Differential effects of 16 α -hydroxyestrone and 2-methoxyestradiol on cyclin D1 involving the transcription factor ATF-2 in MCF-7 breast cancer cells

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Abstract

We studied the effects of 2-methoxyestradiol (2-ME₂) and 16 α -hydroxyestrone (16 α -OHE₁), two metabolites of estradiol (E₂), on DNA synthesis, cell cycle progression and cyclin D1 protein levels in estrogen receptor-positive MCF-7 cells. E₂ and 16 α -OHE₁ stimulated DNA synthesis, and 2-ME₂ inhibited the stimulatory effects of these agents. E₂ and 16 α -OHE₁ stimulated the progression of cells from G₁ to S phase and this effect was attenuated by 2-ME₂. Western blot analysis showed that E₂ and 16 α -OHE₁ increased cyclin D1 protein level by about fourfold compared with control. 2-ME₂ had no significant effect on cyclin D1; however, it prevented the accumulation of cyclin D1 in the presence of E₂ and 16 α -OHE₁. Cells transfected with a cyclin D1 reporter gene and treated with E₂ or 16 α -OHE₁ showed 7- and 9.5-fold increase respectively in promoter activity compared with control. This activity was significantly inhibited by 2-ME₂. Cyclin D1 transactivation was mediated by the cAMP response element (CRE) region, which binds activating transcription factor 2 (ATF-2). DNA affinity assay showed 2.5- and 3.5-fold increases in ATF-2 binding to CRE in the presence of E₂ and 16 α -OHE₁ respectively. The binding of ATF-2 was inhibited by the presence of 2-ME₂. These results show that 2-ME₂ can downregulate cyclin D1 and thereby cell cycle progression by a mechanism involving the disruption of ATF-2 binding to cyclin D1 promoter.

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Introduction

The female sex hormone estradiol (E₂) plays a major role in the development and progression of breast cancer (Henderson *et al.* 1988, Russo *et al.* 2003). E₂ exerts its biologic effects through the estrogen receptors (ER α and ER β), ligand-activated transcription factors present in target tissues (Katzenellenbogen *et al.* 2000, McKenna & O'Malley 2002, Thomas *et al.* 2004). In general, estrogenic action involves the binding of the ligand to the ER, which undergoes conformational changes and dimerization, and is recognized by the estrogen response element (ERE), located at the promoter region of estrogen-responsive genes. The ER–ERE interaction triggers a cascade of cellular events, leading to the transcriptional activation of specific genes responsible for cell proliferation (Nilsson & Gustafsson 2002).

E₂ is metabolized by oxidation and hydroxylation (Martucci & Fishman 1993, Zhu & Conney 1998). The oxidation product of E₂ is estrone (E₁), which is metabolized by two mutually exclusive hydroxylation

pathways: hydroxylation at the C-2 or the 16 α position (Fig. 1). Hydroxylation at the C-2 position yields 2-hydroxyestrone (2-OHE₁) and 2-hydroxyestradiol (2-OHE₂), whereas hydroxylation at the 16 α position yields 16 α -hydroxyestrone (16 α -OHE₁) and 16 α -hydroxyestradiol (16 α -OHE₂). Increased 16 α -hydroxylation in women is associated with increased breast cancer risk (Muti *et al.* 2000). 16 α -Hydroxylated metabolites stimulate breast cancer cell growth, whereas the 2-hydroxylated estrogens inhibit cell growth (Ball *et al.* 1972, Seegers *et al.* 1989, Lottering *et al.* 1992, Zhu & Conney 1998). 2-OHE₁ and 2-OHE₂ are rapidly converted to the monomethyl ethers, 2-ME₁ and 2-ME₂ respectively by the enzyme catechol-*O*-methyltransferase (COMT) (Ball *et al.* 1972, Jefcoate *et al.* 2000). 2-ME₂ inhibits the growth of human mammary cancer cells *in vitro* and *in vivo* (Lottering *et al.* 1992, Fotis *et al.* 1994). The binding affinity 2-ME₂ for ER from MCF-7 cells is about 1% of that of E₂ (Brueggemeier *et al.* 2001). Low concentrations (10 nM) of 2-ME₂ inhibit the growth of ER-positive MCF-7 cells, and cause

Figure 1 Pathways of cellular metabolism of estradiol (E_2). E_2 metabolism occurs primarily through three pathways: C2-hydroxylation (major pathway), C16 α -hydroxylation (major pathway) and C4-hydroxylation (minor pathway). 2-Hydroxyestradiol (2-OHE₂) is further O-methylated by catechol-O-methyltransferase (COMT) to form 2-methoxyestradiol 2-ME₂.

fluctuations in cAMP levels, whereas pharmacologic concentrations (1–10 μ M) inhibit tubulin polymerization and angiogenesis (Lottering *et al.* 1992, D'Amato *et al.* 1994, Cushman *et al.* 1995, Klauber *et al.* 1997, Moobery 2003).

In contrast to the growth-inhibitory effects of 2-ME₂ on MCF-7 cells, 16 α -OHE₁ stimulates the proliferation of these cells (Gupta *et al.* 1998, Suto *et al.* 1999). 16 α -OHE₁ binds to the ER with measurable affinity and exerts uterotrophic activity, comparable to that of E_2 (Fishman & Martucci 1980). We found that 16 α -OHE₁ and E_2 had comparable effects on cell cycle kinetics and in stimulating the expression of cyclin D1 in MCF-7 cells (Lewis *et al.* 2001).

Cyclin D1 is an important regulator of G₁→S phase transition, and its expression in breast cancer cells is sensitive to estrogens and antiestrogens (Musgrove *et al.* 1994, Altucci *et al.* 1996, Foster & Wimalasena 1996, Pacilio *et al.* 1998, Pestell *et al.* 1999). In transgenic mice, overexpression of cyclin D1 led to the development of mammary tumors, while cyclin D1 knockout mice showed impaired development of mammary glands (Wang *et al.* 1994, Sicinski & Weinberg 1997). The

promoter region of cyclin D1 contains binding sites for AP1, SP1, NF- κ B and ATF/CREB, all of which are implicated in its transcriptional activation (Herber *et al.* 1994, Albanese *et al.* 1995, Watanabe *et al.* 1998, Bromberg *et al.* 1999, Guttridge *et al.* 1999). Recent studies indicate that E_2 regulation of cyclin D1 in MCF-7 cells occurs at the transcriptional level, and involves the cAMP response element (CRE) (Altucci *et al.* 1996, Sabbah *et al.* 1999). The CRE element binds bZIP DNA-binding proteins belonging to the ATF/CREB family of transcription factors (Hai *et al.* 1993). ATF-2 is a member of the ATF/CREB family, and stimulates the transcription of cyclin D1 (Beier *et al.* 1999, Sabbah *et al.* 1999). The ability of ATF-2 to bind to the CRE and stimulate CRE-mediated transcription is regulated by phosphorylation (Gupta *et al.* 1995).

In the present study, we examined the effects of E_2 , 16 α -OHE₁ and 2-ME₂ on DNA synthesis and cell cycle progression, cyclin D1 transactivation and ATF-2 DNA-binding activity in MCF-7 breast cancer cells. Our results showed that 2-ME₂ interfered with the growth stimulatory effects of E_2 and 16 α -OHE₁, as did the pure antiestrogen, ICI 182,780. In transient transfection

assays, E₂ and 16 α -OHE₁ enhanced cyclin D1 promoter activity, while 2-ME₂ and ICI 182,780 inhibited this effect. The estrogenic effect on cyclin D1 was mediated through the binding of ATF-2 to the CRE site.

Materials and methods

Chemicals, reagents and antibodies

E₂, 16 α -OHE₁ and 2-ME₂ were purchased from Steraloids (Wilton, NH, USA). The pure antiestrogen ICI 182,780 was purchased from Tocris (Ellisville, MO, USA). Monoclonal antihuman ATF-2, phospho-ATF-2, CREB, ATF-1, c-Jun, c-Fos, p65, p50, p38 and phospho-p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-cyclin D1 antibody was from LabVision (Fremont, CA, USA). DMEM, phenol red-free DMEM and fetal bovine serum were purchased from Sigma. The HexaPlus DNA labeling kit was obtained from MBI Fermentas (Amherst, NY, USA). Antibiotics, trypsin and other additives for cell culture were purchased from Gibco Laboratories (Grand Island, NY, USA). Dynabeads M-280, Streptavidin and Dynal MPC-S were obtained from Dynal Biotechnology (Lake Success, NY, USA). Biotin-16-2'-deoxyuridine-5'-triphosphate (Biotin-16-dUTP) was purchased from Sigma.

Oligonucleotides and plasmids

HPLC-purified oligonucleotides (ODNs) were purchased from Oligos, Etc (Wilsonville, OR, USA). A 74-bp ODN1, with cyclin D1 promoter sequence (−78 to −5) containing the consensus CRE (underlined) and NF- κ B response element (boldface) was used for DNA affinity assays. A mutated CRE/ATF ODN2 with a three-base rearrangement in the consensus sequence (underlined and boldface) was used for determining sequence specificity of the CRE/ATF-2 binding. The base sequence of the top strands of the ODNs used in this study are as follows:

ODN1: 5'-GGGCTTTGATCTTTGCTTAACAAC AGTAACGTCACACGGACTACAGGGGAGTTTTG TTGAAGTTGCAAAGTCCT-3'

ODN2: 5'GGGCTTTGATCTTTGCTTAACAAC AGTAGCGGCACACGGACTACAGGGGAGTTTT GTTGAAGTTGCAAAGTCCT-3'

For transcriptional activation studies, the cyclin D1 luciferase reporter plasmids -1745CD1-LUC, -1745 ATF/CREmut-LUC, -964CD1-LUC, -964AP1 mut-LUC, -66CD1-LUC, -66 ATF/CREmut-LUC and -66CD1 κ Bmut-LUC were used. These reporter gene constructs have been previously described (Albanese *et al.* 1995, Watanabe *et al.* 1999). A mutant and wild-type human ATF-2 cDNA, subcloned into the eukaryotic expression vector pCMV, was used to express

ATF-2 dominant negative mutant (ATF-2 dom neg mutant) and wild-type proteins in transfected cells (Lee *et al.* 1999, D'Amico *et al.* 2000). The pRL-SV40 vector (Promega) containing the *Renilla* luciferase gene was used as an internal control.

Cell culture methods

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 μ g/ml streptomycin, 40 μ g/ml gentamicin, 2 μ g/ml insulin, 0.5 mM sodium pyruvate, 50 mM nonessential amino acids, 2 mM L-glutamine and 10% fetal bovine serum. Two weeks before each experiment, MCF-7 cells were grown in phenol red-free DMEM containing serum treated with dextran-coated charcoal (DCC) to remove serum-derived estrogenic compounds (Berthois *et al.* 1986, Thomas *et al.* 1989, Lewis *et al.* 2001). DCC suspension contained 0.05% dextran, 0.5% charcoal and 25 mM sucrose. Serum was subjected to three 10-min cycles of DCC treatment, centrifugation and filtering through a 0.2 μ m membrane.

For [³H]thymidine incorporation assay, MCF-7 cells (0.5×10^6) were seeded in 35 mm culture dishes in phenol red-free DMEM supplemented with DCC-treated serum and additives. After 24 h of plating, cells were treated with 10 nM E₂, 16 α -OHE₁ or 2-ME₂, as indicated in the text. Control cells received ethanol vehicle, which was maintained at less than 0.1%. DNA synthesis was measured by adding 4 μ Ci/ml [³H]thymidine to cells 1 h before harvest. Cells were washed twice with ice-cold PBS and treated with 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.

For cell cycle analysis, MCF-7 cells (2×10^6) were seeded in 100 mm culture dishes for 24 h and then treated with E₂, 16 α -OHE₁, 2-ME₂ or ICI 182,780 for 24 and 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% DMSO, and stored at −70 °C. On the day of DNA analysis, cells were thawed, and the citrate buffer was removed. Cells were trypsinized for 10 min and then treated with a solution containing a trypsin inhibitor and RNase for 10 min. Cells were then stained by adding propidium iodide solution in sodium citrate buffer and analyzed by a Coulter flow cytometer. Percentage distribution of cells in different phases of cell cycle was calculated with cytologic software (Thomas & Thomas 1994).

Western blot analysis

Cyclin D1 protein levels in MCF-7 cells were determined by Western blot, after treatment with E₂,

16 α -OHE₁, 2-ME₂ or ICI 182,780. Cell lysate was prepared by the procedure previously described (Thomas & Thomas 1994). Briefly, monolayers of cells were washed twice with ice-cold PBS, and lysed by the addition of ice-cold lysis buffer (150 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 2 mM EDTA, 50 mM sodium fluoride, 0.2% SDS, 1 mM sodium vanadate, 2 μ g/ml leupeptin, aprotinin and pepstatin, and 1 mM phenylmethylsulfonyl fluoride). An amount of 30 μ g protein was diluted in 2 $\times$ SDS-PAGE Laemmli buffer (150 mM Tris base (pH 6.8), 30% glycerol, 4% SDS, 7.5 mM dithiothreitol and 0.01% bromophenol blue) and separated on 10% SDS-polyacrylamide gel. Proteins were transferred to PVDF Polyscreen membranes, which were incubated in 5% nonfat milk in Tris-buffered saline, containing 0.1% Tween-20 for 1 h. Membranes were then incubated overnight with a 1:200 dilution of antibodies specific to human cyclin D1, phospho-p38 MAPK (activated) or p38 MAPK (total) respectively. Protein bands were visualized with horseradish peroxidase-conjugated secondary antibody with a chemiluminescence-based detection system. Membranes were stripped and reblotted with anti- β -actin monoclonal antibody (1:5000). Intensity of protein bands was quantified with a Scanjet 4C flatbed scanner (Hewlett Packard) with NIH Image v1.52 software. Lightly exposed films were used for quantification.

Transient transfection assay

MCF-7 cells (1×10^5) were plated in 24-well microtiter plates for 24 h. By the calcium phosphate coprecipitation procedure (Mamalian Transfection Kit; Clontech, Palo Alto, CA, USA), cells were transfected with 4 μ g cyclin D1 promoter plasmid linked to a firefly luciferase reporter gene and 0.4 μ g *Renilla* luciferase plasmid (Shah *et al.* 2001). In some experiments, cells were also transfected with 1–10 μ g ATF-2 dominant negative mutant expression vector (pCMV). After transfection, the medium was refreshed, and cells were treated with 10 nM E₂, 16 α -OHE₁ or 2-ME₂, or 100 nM ICI 182,780 for 7 h. The activities of the cyclin D1 firefly reporter gene and the *Renilla* luciferase internal control were determined in a dual luciferase reporter assay system (Promega). Reporter activity was normalized for each sample by the following formula: normalized luciferase activity = firefly luciferase activity / *Renilla* luciferase activity.

DNA affinity immunoblot assay

Treatments and preparation of cellular extracts were as described under Western blot analysis.

ODNs were biotinylated with the HexaLabel Plus DNA labeling kit. Biotinylated ODNs (1 μ M) were immobilized by incubating with the Dynabeads

(1 mg/ml) in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 M NaCl) for 15 min at 22 °C. The Dynabeads containing the immobilized biotinylated DNA were washed, separated in Dynal MPC-S and incubated with cellular extract (100 μ g) in 8 mM Tris-phosphate at pH 7.4, 0.12 M KCl, 8% glycerol, 4 mM DTT and 0.5% CHAPS for 1 h at 4 °C (Zwijnen *et al.* 1998). Subsequently, beads were washed in 10 mM HEPES at pH 7.7, 50 mM KCl, 20% glycerol and 0.1% NP-40, and boiled in Laemmli buffer (100 μ l), and the DNA-bound proteins were separated on 10% polyacrylamide gel. Proteins were identified by immunoblotting with antibodies specific for ATF-2, phospho-ATF-2, ATF-1, c-Fos, c-Jun, CREB or p65. Membranes were stripped and reprobed, using an antibody to a transcription factor (p50), which did not show any change with treatments, as a control for equal loading and transfer.

Statistical analysis

Results of transient transfection studies are presented as mean $\pm$ S.E. for at least three separate experiments. Statistical difference between control and treatment groups was determined by one-way ANOVA followed by Dunnet's post-test (GraphPad Prism Software program, San Diego, CA, USA). A *P* value of <0.05 was considered to be statistically significant.

Results

Effects of E₂, 16 α -OHE₁ and 2-ME₂ on DNA synthesis and cell cycle

In the first set of experiments, we tested the effects of 10 nM E₂, 16 α -OHE₁ and 2-ME₂ on DNA synthesis in MCF-7 breast cancer cells by the [³H] thymidine incorporation assay. A concentration of 10 nM was used to observe maximal effects of E₂ and 16 α -OHE₁ (Lewis *et al.* 2001) and to avoid the cytotoxic effects of 2-ME₂ observed at μ M concentrations (Brueggemeier *et al.* 2001, Moobery 2003). We treated MCF-7 cells with E₂, 16 α -OHE₁, 2-ME₂ or ICI 182,780 as single agents, or E₂ or 16 α -OHE₁ in combination with 2-ME₂ or ICI 182,780 for 24 and 48 h. There was a significant increase in DNA synthesis by E₂ and 16 α -OHE₁ (Fig. 2). Our results also showed that E₂-induced DNA synthesis was reduced from 500% to 200% at 24 h and from 400% to 200% at 48 h by 10 nM 2-ME₂. Similarly, 16 α -OHE₁-induced DNA synthesis was reduced from 650% to 270% at 24 h and from 500% to 250% at 48 h by 10 nM 2-ME₂. The inhibitory effect of 2-ME₂ was lower than that of the pure antiestrogen, ICI 182,780, which reduced E₂-induced DNA synthesis from 500% to 160% at 24 h and from 400% to 100% at 48 h. These results indicate that 2-ME₂ can interfere with the

Figure 2 Effects of E₂, 16α-OHE₁ or 2-ME₂ on DNA synthesis of MCF-7 breast cancer cells. Cells (0.5 × 10⁶/dish) were treated with the indicated concentration of E₂, 16α-OHE₁ or 2-ME₂ for 24 and 48 h. Results shown are the mean ± S.E. from three separate experiments, and the *P* values were determined by ANOVA followed by Dunnett's or Tukey's post-test.

*Significant stimulation compared with untreated control, *P* < 0.0001; #significant inhibition compared with untreated control, *P* < 0.01; &significant inhibition compared with E₂ or 16α-OHE₁-treated cells, *P* < 0.001; *significant inhibition compared with E₂- or 16α-OHE₁-treated cells, *P* < 0.0001. Fold induction was calculated by dividing the c.p.m. of treated cells by that of untreated control (average c.p.m. for control cells was 52,476 ± 3007; *n* = 3).

growth-stimulatory effects of E₂ and 16α-OHE₁ in MCF-7 cells.

We next examined the effect of this metabolite on cell cycle distribution by flow cytometry. Cells were treated with E₂, 16α-OHE₁ and 2-ME₂ as single agents or in combination, and the percent distribution of cells in different phases of the cell cycle was determined at

24- and 48-h time points by flow cytometry (Table 1). Treatment of cells with E₂ and 16α-OHE₁ reduced the numbers of cells in the G₁ phase from 77.5% to 66.4% and 65.3% respectively, while increasing the numbers of cells in S phase from 11% to 22.6% and 23.3% respectively. In contrast, treatment of cells with 2-ME₂, as a single agent, did not have any significant effect on cell cycle distribution. However, 2-ME₂ blocked the stimulatory effects of E₂ and 16α-OHE₁ on G₁ to S phase transition by 48 h, yielding a cell cycle distribution similar to that of control cells.

Effects of E₂, 16α-OHE₁ and 2-ME₂ on cyclin D1 protein and cyclin D1 transactivation

To investigate the mechanism by which 2-ME₂ inhibited E₂- and 16α-OHE₁-induced G₁ to S phase progression of MCF-7 cells, we tested its effect on cyclin D1 protein levels. Cells were treated with E₂, 16α-OHE₁, 2-ME₂ or ICI 182,780 for 7 h, and the cell lysate was analyzed by Western blotting. The 7-h time point was selected from our previous study showing the maximal induction of cyclin D1 by E₂ and 16α-OHE₁ at 6–8 h (Lewis *et al.* 2001). There was a fourfold increase in cyclin D1 protein level by both E₂ and 16α-OHE₁ at the 7-h time point, compared with control (Fig. 3A). In contrast, there was no significant change in cyclin D1 protein level in cells treated with 4, 10 or 100 nM 2-ME₂ compared with the control group. However, 2-ME₂ was able to prevent the increase in cyclin D1 protein expression in the presence of E₂ and 16α-OHE₁ (Fig. 3B). This effect was comparable to that produced by the pure antiestrogen, ICI 182,780 (Fig. 3B). Thus, a possible mechanism for the ability of 2-ME₂ to retard

Table 1 Percentage distribution of cells in different phases of cell cycle*.

Treatment	G ₁	S	G ₂ /M	G ₁	S	G ₂ /M
	24 h			48 h		
None	77.5 ± 1.2	10.9 ± 0.5	11.6 ± 0.6	82.3 ± 2.3	6.8 ± 0.6	8.3 ± 0.6
E ₂	66.4 ± 0.9	22.6 ± 1.3†	10.1 ± 0.9	68.1 ± 2.1	21.5 ± 1.1†	10.4 ± 0.5
16α-OHE ₁	65.3 ± 1.5	23.3 ± 1.7†	11.4 ± 1.4	69.7 ± 1.7	21.3 ± 0.5†	9.0 ± 0.3
2-ME ₂	76.9 ± 2.7	11.3 ± 1.5	11.8 ± 1.1	80.4 ± 1.4	10.3 ± 1.2	9.0 ± 1.2
ICI 182,780	82.3 ± 1.2	8.4 ± 0.4	9.3 ± 0.3	81.7 ± 2.1	9.1 ± 0.4	10.2 ± 1.1
E ₂ +2-ME ₂	70.5 ± 0.6	14.6 ± 1.2‡	13.9 ± 0.7	79.3 ± 1.7	11.5 ± 1.1	9.2 ± 1.3
16α-OHE ₁ +2-ME ₂	71.8 ± 1.4	15.6 ± 0.7‡	13.6 ± 0.5	79.2 ± 2.1	11.4 ± 1.4	9.4 ± 1.7
E ₂ +ICI 182,780	75.1 ± 0.7	11.2 ± 0.3‡	13.7 ± 0.8	79.9 ± 1.1	9.1 ± 0.3	10.1 ± 0.5
16α+ICI 182,780	74.3 ± 0.6	11.7 ± 0.6‡	14.1 ± 1.1	78.6 ± 1.4	9.4 ± 0.6	10.9 ± 1.2

*MCF-7 cells (in phenol red-free medium) were treated with 10 nM E₂, 16α-OHE₁, 2-ME₂ or 100 nM ICI 182,780, as single agents, or in combination for 24 and 48 h, and DNA was stained with propidium iodide and analyzed by flow cytometry. Percent distribution of cells in the different phases of the cell cycle was calculated with cytologic software program.

†Significantly different from control at *P* < 0.05.

‡Significantly different from E₂/16α-treated cells, *P* < 0.05.

Statistical difference between control and treated samples was determined by ANOVA followed by Dunnett's or Tukey's post-test. Results shown are the mean of three separate experiments ± S.E.

A

B

Figure 3 Western blot analysis of cyclin D1 in MCF-7 cells after treatment with E₂, 16α-OHE₁ or 2-ME₂. Cells were treated with 10 nM E₂ or 16α-OHE₁, or 4, 10 and 100 nM 2-ME₂ for 7 h (A). The effect of E₂ and 16α-OHE₁ on cyclin D1 expression in the presence of 2-ME₂ or ICI 182,780 is also shown (B). To verify equal protein loading and transfer, membranes were stripped and reprobed with anti-β-actin antibody. Results shown represent three separate experiments with comparable results.

cell-cycle progression mediated by E₂ and 16α-OHE₁ might involve the suppression of cyclin D1. We next examined the effects of E₂, 16α-OHE₁ and 2-ME₂ on cyclin D1 transactivation using a full-length cyclin D1 promoter plasmid (-1745CD1 LUC). Treatment of cells with either E₂ or 16α-OHE₁ resulted in 7- and 9.5-fold increases respectively in cyclin D1 promoter (-1745CD1 LUC) activity as compared with the control (Fig. 4A). 2-ME₂ alone did not show any significant effect on cyclin D1 transactivation. When

combined with E₂ or 16α-OHE₁, however, 2-ME₂ significantly reduced the stimulatory effects of these compounds (Fig. 4A). ICI 182,780 also reduced cyclin D1 promoter activity induced by E₂ and 16α-OHE₁ (Fig. 4A). These results indicate that 2-ME₂ can downregulate E₂-induced transactivation of cyclin D1. To test the functional importance of the CRE/ATF motif in cyclin D1 transactivation induced by E₂ or 16α-OHE₁, we performed transfection experiments with a cyclin D1 promoter plasmid containing a mutated

Figure 4 Effects of E₂, 16α-OHE₁ or 2-ME₂ on cyclin D1 promoter activity. MCF-7 cells (1×10^5 cells/dish) were cotransfected with a full-length cyclin D1 promoter plasmid (-1745CD1-LUC) and *Renilla* luciferase control plasmid. After 24 h, cells were treated with 10 nM E₂, 16α-OHE₁ or 2-ME₂, as single agents, or E₂ and 16α-OHE₁ in the presence of 10 nM 2-ME₂ or 100 nM ICI 182,780 for 7 h, and luciferase activity was measured. *Significantly different from untreated control cells at $P < 0.0001$; #significantly different from E₂/16α-treated cells at $P < 0.01$; &significantly different from E₂/16α-treated cells at $P < 0.001$. (B) Effect of ATF/CRE mutation on the activation of the full-length cyclin D1 promoter by E₂ and 16α-OHE₁. These experiments were carried out essentially as described in (A), except that cells were transfected with a mutant cyclin D1 plasmid, -1745 CRE/ATFmut-LUC. Results are shown as the mean $\pm$ S.E. of three separate transfection experiments.

CRE/ATF site (-1745 CRE/ATFmutLUC). Mutation of the CRE/ATF site significantly reduced cyclin D1 transactivation in the presence of E₂ and 16α-OHE₁ (Fig. 4B), as compared with the wild-type plasmid. These results indicate that the CRE/ATF element is essential for the optimal induction of cyclin D1 by E₂ and 16α-OHE₁.

To test the importance of the distal region of the cyclin D1 promoter in its transcriptional activation by E₂ and 16α-OHE₁, we used a cyclin D1 promoter plasmid lacking regions -1745 to -964 (-963CD1 LUC). Our results (Fig. 5, upper panel, compared with Fig. 4A) showed that the removal of the distal region did not have a significant effect on cyclin D1 transcriptional

Figure 5 Transcriptional activation of the cyclin D1 promoter lacking the distal region. Cells were transfected with cyclin D1 promoter plasmid lacking regions -1745 to -964 (-964CD1-LUC) (upper panel) or a plasmid (-964AP1CD1-LUC) with mutation at the AP1 site (lower panel) and the control *Renilla* luciferase plasmid. Subsequently, cells were treated with E₂ or 16α-OHE₁ in the absence or presence of 2-ME₂ or ICI 182,780 for 7 h. *Significantly different from untreated control cells at $P < 0.0001$; #significantly different from E₂/16α-treated cells at $P < 0.01$; &significantly different from E₂/16α-treated cells at $P < 0.001$. The results are shown as the mean $\pm$ S.E. of three separate transfection experiments.

activation by E₂ or 16α-OHE₁. Furthermore, mutation of the AP-1 site had no marked effect on the induction of cyclin D1 by E₂ or 16α-OHE₁ (Fig. 5, lower panel).

To confirm the role of the CRE/ATF site in the regulation of cyclin D1 promoter activity, we transfected MCF-7 cells with a -66 cyclin D1 promoter containing a CRE/ATF and NF-κB site (-66CD1 LUC). Our results showed that transactivation of the -66CD1 LUC promoter increased by sixfold in the presence of E₂ and by 7.5-fold in the presence of 16α-OHE₁, and this induction was significantly inhibited (four- to fivefold) by ICI 182,780 and 2-ME₂ (Fig. 6A). Mutation of the CRE/ATF site on the cyclin D1 promoter (-66 CRE/ATFmutLUC) resulted in ~70% decrease in transactivation, as compared with the wild-type plasmid (-66CD1 LUC) (Fig. 6B). Interestingly, mutation of the NF-κB site did not significantly reduce cyclin D1 promoter activity induced by E₂ or 16α-OHE₁ (Fig. 6C).

Figure 6 Transcriptional activation of the truncated cyclin D1 promoter by E₂ and 16α-OHE₁. (A) Cells (1×10⁵ per dish) were cotransfected with the cyclin D1 promoter (-66CD1 Luc) and *Renilla* luciferase control plasmid. Cells were treated with E₂ or 16α-OHE₁ in the absence or presence of 2-ME₂ or ICI 182,780 for 7 h. (B) Truncated cyclin D1 promoter with ATF/CRE mutation was used for transfection. (C) Truncated cyclin promoter with NF-κB site mutation was used for transfection. *Significantly different from untreated control cells at P<0.0001; #significantly different from E₂/16α-treated cells at P<0.01; &significantly different from E₂/16α-treated cells at P<0.001. The results are shown as the mean±S.E. of three separate transfection experiments.

Figure 7 ATF-2 binding to the CRE/ATF site of cyclin D1 in the presence of E₂, 16α-OHE₁, 2-ME₂ or ICI 182,780. MCF-7 cells (2.5×10⁶ cells/dish) were treated with 10 nM E₂, 16α-OHE₁ and 2-ME₂ for 7 h. Cellular extracts were incubated with ODN1 for 1 h at 4 °C. The DNA-bound proteins were separated on a 10% SDS-PAGE and identified by immunoblotting with antibodies specific for ATF-2 and phosphorylated ATF-2 (phospho-ATF-2). (A) Effect of 2-ME₂ on E₂ and 16α-OHE₁-mediated increase in ATF-2 binding to cyclin D1 promoter. (B) Effect of ICI 182,270 on E₂ and 16α-OHE₁-mediated increase in ATF-2 binding to cyclin D1 promoter. (C) Effect of 2-ME₂ on E₂ and 16α-OHE₁-mediated increase in phospho-ATF-2 binding to cyclin D1 promoter. The results presented here represent three separate experiments.

Effects of E₂, 16α-OHE₁ and 2-ME₂ on ATF-2 binding to CRE/ATF element

In this series of experiments, we examined the effects of E₂, 16α-OHE₁ and 2-ME₂ on the binding of ATF-2 to a 74-mer ODN1 from cyclin D1 promoter. Cells were treated with 10 nM E₂, 16α-OHE₁ or 2-ME₂, as single agents, or E₂ and 2-ME₂ combinations for 7 h. A cellular extract was prepared, and binding to ODN1 determined by DNA affinity assay. (The specificity of the assay was verified with a mutant 74-mer ODN with a 3 base pair mutation at the ATF-2 binding site.) Figure 7 shows our results from DNA affinity assay with the wild-type 74-mer ODN. There was very little constitutive binding of ATF-2 to ODN1 in the absence of treatment. Treatment of cells with E₂ resulted in a two- to threefold increase (n=3) in ATF-2 binding to ODN1, while 16α-OHE₁ caused a fourfold increase (n=3) (Fig. 7). In contrast, treatment of cells with 2-ME₂ resulted in a relatively minor band of ATF-2 binding. Importantly, 2-ME₂ blocked the effects of both E₂

Figure 8 Binding of ATF-1, CREB, AP-1 and NF- κ B proteins to the cyclin D1 CRE/ATF site. MCF-7 cells (2.5×10^6 cells/dish) were treated with E₂, 16 α -OHE₁ or 2-ME₂ for 7 h, and cellular extract was incubated with ODN1 (1 μ M) for 1 h at 4 °C. The DNA-bound proteins were separated on a 10% SDS-polyacrylamide gel and identified by immunoblotting with antibodies specific for ATF-1, CREB, c-Jun, c-Fos, p50 and p65. The results shown represent three separate binding experiments.

and 16 α -OHE₁ treatment on ATF-2 binding to ODN1 (Fig. 7A). This inhibitory effect was similar to that observed with ICI 182,780 (Fig. 7B). To verify that the increase in ATF-2 binding was due to phosphorylation, membrane (Fig. 7A) was stripped and reblotted with a phospho-ATF-2 specific antibody. The binding of phosphorylated ATF-2 to ODN1 was increased by 2.5-fold in the presence of E₂ and by fourfold in the presence of 16 α -OHE₁, compared with control cells. In the presence of 2-ME₂, the effects of E₂ and 16 α -OHE₁ were significantly reduced (Fig. 7C). These results show that both E₂ and 16 α -OHE₁ enhanced the DNA-binding activity of ATF-2 through its phosphorylation, and that 2-ME₂ downregulated this process.

We also examined the DNA-binding activity of c-Jun, c-Fos, ATF-1, CREB and NF- κ B-p65/p50 in MCF-7 cells to test the effects of E₂, 16 α -OHE₁ and 2-ME₂. Our results showed that these transcription factors bound to ODN1 constitutively; however, c-Jun and c-Fos binding was altered by E₂ or 16 α -OHE₁ treatment (Fig. 8). With E₂, binding of c-Fos and c-Jun to ODN1 increased by 2- and 1.3-fold respectively, whereas with 16 α -OHE₁ the binding of c-Jun increased by twofold over the control.

10 nM E ₂	—	+	—	+	—	—	+	—	—	+	—	+	—
10 nM 16 α	—	—	+	—	+	—	—	+	—	—	+	—	+
10 nM 2-ME ₂	—	—	—	—	—	+	+	+	—	—	—	+	+
100 nM ICI	—	—	—	—	—	—	—	+	+	+	—	—	—
10 μ g AFT-2 dom neg mutant	—	—	—	+	+	+	+	+	—	+	+	—	—
10 μ g AFT-2 wild-type	—	—	—	—	—	—	—	—	—	—	—	+	+

Figure 9 Effect of ATF-2 dominant negative mutant (dom neg mut) on E₂ and 16 α -OHE₁-stimulated cyclin D1 transactivation. MCF-7 cells were cotransfected with the full-length cyclin D1 luciferase reporter plasmid (-1745CD1-LUC), 10 μ g expression vector for ATF-2 dominant negative mutant or ATF-2 wild-type, and *Renilla* luciferase control. Cells were treated with 10 nM E₂, 16 α -OHE₁ or 2-ME₂ as separate agents or in combination for 7 h, and luciferase activity was determined. The results shown are the mean $\pm$ S.E. of three separate experiments. *Significant ($P < 0.0001$) induction compared with untreated control; **significant inhibition compared with E₂/16 α treatment groups ($P < 0.001$); #significantly different from E₂/16 α -OHE₁ and ATF-2 (dom neg mut) treated cells ($P < 0.05$); &significantly different from E₂/16 α -treated cells ($P < 0.01$).

Treatment with 2-ME₂, as a single agent, or in combination with E₂ or 16 α -OHE₁, caused a significant reduction of both c-Fos and c-Jun binding to ODN1.

Effect of ATF-2 dominant negative mutant on cyclin D1 transactivation

We next performed transfection studies using an ATF-2 dominant negative mutant to verify the importance of ATF-2 protein in cyclin D1 activation. Cells were transfected with the full-length cyclin D1 reporter plasmid along with dominant negative mutant expression vector. After transfection, cells were treated with 10 nM E₂ or 16 α -OHE₁ in the presence or absence of 2-ME₂ for 7 h, and cyclin D1 luciferase activity was measured. Our results (Fig. 9) showed that transfection of cells with the ATF-2 dominant negative mutant resulted in a significant reduction (four- to sixfold) of E₂- and 16 α -OHE₁-induced cyclin D1 transactivation. ATF-2 dominant negative mutant in the presence of

Figure 10 Effects of E₂, 16α-OHE₁ and 2-ME₂ on p38 MAP kinase activity in MCF-7 breast cancer cells. MCF-7 cells (2.5×10⁶ cells/dish) were treated with 10 nM E₂ or 16α-OHE₁ in the presence or absence of 10 nM 2-ME₂ or 100 nM ICI 182,780 for 7 h. Cells were then lysed, and active p38 MAP kinase (phospho-p38) was detected by immunoblotting. To ensure equal protein loading, the membrane was stripped and reprobed for total p38 MAP kinase. The result presented here represents three separate experiments.

2-ME₂ further suppressed cyclin D1 promoter activity. In contrast, overexpression of the ATF-2 wild-type protein partially reversed the inhibitory effects of 2-ME₂ on cyclin D1 transactivation, confirming the importance of ATF-2 in the action of 2-ME₂.

Effects of E₂ and its metabolites on p38 MAPK activation in MCF-7 cells

ATF-2 is activated by phosphorylation, in part, by p38 MAPK (Waas *et al.* 2001, Recio *et al.* 2002). We therefore examined phosphorylation of p38 MAPK in MCF-7 cells. Cells were treated with 10 nM E₂ or 16α-OHE₁, in the presence or absence of 10 nM 2-ME₂ or 100 nM ICI 182,780, and p38 MAPK was detected by Western blotting. Our results (Fig. 10) show that E₂ or 16α-OHE₁ caused a threefold increase in the level of phospho-p38 MAPK as compared with the control. In contrast, 2-ME₂ when combined with E₂ or 16α-OHE₁, significantly reduced the level of phospho-p38 MAPK. ICI 182,780 also significantly reduced the effect of E₂ and 16α-OHE₁ on phospho-p38 levels (Fig. 10). These results indicate that E₂ and its metabolites altered p38 MAPK activity, and suggest a mechanism for the activation of ATF-2.

Discussion

In this report, we present evidence for the growth-inhibitory effect of low concentrations of 2-ME₂ on MCF-7 breast cancer cells. We found that E₂ and 16α-OHE₁ significantly enhanced cyclin D1 protein level compared with the control. Transient transfection experiments, using a full-length cyclin D1 promoter

construct, show that E₂ and 16α-OHE₁ increased cyclin D1 promoter activity by 7.5- and 9.5-fold respectively, whereas 2-ME₂ reduced the stimulatory effects of these compounds. Our results also show that transactivation of cyclin D1 by E₂ and 16α-OHE₁ is mediated by the CRE/ATF motif. E₂ and 16α-OHE₁ facilitated the binding of ATF-2 to the CRE/ATF motif. Increase in DNA binding of ATF-2 was associated with phosphorylation of ATF-2 and p38 MAPK, events that were significantly inhibited by 2-ME₂ and ICI 182,780. These results indicate that estrogenic induction of cyclin D1 transcription involves the p38 phosphorylation cascade that is inhibited by 2-ME₂.

E₂ was shown to upregulate the levels of cyclin D1 at the mRNA and protein levels along with other critical mediators of cell cycle progression (Altucci *et al.* 1996, 1997, Foster & Wimalasena 1996, Prall *et al.* 1997, Doisneau-Sixou *et al.* 2003). Although E₂-induced regulation of cyclin D1 transcription has been established by these and other studies, the molecular mechanism or mechanisms are complex and poorly defined (Planas-Silva *et al.* 1999, Sabbah *et al.* 1999, Foster *et al.* 2001). It is important to note that the cyclin D1 promoter does not contain an ERE element; however, it contains regulatory elements for ATF-2, AP-1, CREB, NF-κB, E2F and SP-1, all of which have been shown to regulate its gene expression (Pestell *et al.* 1999, Sutherland & Musgrove 2004).

We found that the CRE/ATF site, located at position -54 from the transcriptional start site of the cyclin D1 promoter was required for the induction of cyclin D1 by E₂ and 16α-OHE₁. Mutation of this site led to a dramatic reduction (60–80%) in the cyclin D1 promoter activity induced by these agents (Figs 4B and 6B). The marked increase in the DNA binding of ATF-2 in the presence of E₂ and 16α-OHE₁ and its inhibition by 2-ME₂ support the role of CRE/ATF-2 site. However, there was about a twofold induction of cyclin D1 promoter activity in ATF/CRE mutant constructs in the presence of E₂. The low level of promoter activity in ATF/CREMUT constructs could be attributed to the binding of AP-1 proteins through consensus or AP-1-like elements. Increased binding of c-Jun and c-Fos to the cyclin D1 promoter fragment supports the role of AP-1 proteins in E₂-induced cyclin D1 transactivation. In contrast, the binding of ATF-1, CREB or NF-κB proteins to the cyclin D1 promoter fragment did not show any change in the presence of E₂, 16α-OHE₁ or 2-ME₂. These results are consistent with previous reports on the roles of the CRE/ATF and AP-1 sites in cyclin D1 gene activation (Brown *et al.* 1998, Beier *et al.* 1999, Lee *et al.* 1999, Liu *et al.* 2002). However, mutation of the AP-1 site, while retaining the ATF/CRE site, did not affect cyclin D1 promoter activity. The ability of ATF/CREB proteins to form selective heterodimers with AP-1 proteins (Uht *et al.* 1997,

Chinenov & Kerppola 2001), the presence of AP-1-like elements (GACTA versus the consensus AP-1 element TGACTAA) close to the ATF/CRE site and the ability of ER α to interact with the Jun protein (Teyssier *et al.* 2001) might be important factors in the flexibility of the cyclin D1 transcriptional response.

ICI 182,780 could inhibit cyclin D1 promoter activity induced by E₂ and 16 α -OHE₁, suggesting that activation of cyclin D1 involves the function of the ER. ICI 182,780 is able to inhibit nongenomic action of E₂, such as the activation of the phosphorylation cascade (Wade *et al.* 2001, Kinoshita & Chen 2003). ICI 182,780 binding of ER α provides it with a unique conformational state, interrupting protein-protein associations (Weatherman *et al.* 2002, Margeat *et al.* 2003). In addition, ligand-induced alterations in signaling pathways determine the availability and homo/heterodimer formation between ATF-2, c-Jun and c-Fos, leading to their binding to the CRE/ATF motif of the cyclin D1 promoter. The formation of these multiprotein complexes and their DNA binding appears to be facilitated by the presence of E₂ and 16 α -OHE₁, but disrupted by 2-ME₂ and ICI 182,780.

While the ability of 2-ME₂ to inhibit E₂-induced growth stimulation suggests that it might be functioning like an antiestrogen, 2-ME₂ does not have the properties of ICI 182,780 or other antiestrogens. 2-ME₂ has very low binding affinity for ER ($\sim 1\%$ that of E₂) (Dubey *et al.* 2000, Brueggemeier *et al.* 2001) and inhibits both ER-positive and ER-negative tumor growth (Robertson 2001, Schumacher & Neuhaus 2001). However, binding affinity does not always correlate with biologic activity. Previous studies have indicated that 16 α -OHE₁ binds to the classical ER α with an affinity $\sim 10\%$ of that of E₂; however, it is as potent as E₂ in stimulating uterine growth (Suto *et al.* 1999). 16 α -OHE₁ was slightly more potent than E₂ in stimulating DNA synthesis, cell cycle progression and cyclin D1 expression in MCF-7 cells (Lewis *et al.* 2001). Hence, the antiestrogenic effects of 2-ME₂ might not be dependent on its high affinity binding to the classical ER. Alternatively, as suggested by Kousteini *et al.* (2001), the classical binding assays may not capture the efficiency of ligand association rates, but rather the ratio of association to dissociation rates.

It is also possible that 2-ME₂ may utilize a receptor that is distinct from the classical ER. Potential receptors include: 1. the type II ER (Markaverich & Clark 1987, Shoulars *et al.* 2002); 2. one or more of the variants of ER α or ER β (Fuqua *et al.* 1991) and 3. certain members of the nuclear orphan receptor family (Laudet *et al.* 1992). Plasma membrane ER that mediates rapid activation of signaling pathways in response to E₂ is found in target cells (Migliaccio *et al.* 1996, Falkenstein *et al.* 2000, Kousteni *et al.* 2001). Although plasma membrane ER is reported to be similar to the classical

nuclear ER α , unique ligand specificity might exist due to ER association with scaffold proteins, such as caveolin (Razandi *et al.* 1999, Marquez & Pietras 2001, Song *et al.* 2004). Therefore, E₂ and 16 α -OHE₁ can utilize a number of signaling pathways other than the classical ER pathway to stimulate the transcription of cyclin D1. The mechanism of 2-ME₂ in antagonizing the effects of E₂ appears to involve a blockade of estrogenic signaling through p38 MAPK and ATF-2 phosphorylation.

ATF-2 is phosphorylated by JNK/SAPK and p38 MAP kinases, in response to cellular stress (van Dam *et al.* 1995, Raingeaud *et al.* 1996), and by extracellular signal-regulated kinases (ERK), in response to growth factors (Albanese *et al.* 1995, Watanabe *et al.* 1996). Phosphorylation of ATF-2 increases its transcriptional activity by relieving intramolecular inhibitory interaction between the DNA-binding domain and the transactivating domain (Abdel-Hafiz *et al.* 1992, Kawasaki *et al.* 2000). In our experiments, increased ATF-2 binding to DNA was facilitated by ATF-2 phosphorylation and associated cyclin D1 transcriptional activation. Activation of p38 MAPK appears to be responsible for ATF-2 phosphorylation, since a three- to fourfold higher level of phosphorylated p38 MAPK is found in cells treated with E₂ and 16 α -OHE₁ than in control cells. 2-ME₂ blocked the stimulatory effects of E₂ and 16 α -OHE₁ on ATF-2 phosphorylation and cyclin D1 promoter activation. The inhibitory effect of 2-ME₂ might be due to reduced phosphorylation of p38 MAPK. However, it is not known what step of the signaling cascade, leading to the phosphorylation of p38 MAPK, is inhibited by 2-ME₂.

Activation of p38 MAPK might be mediated by nongenomic action of estrogens. Recent studies reveal many instances of estrogenic action with activation of Src/Shc/ERK signaling pathways by ER α located on membrane sites of target cells (Castoria *et al.* 2001, Kousteni *et al.* 2001, Song *et al.* 2002, 2004). Estrogenic action may include ER interaction with the G protein-coupled receptor, GPCR30, which stimulates adenylyl cyclase activity, leading to the production of cAMP and associated signaling (Aronica *et al.* 1994, Filardo *et al.* 2002). Alternatively, E₂ stimulation of MCF-7 cells may induce a direct interaction between ER α , Src and the p85 subunit of phosphatidylinositol (PI)3-kinase, thereby activating the PI3K/Akt pathway (Migliaccio *et al.* 1996). In another study, the HER-2 signaling pathway was implicated in E₂-induced activation of Akt (Stoica *et al.* 2003). Akt may activate p38 MAPK (Madrid *et al.* 2001). Further studies are needed to validate the involvement of these pathways in the activation of p38 MAPK by E₂.

Previous studies indicated that 2-ME₂ inhibits DNA synthesis in MCF-7 and MDA-MB-231 breast cancer cells (Seegers *et al.* 1989, Brueggemeier *et al.* 2001, Schumacher & Neuhaus 2001). In human prostate

cancer, 2-ME₂ inhibited tumor growth by arresting cells in the G₂/M phase of the cell cycle (Kumar *et al.* 2001). The 2-ME₂-induced G₂/M block, occurring at 3 µM concentration, was associated with a significant increase in p21 and cdc2 expression. An effect of 2-ME₂ on tubulin polymerization has been reported at 3 µM or higher concentration (Cushman *et al.* 1995, Klauber *et al.* 1997, Pribluda *et al.* 2000). In the osteosarcoma 143 B cell line, a dual effect of 2-ME₂ on cell cycle was observed with G₁ arrest at 1 µM level and G₂/M arrest at 10 µM level (Golebiewska *et al.* 2002). We found that 2-ME₂ is effective in blocking the stimulatory effects of E₂ and 16α-OHE₁ on DNA synthesis, and G₁ to S phase progression. The increased expression of cyclin D1 caused by E₂ and 16α-OHE₁ is downregulated by 2-ME₂. In the absence of E₂, the low level of DNA synthesis found in phenol red-free cells is inhibited by 2-ME₂, although this was not sufficient to be detected as a cell cycle arrest by flow cytometry. Thus, in the absence of E₂, MCF-7 cells remain largely (77–82%) in the G₁ phase in the presence or absence of low concentrations of 2-ME₂. This may be because breast cancer cells with low growth fraction are more resistant to the action of antiproliferative agents than log-phase cells (Carlisle *et al.* 2002).

In summary, our results show that E₂ and 16α-OHE₁ stimulate cell growth and cell cycle progression by upregulating cyclin D1 expression, whereas 2-ME₂, like an antiestrogen, blocks the stimulatory effects of E₂ and 16α-OHE₁. Estrogenic regulation of the cyclin D1 gene occurs at the transcriptional level and is mediated by the CRE/ATF-2 element, located at -54 upstream of the cyclin D1 promoter transcription start site. Transcriptional activation of cyclin D1 by E₂ and 16α-OHE₁ is dependent on phosphorylation and binding of ATF-2 to the CRE/ATF motif. Our results demonstrate an antiestrogenic effect of 2-ME₂ in MCF-7 breast cancer cells, and suggest that cyclin D1 might be a key target in the growth-inhibitory actions of 2-ME₂. Our results also indicate that transduction of the estrogenic signal for cell proliferation includes phosphorylation cascades involving the activation of p38 MAP kinase.

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