target of CDK-activating kinase (CAK), is known to increase cyclin E/Cdk2 activity (Gu *et al.* 1992, Chiariello *et al.* 2000). Other pathways for the enhanced activation of cyclin E/Cdk2 complex may involve the re-distribution of p21 and p27 (Planas-Silva & Weinberg 1997, Prall *et al.* 1997). Both p21 and p27 have been shown to prevent CAK-mediated phosphorylation of Thr-160 on Cdk2 (Aprelikova *et al.* 1995); therefore, their absence from the cyclin E/Cdk2 complexes would allow for CAK-mediated phosphorylation of Cdk2 and activation. It is possible that these multiple pathways contribute to the robust activation of Cdk2 and phosphorylation of RB in the present study.

The importance of cyclin D1 in the regulation of breast cancer cell growth is substantiated by the observation that the cyclin D1 gene (PRAD1) is amplified or overexpressed in a number of primary breast cancers and in tumor-derived cell lines (Bartkova *et al.* 1995). Our results (Fig. 8A) and those from other investigators (Altucci *et al.* 1996,



Musgrove *et al.* 1996) suggest that a major part of estrogenic regulation of cyclin D1 gene seems to be at the transcriptional level. An estrogen-responsive region was previously identified within the first 944 base pairs upstream of the cyclin D1 transcriptional start site (Altucci *et al.* 1996). More recently, Sabbah *et al.* (1999) identified a putative cAMP response element in the proximal promoter of cyclin D1 which is activated by estradiol and inhibited by the antiestrogen ICI 182,780. This required both the AF-1 and AF-2 domains of the estrogen receptor but not direct DNA binding (Sabbah *et al.* 1999). In the classical model of E<sub>2</sub>-induced regulation of responsive genes, estradiol binds to ER and causes conformational changes, thus allowing the receptor to dimerize and bind to the estrogen response element. The promoter region of the cyclin D1 gene that is responsive to estrogen, however, does not contain a classical estrogen response element. It contains an AP-1 site and potential recognition sequences for several transcription factors, including Sp1, E2F, Myc, activating transcription factor/cAMP response element and nuclear factor  $\kappa$ B (Herber *et al.* 1994, Guttridge *et al.* 1999, Sabbah *et al.* 1999). Recently, it has been shown that ER physically interacts with the AP-1 heterodimeric proteins c-Fos/c-Jun at their cognate AP-1 site in the presence of E<sub>2</sub> (Webb *et al.* 1995, Paech *et al.* 1997). ER also interacts with the nuclear transcription factor Sp1, thus enhancing Sp1-driven gene transcription in the absence of ER $\alpha$ -DNA binding (Paech *et al.* 1997, Wang *et al.* 1999). Thus, a potential mechanism of 16 $\alpha$ -OHE<sub>1</sub> induction of cyclin D1 gene may involve interaction between ER and AP-1/Sp1 proteins rather than ER interaction with its cognate ERE.

In summary, our results demonstrate that 16 $\alpha$ -OHE<sub>1</sub> is a potent stimulator of DNA synthesis in ER-positive breast cancer cells and it enhances cell cycle progression by modulating the expression and activities of several cell cycle regulatory proteins. 16 $\alpha$ -OHE<sub>1</sub> increased cyclin D1 expression (protein and mRNA) and pRB phosphorylation, similar to the effects observed with E<sub>2</sub>. 16 $\alpha$ -OHE<sub>1</sub> also caused significant increases in cyclin A expression and Cdk2 and cyclin E/Cdk2 activities. The ability of 16 $\alpha$ -OHE<sub>1</sub> to alter the expression of cyclins provides a possible mechanism for the growth stimulatory effect of this compound in breast cancer cells. Our results, in conjunction with the reported ability of 16 $\alpha$ -OHE<sub>1</sub> to form adducts with DNA and proteins (Liehr 1998), give credence to the hypothesis that elevated levels of 16 $\alpha$ -OHE<sub>1</sub> may play an important role in the development and/or progression of breast cancer.

## ACKNOWLEDGEMENTS

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