Regulation of cell cycle and cyclins by 16α-hydroxyestrone in MCF-7 breast cancer cells

J S Lewis1,2,5, T J Thomas1,6, C M Klinge4, M A Gallo2,3,6 and T Thomas2,3,6

1Department of Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, New Jersey 08903, USA
2Department of Environmental and Community Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, New Jersey 08903, USA
3Environmental and Occupational Health Sciences Institute, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854, USA
4Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, Kentucky 40292, USA
5Nutritional Sciences Graduate Program, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08903, USA
6The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, New Brunswick, New Jersey 08903, USA

(Requests for offprints should be addressed to T Thomas, Clinical Academic Building, Room 7090, UMDNJ-Robert Wood Johnson Medical School, 125 Paterson Street, New Brunswick, New Jersey 08903, USA; Email: thomasth@UMDNJ.edu)

ABSTRACT

It has been suggested that alterations in estradiol (E2) metabolism, resulting in increased production of 16α-hydroxyestrone (16α-OHE1), is associated with an increased risk of breast cancer. In the present study, we examined the effects of 16α-OHE1 on DNA synthesis, cell cycle progression, and the expression of cell cycle regulatory genes in MCF-7 breast cancer cells. G1 synchronized cells were treated with 1 to 25 nM 16α-OHE1 for 24 and 48 h. [3H]Thymidine incorporation assay showed that 16α-OHE1 caused an 8-fold increase in DNA synthesis compared with that of control cells, whereas E2 caused a 4-fold increase. Flow cytometric analysis of cell cycle progression also demonstrated the potency of 16α-OHE1 in stimulating cell growth. When G1 synchronized cells were treated with 10 nM 16α-OHE1 for 24 h, 62 ± 3% of cells were in S phase compared with 14 ± 3% and 52 ± 2% of cells in the control and E2-treated groups, respectively. In order to explore the role of 16α-OHE1 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 16α-OHE1 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 16α-OHE1 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 (E2) is metabolized primarily...
by hydroxylation at either the C-2 or the C-16α position (Fig. 1). Hydroxylation at the C-2 position yields the catecholestrogens, 2-hydroxyestrone (2-OHE1) and 2-hydroxyestradiol (2-OHE2), while hydroxylation at the C-16α position yields 16α-hydroxyestrone (16α-OHE1) and estriol (E3) (Ball & Conney 1998). The products of these two hydroxylation pathways exert markedly different biological properties: the 16α-metabolites are active estrogens whereas the 2-hydroxy metabolites are not (Martucci & Fishman 1977, 1979, Fishman & Martucci 1980, Swanek & Fishman 1988). Epidemiological and animal studies suggest that increased production of 2-hydroxyestrone confers a decreased risk for breast cancer, whereas elevated levels of 16α-OHE1 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, ERα and ERβ (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). ERα and ERβ 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 E2, 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). 16α-OHE1 binds to ERα 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 G1 progression. Cyclin A in complex with Cdk2 is important for S phase progression and S→G2 transition while cyclin B1 in complex with its catalytic subunit Cdk1/Cdc2 is essential for cellular entrance into G2/M phase and progression through mitosis. Estrogens, anti-estrogens, and other steroid hormones initiate their cell cycle effects in the G1 phase (Taylor et al. 1983, Prall et al. 1998). Estradiol stimulates G1 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 & Wimalesena 1996, Planas-Silva & Weinberg 1997, Prall et al. 1997).

We examined the effects of 16α-OHE1 on DNA synthesis, cell cycle kinetics, cyclin/CDK expression, and ER-mediated transactivation in MCF-7 breast cancer cells. Our results showed that 16α-OHE1 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 16α-OHE1 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 16α-OHE1 induced ER-mediated gene transcription, causing a 15-fold increase in the expression of an ER-driven

![Figure 1](https://www.endocrinology.org/downloadedfrombiorxiv.com.at/06/14/20222036.53PM)

**FIGURE 1.** Pathways of endogenous estradiol (E2) metabolism. E2 is metabolized by one of three pathways: C2-hydroxylation (major pathway), C16α-hydroxylation (major pathway), and C4-hydroxylation (minor pathway).
luciferase gene. These results show that 16α-OHE₁ 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

17β-Estradiol (E₂) and 16α-OHE₁ 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) was purchased 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. 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 × 10⁶) 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 G₁ 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 E₂ or 16α-OHE₁. Control cells received ethanol vehicle, which was maintained at less than 0·1%. DNA synthesis was measured by adding 4 microCi/ml [³H]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 × 10⁶) were seeded in 100-mm culture dishes and after 24 h, cells were treated with 10 nM 16α-OHE₁ or E₂. Cells were then harvested in 1 × 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 × 10⁶) 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 E₂ or 16α-OHE₁. 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–HCl–EDTA buffer (150 mM Tris–HCl (pH 6.8), 30% glycerol, 6% SDS, 15% (v/v) 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 horse-radish 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 an anti-α-tubulin 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 MgCl2, 1 mM EGTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 200 µM 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 µl kinase buffer (50 mM HEPES (pH 7.5), 1 mM DTT, 2.5 mM EGTA, 10 mM MgCl2, 20 µM ATP, 10 µCi \([\gamma-32P]ATP, 0.1-1\) mM orthovanadate, 1 mM NaF, 10 µM β-glycerophosphate) supplemented with either 3 µg histone H1 (Cdk2 immunoprecipitates) or 10 µg histone H1 (cyclin E immunoprecipitates) as a substrate for 30 min at room temperature. The reaction was terminated with the addition of 20 µl of 3X SDS sample buffer (187 mM Tris–HCl (pH 6.8), 30% (v/v) glycerol, 6% SDS, 15% (v/v) β-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). \(G_1\) synchronized cells (\(2 \times 10^6\)) were treated with 10 nM E2, 16α-OHE1, 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 µg/lane) was separated by 1% agarose gel electrophoresis under denaturing conditions and transferred onto a Sure Blot Hybridization membrane (Oncor, Gaithesburg, MD, USA) or an anti-cyclin E 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 µl kinase buffer (50 mM HEPES (pH 7.5), 1 mM DTT, 2.5 mM EGTA, 10 mM MgCl2, 20 µM ATP, 10 µCi \([\gamma-32P]ATP, 0.1-1\) mM orthovanadate, 1 mM NaF, 10 µM β-glycerophosphate) supplemented with either 3 µg histone H1 (Cdk2 immunoprecipitates) or 10 µg histone H1 (cyclin E immunoprecipitates) as a substrate for 30 min at room temperature. The reaction was terminated with the addition of 20 µl of 3X SDS sample buffer (187 mM Tris–HCl (pH 6.8), 30% (v/v) glycerol, 6% SDS, 15% (v/v) β-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.
was labeled with \([\alpha\cdot^{32}\text{P}]\text{dCTP}\) (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 \(^\circ\)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 \(^\circ\)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 \(^\circ\)C before development. To verify equal RNA loading, membranes were stripped and rehybridized with a \(^{32}\text{P}\)-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 (\(5 \times 10^5\)) 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 E2 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 Dunnet'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 E2 on DNA synthesis in MCF-7 breast cancer cells using the \([\text{H}]\text{thymidine}\) incorporation assay. G1 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 E2 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 E2 also showed a dose-dependent increase of DNA synthesis in MCF-7 cells; however, E2 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 \([\text{H}]\text{thymidine}\) 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 E2 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 E2 and 16\(\alpha\)-OHE\(_1\) on cell number. MCF-7 cells (\(0-25 \times 10^6\)) were treated with 10 nM E2 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 (\(P<0.001\))
increase in cell numbers on days 6, 8, and 10 with the most dramatic increases observed on days 8 and 10 (Table 1). With 16α-OHE1 treatment, cell numbers increased ~6.3-fold above control by day 8 and 5.1-fold by day 10. In comparison, E2 treatment caused a 5.1-fold increase by day 8 and a 4.1-fold increase by day 10. Thus, the effects of 16α-OHE1 and E2 on DNA synthesis are reflected in cell growth.

**Effects of 16α-OHE1 on cell cycle progression in MCF-7 cells**

We next examined the effect of 16α-OHE1 on cell cycle progression in MCF-7 cells. G1 synchronized cells were treated with 10 nM 16α-OHE1 or E2, 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 16α-OHE1 or E2 is shown in Fig. 3. Treatment of G1 synchronized cells with 16α-OHE1 caused a significant decline (from 80 to 23%) in the proportion of cells in the G1 phase and a concomitant 5-fold increase in the amount of S phase cells. At 24 h, 62 ± 3% of 16α-OHE1-treated cells were in the S phase compared with 14 ± 3% of untreated cells (Fig. 3A and B). A significant increase (2-fold) in G2/M cells was also observed following 16α-OHE1 treatment (30–36 h) as compared with untreated cells. Similarly, treatment of cells with E2 caused a 4-fold increase in the proportion of cells in S phase – from 14 ± 3% to 52 ± 2% at 24 h (Fig. 3C). E2 treatment also caused an increase in the percentage of cells in G2/M phase (1.3-fold). This result shows that 16α-OHE1 is an effective inducer of G1 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 16α-OHE1 on cyclin and CDK expression**

To investigate the possible mechanism by which 16α-OHE1 enhances G1 to S phase progression, we examined its effect on cyclin D1, an important regulator of early G1 progression. G1 synchronized MCF-7 cells were treated with 10 nM 16α-OHE1 or E2 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 16α-OHE1 or E2 for 2, 4, 6, and 8 h. Treatment of cells with 10 nM 16α-OHE1 caused a significant time-dependent increase in cyclin D1 protein, with maximum induction (~4-fold above control) observed between 4 and 8 h after the initiation of the cell cycle. E2 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 16α-OHE1 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 16α-OHE1-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
a time lag in the suppressive effects of ICI 182,780 on 16a-OHE1. However, we did not observe any change in cyclin E protein levels following 16α-OHE1 or E2 treatment (data not shown).

Next, we examined the effects of 16α-OHE1 on cyclin A and cyclin B1, important regulators of S and G2/M phase progression. We found that treatment of cells with 10 nM 16α-OHE1 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 E2, a less than 25% increase in cyclin A was observed at comparable time points (Fig. 4D). 16α-OHE1 treatment caused a 1.4-fold increase in cyclin B1 protein at 16 h and a ~2-fold increase at 24 and 36 h (Fig. 5), a finding consistent with its established role as a regulator of G2/M progression. In contrast, E2 treatment caused only a 20% increase at 16 h and a 30–40% increase at 24 h (Fig. 5).

The effects of 16α-OHE1 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 16α-OHE1-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 16α-OHE1 on pRB phosphorylation**

Retinoblastoma (pRB) is a tumor suppressor protein that inhibits progression through the G1 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 G1 checkpoint of the cell cycle. We investigated the effects of 16α-OHE1 on pRB phosphorylation in G1 arrested MCF-7 cells using Western blot analysis. In G1 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 16α-OHE1 or E2, pRB remained primarily in the hypophosphorylated state up to 24 h (Fig. 6A). Treatment of cells with 16α-OHE1 resulted in a significant time-dependent increase in pRB phosphorylation, with initial induction observed at 2 h (Fig. 6C). E2 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 16α-OHE1 is capable of inducing pRB phosphorylation, and that this induction follows a time-course that is slightly different from that of E2.

**Effects of 16α-OHE1 on Cdk2 activity**

Although E2 and 16α-OHE1 did not change cyclin E protein levels, E2 has been reported to alter Cdk2 activity. Therefore, we examined the effect of 16α-OHE1 on Cdk2 activity in MCF-7 cells. Cdk2 activity was measured by immune-complex kinase assays using histone H1 as the substrate (Fig. 7).

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**Table 1. Effects of E2 and 16α-OHE1 on MCF-7 cell number.** Data shown are the means ± 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.

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>Control (x 10^{-5})</th>
<th>E2 (x 10^{-5})</th>
<th>16α-OHE1 (x 10^{-5})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>5.3 ± 0.13</td>
<td>8.1 ± 0.14 (1.5) NS</td>
<td>7.3 ± 0.17 (1.4) NS</td>
</tr>
<tr>
<td>4</td>
<td>9.4 ± 0.19</td>
<td>24.1 ± 0.22 (2.6)*</td>
<td>22.8 ± 0.52 (2.3)*</td>
</tr>
<tr>
<td>6</td>
<td>11.3 ± 0.30</td>
<td><strong>46.4 ± 0.53 (4.1)</strong></td>
<td><strong>53.1 ± 0.54 (4.7)</strong>*</td>
</tr>
<tr>
<td>8</td>
<td>24.6 ± 0.29</td>
<td>126.7 ± 0.44 (5.1)**</td>
<td>156.1 ± 0.67 (6.3)**†</td>
</tr>
<tr>
<td>10</td>
<td>45.0 ± 0.39</td>
<td>168.0 ± 0.60 (4.1)**</td>
<td>244.0 ± 0.84 (5.0)**†</td>
</tr>
</tbody>
</table>

Cells were treated with 10 nM E2 or 16α-OHE1 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<0.01 compared with control; †P<0.001 compared with E2-treated samples. P values were determined by ANOVA followed by Dunnet’s test.

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Treatment with 16α-OHE1 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 5-fold higher than that observed with E2 (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 16α-OHE1 on Cdk2 activity are mediated by the ER, cells were treated with 16α-OHE1 plus 100 nM ICI 182,780, when added together with 16α-OHE1, 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 16α-OHE1.

Effects of 16α-OHE1 and E2 on cyclin D1, cyclin A, and cyclin B1 mRNA expression

Since the earliest and largest effect on G1 cyclin protein expression following 16α-OHE1 treatment was that of cyclin D1, we further examined the effect of 16α-OHE1 on cyclin D1 mRNA level. In the presence of 16α-OHE1, cyclin D1 mRNA level increased 3-fold at 6 h and 4-fold at 8 h (Fig. 8A) compared with controls. Similarly, E2 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 16α-OHE1 on cyclin A and cyclin B1 mRNA levels (Fig. 8B and C). 16α-OHE1 stimulated cyclin A mRNA expression at 12, 16, 24, and 36 h. Densitometric scanning and normalization with GAPDH indicated 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 E2 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, 16α-OHE1 caused a 40% increase in cyclin B1 mRNA levels at 36 h and E2 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 16α-OHE1 on ERE-mediated gene transactivation

The transcription of a number of genes is under the control of ERs in the genome. Previous studies have shown that 16α-OHE1 binds the ER with lower affinity than that of E2 (Swaneck & Fishman 1988). Since our studies suggested that the action of 16α-OHE1 on the molecular mechanisms controlling the cell cycle was mediated through the ER, we examined the ability of 16α-OHE1 to stimulate transcription of genes controlled by the ERE. We performed transient transfection experiments in
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 16α-OHE_1 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_2 (at similar concentrations) caused a 10-, 13-, and 14-fold increase respectively. To determine the specificity of 16α-OHE_1/E_2 in activating ER-mediated gene

**FIGURE 5.** Effects of 16α-OHE_1 on cyclin B1 protein expression in MCF-7 cells. G_1 synchronized cells were treated with <0.1% ethanol (control, C), 10 nM 16α-OHE_1 or E_2 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 reprobed with anti-β-actin antibody. Results shown are representative of two separate experiments producing identical results.

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**FIGURE 4.** Effects of 16α-OHE_1 and E_2 on cyclin D1 and cyclin A protein expression. G_1 synchronized MCF-7 cells were treated with <0.1% ethanol (control, C), 10 nM 16α-OHE_1 or E_2. Representative Western blots are shown for cyclin D1 (A) and cyclin A (D). To verify equal protein loading, membranes were stripped and reprobed with anti-β-actin antibody, and cyclin D1 and cyclin A protein bands were normalized to the density of the β-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 ± 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).
transcription, we tested whether the pure antiestro-
gen ICI 182,780 could block this response. The
addition of ICI 182,780 (100 nM) simultaneously
with E2, or 16α-OHE1, completely blocked the
estrogen-induced stimulation (Fig. 9B). These data
indicate that ER is required for the effects of
16α-OHE1 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 16α-hydroxyestrone on cell cycle regulation of estrogen-
responsive MCF-7 breast cancer cells. Our results show that 16α-OHE1 causes a significant increase in
DNA synthesis in MCF-7 cells and stimulates the progression of cells from G1 to S phase of the cell
cycle. Further analysis shows that the acceleration of G1 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 16α-OHE1 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 16α-OHE1.

16α-Hydroxyestrone is found in the plasma in pg/ml levels and in bile and urine in µg/ml levels
(Ikegawa et al. 1983, Naganuma et al. 1989). Several
studies have shown that the 16α-hydroxylation
pathway (yielding 16α-OHE1 and estriol) is signifi-
cantly 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 16α-OHE1 is
not known, this metabolite exhibits estrogenicity
comparable to that of E2 (as measured by stimulation of uterine growth). 16α-OHE1 is known
to have low binding affinity to sex hormone-binding
globulin (Fishman & Martucci 1980) and it binds

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**Figure 6.** Effects of 1α-OHE1 and E2 on pRB phosphorylation in MCF-7 cells. G1 synchronized
MCF-7 cells were treated with 10 nM 1α-OHE1 or E2 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) E2, or (C) 16α-OHE1. 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-β-actin antibody. Results shown are
representative of three separate experiments.

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**Figure 7.** Effects of 16α-OHE1 on Cdk2 kinase
activation in MCF-7 cells. Growth arrested cells were
treated with 10 nM 16α-OHE1 or 10 nM 16α-OHE1
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 16α-OHE1 treatment. The bottom
panel shows Cdk2 activation following 16α-OHE1 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.
irreversibly to the estrogen receptor and up-regulates c-myc oncogene expression (Swaneck & 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 E₂ 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 E₂-induced G₁ progression. Foster & Wimalasena (1996) reported that the synthesis of cyclin D1 protein increased within 3 h after E₂ treatment of growth arrested MCF-7 cells and peaked at 6 h, while Cdk4-associated RB kinase activity was evident within 6 h after E₂ treatment. In our study, cyclin D1 protein level increased within the first 2 to 4 h of E₂ treatment and peaked at 6 h which is consistent with previous findings. We found that 16α-OHE₁ emulated (to some extent) the effects of E₂ on cyclin D1 protein expression with initial induction occurring at 4 h. The effects of 16α-OHE₁ on cyclin D1 were inhibited by the pure antiestrogenICI 182,780. Although cyclin D1 induction was detected at both mRNA and protein levels, data on 16α-OHE₁ 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...
such as insulin-like growth factor-I (IGF-I) and E2 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 $G_1$ 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 G1 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 16α-OHE1 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. 16α-OHE1 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 16α-OHE1 and E2 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 16α-OHE1 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 cyclins in S phase of the cell cycle. In addition, a phosphorylated fast migrating form of Cdk2 was observed following 16α-OHE1 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 required for late stage pRB phosphorylation and G1 to S transition (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 $E_2$-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 kB (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 $E_2$ (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 ERα-DNA binding (Paech et al. 1997, Wang et al. 1999). Thus, a potential mechanism of $16\alpha$-OHE1 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 $16\alpha$-OHE1 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. $16\alpha$-OHE1 increased cyclin D1 expression (protein and mRNA) and pRB phosphorylation, similar to the effects observed with $E_2$. $16\alpha$-OHE1 also caused significant increases in cyclin A expression and Cdk2 and cyclin E/Cdk2 activities. The ability of $16\alpha$-OHE1 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 $16\alpha$-OHE1 to form adducts with DNA and proteins (Liehr 1998), give credence to the hypothesis that elevated levels of $16\alpha$-OHE1 may play an important role in the development and/or progression of breast cancer.

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