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

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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% 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...
hydroxyestrone (16\textsuperscript{a}-OHE\textsubscript{1}) and 2-hydroxyestradiol (2-OHE\textsubscript{2}), while hydroxylation at the C-16\textsuperscript{a} position yields 16\textsuperscript{a}-hydroxyestrone (16\textsuperscript{a}-OHE\textsubscript{1}) and estriol (E\textsubscript{3}) (Ball & Conney 1998). The products of these two hydroxylation pathways exert markedly different biological properties: the 16\textsuperscript{a}-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 16\textsuperscript{a}-OHE\textsubscript{1} 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\textalpha and ER\textbeta (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\textalpha and ER\textbeta 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\textsubscript{2}, 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\textsuperscript{a}-OHE\textsubscript{1} binds to ER\textalpha 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\textsubscript{1} progression. Cyclin A in complex with Cdk2 is important for S phase progression and S→G\textsubscript{2} transition while cyclin B1 in complex with its catalytic subunit Cdk1/Cdc2 is essential for cellular entrance into G\textsubscript{s}/M phase and progression through mitosis. Estrogens, anti-estrogens, and other steroid hormones initiate their cell cycle effects in the G\textsubscript{1} phase (Taylor et al. 1983, Prall et al. 1998). Estradiol stimulates G\textsubscript{1} 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 16\textsuperscript{a}-OHE\textsubscript{1} on DNA synthesis, cell cycle kinetics, cyclin/CDK expression, and ER-mediated transactivation in MCF-7 breast cancer cells. Our results showed that 16\textsuperscript{a}-OHE\textsubscript{1} 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\textsuperscript{a}-OHE\textsubscript{1} 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\textsuperscript{a}-OHE\textsubscript{1} induced ER-mediated gene transcription, causing a 15-fold increase in the expression of anERE-driven...
luciferase gene. These results show that 16α-OHE$_1$ 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 (E$_2$) and 16α-OHE$_1$ 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 (G3-31+DCS-35) antibodies were purchased from PharMingen (San Diego, 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 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 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 Immobilin 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 µg/ml streptomycin, 40 µg/ml gentamicin, 2 µg/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 × 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$_1$ 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$_2$ or 16α-OHE$_1$. Control cells received ethanol vehicle, which was maintained at less than 0·1%. DNA synthesis was measured by adding 4 µCi/ml $^3$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 × 10$^6$) were seeded in 100-mm culture dishes and after 24 h, cells were treated with 10 nM 16α-OHE$_1$ or E$_2$. Cells were then harvested in 1 × PBS on days 2, 4, 6, 8, and 10 and counted in a hemocytometer using the trypsin blue exclusion.

Cell cycle analysis/flow cytometry

MCF-7 cells (2 × 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$_2$ or 16α-OHE$_1$. 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 cytoplogic 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 \(-20^\circ\)C in aliquots. Thirty micrograms protein (determined by the Bradford protein assay) for molecular weight determinations, we used multicolored protein markers (NEN Life Science Products, Boston, MA, USA) or an anti-cyclin E monoclonal antibody (Neomarkers, Union City, CA, USA) or an anti-cyclin A 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 MgCl_2, 20 µM ATP, 10 µCi \([\gamma-^3P]ATP, 0.1\) mM orthovanadate, 1 mM NaF, 10 mM 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 \( E_2 \), 16α-OHE\(_1\), 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 SureBlot 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 NotI 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 0.2–0.5 µg/µl. The DNA

\[ \text{phenylmethylsulfonyl fluoride, 200} \]
was labeled with [α-32P]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 × 10^8 c.p.m./µg 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 32P-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 × 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 µg 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 16α-OHE1, 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:

normalized luciferase activity = observed firefly luciferase activity/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 16α-OHE1 on DNA synthesis and growth of ER-positive MCF-7 cells

In the first set of experiments, we examined the effects of 16α-OHE1 and E2 on DNA synthesis in MCF-7 breast cancer cells using the [3H]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 16α-OHE1 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 16α-OHE1 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 16α-OHE1 in stimulating DNA synthesis, causing a maximum 4.5-fold increase at these time points (Fig. 2B).

We also performed the [3H]thymidine incorporation assay on an ER-negative breast cancer cell line, MDA-MB-468, to test whether the stimulatory effect of 16α-OHE1 was mediated by the ER. Our results showed that 16α-OHE1 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 16α-OHE1 in MCF-7 cells.

We also examined the effects of E2 and 16α-OHE1 on cell number. MCF-7 cells (0–25 × 10^5) were treated with 10 nM E2 or 16α-OHE1, 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. 16α-OHE1 caused a significant (P<0.001)
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 immuno-blotted 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

**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

<table>
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<th>Time of treatment, h</th>
<th>24</th>
<th>48</th>
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<tr>
<td>[3H]-Thymidine Incorporation, cpm x 10^-5</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

| [3H]-Thymidine Incorporation, cpm x 10^-5 | 1 | 2 |

| [3H]-Thymidine Incorporation, cpm x 10^-5 | 1 | 2 |

**Figure 2.** Thymidine incorporation assay of MCF-7 breast cancer cells in the presence of 16α-OHE1 and E2. G1 synchronized MCF-7 cells (0·5 × 10^6) were treated with 1, 4, 10 or 25 nM 16α-OHE1 (A) or E2 (B) for 24 and 48 h. One hour prior to the 24 and 48 h time points, cells were treated with 4 µCi [3H]thymidine and then processed according to procedures set out in the Materials and Methods section. Results shown are the means ± s.e. based on three separate experiments, and the P values were determined using ANOVA followed by Dunnet’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 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
progression. In contrast, E₂ treatment caused only a 20% increase at 16 h and a 30% change in cyclin E protein levels following its established role as a regulator of G₂/M phase progression. We found that cyclin A and cyclin B1, important regulators of S phase of the cell cycle in 16α-OHE₁-treated cells at 12, 16, and 24 h (Fig. 4D). This increase in cyclin A correlated with 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.

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>Cell number (× 10⁻⁵)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>2</td>
<td>5.3 ± 0.13</td>
</tr>
<tr>
<td>4</td>
<td>9.4 ± 0.19</td>
</tr>
<tr>
<td>6</td>
<td>11.3 ± 0.30</td>
</tr>
<tr>
<td>8</td>
<td>24.6 ± 0.29</td>
</tr>
<tr>
<td>10</td>
<td>45.0 ± 0.39</td>
</tr>
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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<0.001 compared with control; †P<0.001 compared with E₂-treated samples. P values were determined by ANOVA followed by Dunnett’s test.

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).
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 2-3 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 10 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 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-OHE₁ 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 E₂ (at similar concentrations) caused a 10-, 13-, and 14-fold increase respectively. To determine the specificity of 16α-OHE₁/E₂ in activating ER-mediated gene

**FIGURE 4.** Effects of 16α-OHE₁ and E₂ on cyclin D₁ and cyclin A protein expression. G₁ synchronized MCF-7 cells were treated with <0·1% ethanol (control, C), 10 nM 16α-OHE₁ or E₂. 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).

**FIGURE 5.** Effects of 16α-OHE₁ on cyclin B₁ protein expression in MCF-7 cells. G₁ synchronized cells were treated with <0·1% ethanol (control, C), 10 nM 16α-OHE₁ or E₂ 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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transcription, we tested whether the pure antiestrogen ICI 182,780 could block this response. The addition of ICI 182,780 (100 nM) simultaneously with 16α-OHE1 or E2, 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 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 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...
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 antiestrogen ICI 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

**FIGURE 8.** Effects of 16α-OHE₁ and E₂ on cyclin D1, cyclin A, and cyclin B1 mRNA expression in MCF-7 cells. G₁ synchronized cells were treated with 10 nM 16α-OHE₁ or E₂ 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 16α-OHE₁ - and E₂-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 A¹ and B¹. Error bars represent s.e. for three separate experiments. *Significantly different from control (P<0·01).
such as insulin-like growth factor-I (IGF-I) and E$_2$ 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 G$_1$ 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 16a-OHE$_1$ 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. 16a-OHE$_1$ 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 16a-OHE$_1$ and E$_2$ 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 16a-OHE$_1$ 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 16a-OHE$_1$ 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, etc.)
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 E2-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 E2 (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 16α-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 16α-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. 16α-OHE1 increased cyclin D1 expression (protein and mRNA) and pRB phosphorylation, similar to the effects observed with E2. 16α-OHE1 also caused significant increases in cyclin A expression and Cdk2 and cyclin E/Cdk2 activities. The ability of 16α-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 16α-OHE1 to form adducts with DNA and proteins (Liehr 1998), give credence to the hypothesis that elevated levels of 16α-OHE1 may play an important role in the development and/or progression of breast cancer.

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