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Estrogen-induced mitogenesis of MCF-7 cells does not require the induction of mitogen-activated protein kinase activity

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Abstract

Estrogen mediates the transcription of responsive genes via its interaction with the estrogen receptor (ER). This ligand-dependent transcriptional activity has been the mechanistic basis for understanding estrogen-induced proliferation. However, recent reports suggest that estrogen stimulation results in activation of the mitogen-activated protein kinase (MAPK) cascade in an ER-dependent manner suggesting that mitogenesis may be mediated through this cytoplasmic signaling cascade. In this study, we demonstrate that estrogen stimulation of MCF-7 cells does not activate MAPK regardless of hormone concentration, serum concentration, or cell density. We also excluded the activation of MAPK through autocrine effects after estrogen treatment. Finally, concentrations required for estrogen-induced mitogenesis and estrogen-mediated transcription were shown to be the same. These results support transcriptional activation as the primary mechanism for estrogen-mediated mitogenesis. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Estrogen-induced mitogenesis; MCF-7; Mitogen-activated protein kinase

1. Introduction

Breast epithelial and uterine cells proliferate in response to estrogen (reviewed in [1]). 17β-Estradiol (E_2), the most prevalent estrogen produced by the ovaries, diffuses through the plasma membrane and into the nucleus to bind the ER. Hormone binding results in the dissociation of heat shock proteins from ER followed by receptor dimerization. The dimer contacts DNA at specific palindromic sequences, known as estrogen response elements (EREs). ER modulates gene expression of ERE-containing genes by functioning as a ligand-dependent transcription factor. EREs have been identified in several genes involved in cell cycle progression, including the immediate-early gene c-*myc* [2]. The classical model of E_2-induced proliferation is based on the ability of ER to coordinate expression of genes essential for mitogenesis.

Recent findings have called this model into question. Several studies have demonstrated that E_2 can activate several different cytoplasmic signaling cascades. E_2 stimulates the activity of protein kinase C in endometrial fibroblasts [3]. In a malignant breast epithelial cell line, E_2 increases synthesis of phosphatidylinositol and activates phosphatidylinositol kinases [4]. Finally, E_2 activation of the mitogen-activated protein kinases (MAPKs), Erk1 and Erk2 occurs in a variety of cell types, including breast epithelium (reviewed in [5]). Support for the existence of this pathway in vivo was demonstrated in a rat mammary carcinoma model. In this system, oophorectomy diminished the quantity of total and active MAPK present in the cancer cells [6]. Administration of E_2 increased both of these levels, suggesting that MAPK expression and activation can be regulated by E_2.

Activation of the MAPK cascade raises the possibility that some or all of the proliferative effect of E_2 may be mediated through this established mitogenic pathway. This theory was supported by the finding that

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PD098059, an inhibitor of MAPK activation, prevents E_2-induced mitogenesis in cardiac fibroblasts and transformed breast epithelial cells [7,8]. Furthermore, E_2 was shown to stimulate cell cycle progression in NIH-3T3 cells transiently transfected with an ER mutant that had no transcriptional activity, presumably by activating MAPK [9]. Cumulatively, these studies indicate an involvement of the MAPK cascade in the proliferative response to E_2 stimulation.

In the current study, we further explore the relationship between MAPK and E_2-induced mitogenesis. We found no evidence that mitogenic concentrations of E_2 specifically activate MAPK in breast cancer cells. We observed neither immediate activation nor delayed activation due to stimulation of an autocrine loop. Inhibition of mitogenic growth factors by neutralizing antibodies failed to inhibit the proliferative effects of E_2. In addition, we demonstrate that E_2-induced transcription cannot be uncoupled from E_2-stimulated proliferation. Cumulatively, these findings indicate that activation of MAPK is not an essential feature of E_2-induced cell cycle progression.

2. Results

2.1. Phosphorylation status of MAPK in the presence of E_2

Activation of MAPK family members Erk1 and Erk2 is triggered by the phosphorylation of a threonine and a tyrosine residue in their regulatory site (reviewed in [10]). Contradictory data has been reported regarding the ability of E_2 to stimulate activation of Erk1 and Erk2 in breast epithelial cells [8,11–13]. Variations in the methodology detailed in these studies include E_2 and serum concentrations, cell density, and the method of hormone delivery [8,11–16]. We undertook a series of studies to determine which, if any, of these parameters could account for the varied results.

To address conflicting reports of MAPK activation, we assayed the effect of E_2 on the phosphorylation of Erk1 and Erk2 in the ER+ breast cancer cell line, MCF-7 (Fig. 1A). Cells were starved for 24 h and then treated with varying amounts of E_2 by diluting the hormone in 0.1 ml of fresh starvation medium and then adding this to the existing culture media. Cells were lysed after 5 min of treatment and total MAPK expression levels and MAPK phosphorylation were assessed by Western blotting. EGF, a well-documented activator of MAPK, induced phospho-Erk1 and -Erk2 levels as compared with the basal levels found in serum-starved cells. However, concentrations of E_2 ranging from 2×10^{-7} to 10^{-9} M did not elevate phosphorylated Erk1 and Erk2 above basal levels.

Several studies have detailed varying durations of starvation prior to hormone stimulation [12,13]. Increasing the period of starvation to 72 h did not affect the levels of phospho-Erk1 or -Erk2 present after 5 min of E_2 treatment. Previously, Strobl and Lippman demonstrated that 3.3 nM E_2 was still retained within the cell after this extended starvation period, indicating that MCF-7 cells may never be complete devoid of E_2 [17]. Though residual E_2 may be present in these cells, the addition of exogenous E_2 is sufficient to increase the rate of proliferation as well as transcriptional activity in serum-starved cells presumably in the absence of increased MAPK activity. These results are consistent

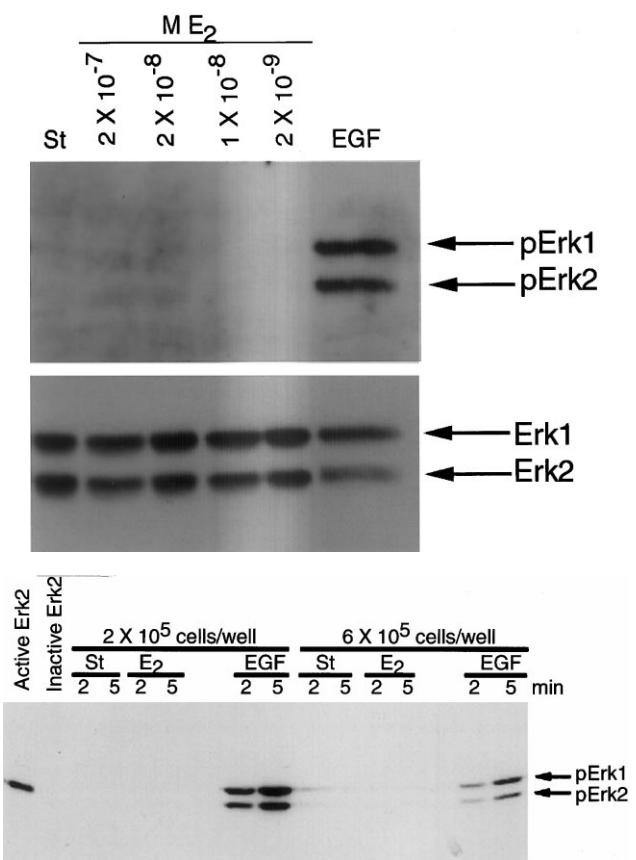


Fig. 1. E_2 does not activate MAPK in MCF-7 cells. (A) MCF-7 cells were stimulated (using the 'add-to' method) with 10 ng/ml EGF or variable concentrations of E_2 (ranging from 2×10^{-9}–2×10^{-7} M) for 5 min. Cell lysates were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a monoclonal antibody against diphosphorylated Erk1 and Erk2 (pErk1 and pErk2). The blot was stripped and total levels of Erk1 and Erk2 were detected using an antibody recognizing Erk2 that has cross-reactivity with Erk1. The serum-starved (St) negative control was mock treated with 100 μl of starvation medium. (B) MCF-7 cells were seeded either at a low density (2×10^5 cells per well in a six-well dish) or a high density (6×10^5 cells per well). Cells were treated (using the 'add-to' method) with EGF or E_2. Cells were lysed after 2 or 5 min of stimulation and total levels of Erk expression and the amount of phosphorylation was determined as described in (A). Commercially available positive and negative controls for MAPK phosphorylation, active Erk2 and inactive Erk2 (New England Biolabs), were included on this Western.

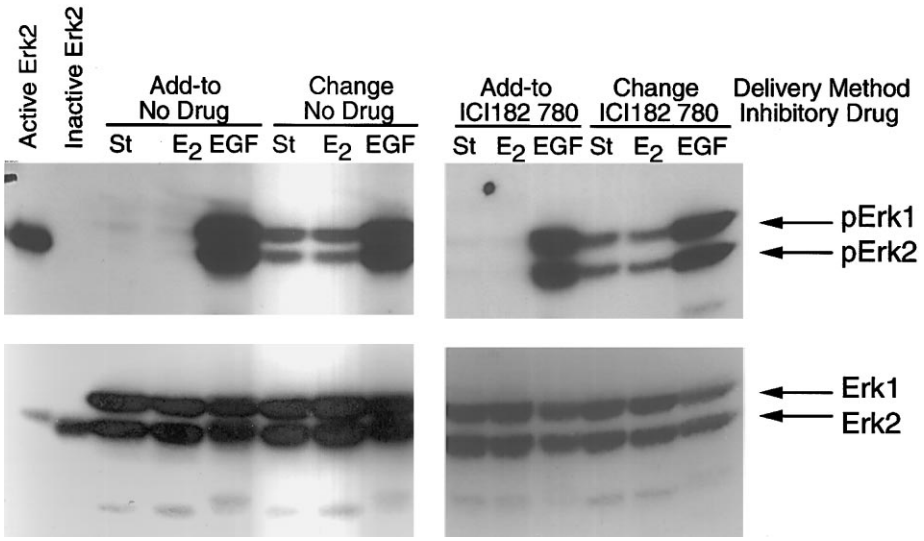


Fig. 2. Effect of an ER antagonist on EGF- or E_2-induced phosphorylation of Erk1 and Erk2. Serum-starved MCF-7 cells were pre-treated with 2 μM ICI 182 780 (ER antagonist) for 2 h prior to hormone or growth factor stimulation. For 'add-to' lanes, 100 μl of fresh starvation medium containing sufficient EGF or E_2 to generate a final concentration of 10 ng/ml or 2×10^{-8} M, respectively, was pipetted into each well. For 'change' samples, starvation medium was aspirated and replaced with fresh medium containing the appropriate concentrations of both mitogen and inhibitor. After 5 min of stimulation, cells were lysed. Total and phosphorylated levels of Erk1 and Erk2 were visualized using Western analysis.

with our previous observations [8] but contradictory published reports led us to search further for activation.

The activity of p38 kinase (a MAPK family member) may be modulated by cell density [18]. Therefore, we explored the effect of density on the ability of E_2 to activate Erk1 and Erk2 (Fig. 1B). MCF-7 cells were seeded at either 2×10^5 or 6×10^5 (confluent) cells per well in six-well dishes. Each well was treated with 2×10^{-8} M E_2 or 10 ng/ml EGF for 2 or 5 min. EGF stimulated the phosphorylation of Erk1 and Erk2 regardless of cell density though the magnitude of induction was significantly greater in the subconfluent cultures. E_2 did not measurably stimulate MAPK activity at either density; however, the basal levels of phospho-Erk1 and 2 were higher in confluent cultures.

In all of our experiments to date, we have stimulated in serum-free medium with no added growth factors. In other similar studies, hormone-depleted (charcoal-stripped) fetal bovine serum (CS-FBS) was present in culturing medium during E_2 stimulation raising the possibility that co-factors are needed for the MAPK response [12,13]. To test this possibility, we starved and treated MCF-7 cells in medium containing 0, 0.05, or 5% CS-FBS (concentrations used in other studies). Under these conditions, EGF increased phospho-Erk1 and -Erk2 to identical levels regardless of serum concentrations; however, E_2 failed to stimulate MAPK activation with or without serum in the medium (data not shown).

In searching for differences that might account for the discrepant observations, we also investigated the method of hormone delivery. In our previous experiments, the hormone was added without changing the medium ('add-to'). We compared this mode of delivery to replacing the medium at the time of activation with pre-warmed medium containing the hormone ('change'). Using the 'change' technique, we observed increasing levels of active MAPK (Fig. 2). The appropriate control for this experiment is cells that were stimulated by replacing their medium with starvation medium in the absence of any hormone or inhibitory compounds (St, No Drug, Change). When compared with the control, it is apparent that the increase in MAPK phosphorylation is not a result of E_2 stimulation, but rather an effect generated by replacing the medium. The increase in basal levels of phosphorylated MAPK was consistently observed, though the absolute level of increase was variable between experiments. Induction of MAPK due to media change, either with or without E_2, was not inhibited by the pure antiestrogen, ICI 182 780 further indicating that this response is independent of ER signaling. Without appropriate controls, adding E_2 by changing the culture medium may be mistaken for specific activation of MAPK.

2.2. The role of autocrine loops in E_2-induced proliferation

Previously, we demonstrated that the addition of pharmacological inhibitors of MAPK activation (PD098059 and U0126) up to 6 h after E_2 stimulation prevents mitogenesis [8]. Considering the absence of measurable MAPK activation by E_2, we theorized that MAPK activity might be necessary as a delayed early event induced via an autocrine loop similar to that

observed in human umbilical vein endothelial cells (HUVEC) [19]. To test this hypothesis, we used medium taken from hormone stimulated cells to re-stimulate fresh cultures (Fig. 3). Consistent with the transient nature of the mitogenic MAPK response, after 3 h of stimulation, we observed a very minor increase in phospho-Erk1 and -Erk2 levels only in the EGF-stimulated cultures. When EGF-conditioned medium was transferred to untreated cells, a dramatic increase in the level of active MAPK occurred, likely due to the continued presence of EGF in the medium. The transfer of conditioned medium from serum-starved cells stimulated a minor increase in the basal levels of active Erk1 and Erk2. This activation is likely an artifact resulting from the medium change (as shown in Fig. 2) rather than the presence of a specific activator of MAPK in the starvation medium. Conditioned medium from E_2-treated cells had no effect on the levels of phospho-Erk1 and Erk2 when compared with the levels present in cells treated with conditioned starvation medium for 5 min. Conditioned medium from the E_2-stimulated cells was still capable of stimulating ER-mediated transcription and inducing proliferation (data not shown). The time course for activation was further explored by inducing MCF-7 cells with E_2 and harvesting cell lysates every 15 min for 4 h (data not shown). At no time were the levels of phospho-Erk1 and -Erk2 elevated beyond basal levels. These data indicate that there is not a significant MAPK activation resulting from autocrine loops or directly from long-term E_2 treatment.

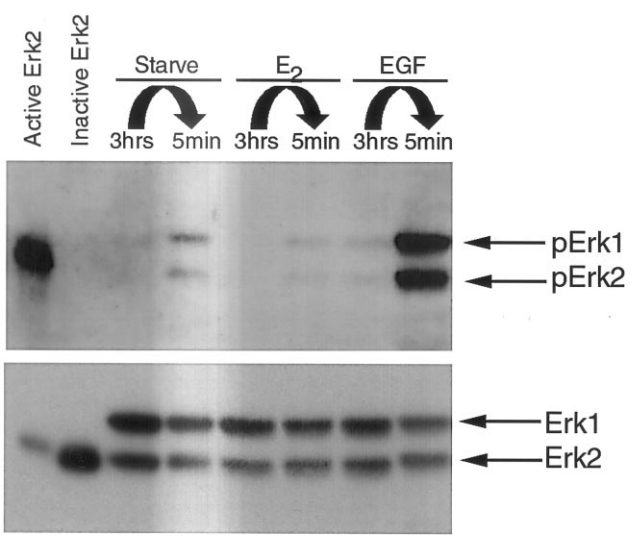


Fig. 3. Conditioned medium from MCF-7 cells treated for 3 h with E_2 does not stimulate the phosphorylation of Erk1 and Erk2. Serum-starved MCF-7 cells were stimulated with 10 ng/ml EGF or 2×10^{-8} M E_2 at 37°C in 95% O_2/5% CO_2. After 3 h this conditioned medium was transferred to a fresh well of cells and incubated at room temperature. Five minutes later cells were lysed and the total and phosphorylated levels of Erk1 and Erk2 were assessed using Western analysis. The minor increase in active Erk1 and Erk2 levels present in cells treated for 5 min with conditioned medium from starved or E_2-treated cells is likely to be an artifact of the media change (as seen in Fig. 2) rather than the presence of a specific activator in the medium.

Previous work demonstrated that an IGF-I antibody could prevent IGF-I-induced mitogenesis, but did not inhibit the proliferative effect of E_2 on MCF-7 cells [20]. However, IGF-I is not the only growth factor expressed as a result of E_2 exposure in MCF-7 cells. Others include EGF and bFGF [21,22]. We tested the ability of neutralizing antibodies directed against these factors to inhibit E_2-mediated growth (Fig. 4). MCF-7 cells treated with EGF increased the population of cells in S-phase greater than 4-fold. When co-incubated with either a neutralizing EGF antibody or an antibody recognizing the EGF receptor (EGFR), proliferation rates were maintained at basal or near-basal (starved) levels. Consistent with published data, an IGF-I neutralizing antibody prevented IGF-I induced mitogenesis, as did a bFGF antibody on bFGF-stimulated growth [20]. However, none of these antibodies demonstrated a statistically significant affect on E_2's ability to stimulate mitogenesis. While these studies do not exclude an autocrine growth factor loop as the basis for E_2-induced proliferation, the weight of evidence indicates that it is unlikely to be a major component of this process.

An additional site for potential interaction between E_2 and the cell surface exist. A small subpopulation of ER may localize to the plasma membrane [23]. However, an antibody recognizing ER did not effect E_2-mediated proliferation (Fig. 4).

2.3. Concentration of E_2 necessary for transcription and proliferation

It has been reported that NIH-3T3 cells expressing transcriptionally inactive ER still proliferate in response to E_2 [9], uncoupling ER-mediated transcription from proliferation. Therefore, we might expect to observe a different dose–response for these two activities (Fig. 5). Consistent with published reports, concentrations greater than 2×10^{-11} M increased the number of cells in S-phase approximately 2.5-fold [24]. A minor, yet statistically significant, increase in S-phase population was observed in cells treated with 2×10^{-12} M E_2. This demonstrates that MCF-7 cells respond mitogenically to physiologic doses of E_2 though activation of MAPK is not detected (Fig. 1a).

To assess the concentration of E_2 necessary for a transcriptional response, we measured the dose–response using three independent measures of ER-mediated transcription, c-*myc* expression, ERE-luciferase reporter gene activity, and pS2 levels. c-*myc* is an immediate-early gene that contains an ERE in its promoter [2]. Using Northern analysis, we examined c-*myc*

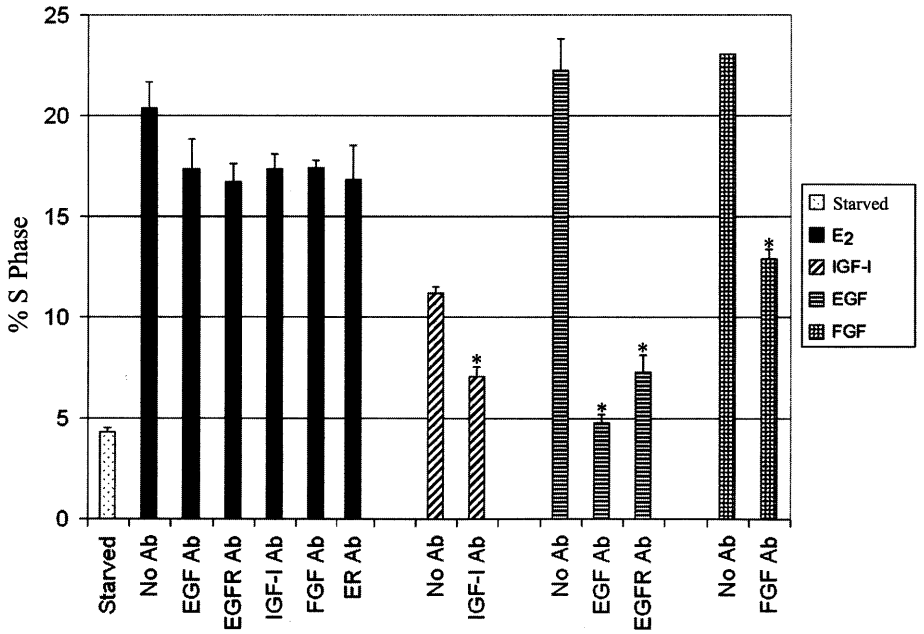


Fig. 4. Antisera against growth factors expressed in response to E_2-stimulation do not inhibit E_2-induced cell cycle progression. Serum-starved MCF-7 cells were stimulated with 2×10^{-8} M E_2 in the presence or absence of 10 μg/ml EGF, EGFR, IGF-I, FGF, or ER antisera for 24 h. Cells were harvested, fixed, and stained. The S-phase population was determined using flow cytometry. The data represent an average of results obtained from three experiments. Bars, S.D. Statistically significant repression of mitogen-induced proliferation was determined by performing a Student's *t*-test. * Denotes a *P*-value < 0.05.

transcript levels in MCF-7 cells after 1 h of hormone stimulation. A typical blot is illustrated in Fig. 6A. Treatment with concentrations between 2×10^{-12} and 2×10^{-8} M E_2 consistently resulted in increased c-*myc* expression as compared with the basal levels found in serum-starved cells. Less concentrated doses had no transcriptional effects, consistent with mitogenic concentrations of the hormone.

Since c-*myc* is an immediate early gene, its expression is upregulated in response to a variety of mitogenic signals and pathways. Therefore, expression of this gene may be indirectly responsive to E_2. For this reason, we measured the transcriptional response using a transient reporter system. MCF-7 cells were transfected with one of two luciferase constructs containing E_2-responsive promoters (TK-ERE, vitellogenin promoter, or 3X-ERE, three consensus EREs). Transfected cells were treated with serial dilutions of E_2 (2×10^{-8}–2×10^{-15} M) for 24 h prior to lysis and assay of luciferase activity. E_2 concentrations greater than 2×10^{-10} M stimulated a 3.5–4-fold increase in luciferase activity compared with serum-starved cells. 2×10^{-11} ME_2 almost doubled the amount of luciferase activity, whereas lower concentrations had no effect.

The pS2 gene was identified as being E_2-responsive in a screen of rapidly induced transcripts in E_2-treated MCF-7 cells, does not require de novo protein synthesis for its expression, and contains a functional ERE in its promoter [25–28]. In clinical breast cancer samples, pS2 expression is correlated with responsiveness to hormone therapy and favorable tumor characteristics [29]. We measured pS2 transcript levels after 24 h of stimulation with variable concentrations of E_2. A typical blot is shown in Fig. 6C. Expression of pS2 more than doubled in response to concentrations of E_2 greater than 2×10^{-11} M. Consistent with proliferation and c-*myc* expression, 2×10^{-12} M reproducibly generated a small but significant effect on pS2 transcription. These three independent assays of transcriptional activity indicate that the minimal concentration of E_2 sufficient to modulate gene expression as well as the lowest dose capable of inducing cell cycle progression are equivalent in wild-type MCF-7 cells suggesting that these two processes are associated.

3. Discussion

ER-mediated transcription is generally accepted as the molecular mechanism that enables a proliferative response to E_2 stimulation [1]. However, the recent report by Castoria et al. raises significant questions about this model [9]. That study used NIH-3T3 cells transiently transfected with a transcriptionally inactive ER construct (lacking the DNA binding domain). Surprisingly, these cells proliferated when treated with E_2, suggesting either an error in the existing model or the presence of an alternative pathway capable of mediating cell cycle progression. Activation of MAPK has been proposed as this alternate pathway.

In this report, we investigate the role of MAPK in E_2-induced mitogenesis. We demonstrate that E_2 treatment of MCF-7 cells stimulates cell cycle progression in the absence of detectable MAPK activation regardless of E_2 or serum concentrations, cell density, or the method of hormone delivery. Also, we add increasing support to the theory that growth factor production resulting from E_2 stimulation does not stimulate an autocrine loop that is essential for the mitogenic response in MCF-7 cells (reviewed in [30]). Since ER-mediated transcription may not be necessary for E_2-induced mitogenesis, as proposed by Castoria et al., we attempted to uncouple the transcriptional activity of E_2 from its proliferative action based on concentration differences. We found that 2×10^{-12} M is the minimal concentration of E_2 capable of inducing measurable transcription as well as stimulating cell cycle progression. Cumulatively, these findings support the theory that ER-mediated transcription directly regulates E_2-stimulated mitogenesis.

We, and others, have previously reported that E_2 stimulation does not activate MAPK in MCF-7 cells [8,11]. However, activation has been described by others [9,12,13]. The reported discrepancy may be the result of genetic differences between MCF-7 cells maintained in different laboratories. We have tested a culture of MCF-7 cells maintained in a separate laboratory (originally obtained from the ATCC) with identical results (data not shown). Under all conditions examined we did not detect activation of MAPK in response to E_2 treatment though mitogenesis still resulted.

We have reported previously that inhibition of the MAPK cascade arrests E_2-stimulated MCF-7 cells in the G1 stage of the cell cycle even though there was no immediate induction of the MAPK cascade [8]. The pharmacological inhibitors of MAPK did not effect the transcription of immediate early genes that have been reported to be transcriptional targets of ER [2,31]. However, the expression of cyclin D1, which is not regulated by ER-mediated transcription, was inhibited [32]. This suggested two alternate hypotheses (1) MAPK was activated in a delayed fashion, perhaps through the stimulation of growth factor synthesis inducing an autocrine loop resulting in the increased expression of cyclin D1 or (2) basal activity of MAPK is necessary for cell cycle progression in MCF-7 cells. From this study, we conclude that the first hypothesis is unlikely to be true. We observed no stimulation of MAPK activity with conditioned medium from E_2-treated cells. Further, neutralizing antibodies to IGF, EGF, EGFR, and FGF failed to inhibit E_2 mediated growth. These findings led us to believe that basal levels of active MAPK are essential to maintain cells in a 'receptive state' for mitogenic stimulation and that depletion of these signaling intermediates functions as a dominant negative influence on cell cycle progression.

Our studies demonstrate that activation of MAPK does not occur in response to E_2 stimulation of MCF-7 cells. Furthermore, the production of IGF-I, bFGF, and EGF does not play an essential role in E_2-induced mitogenesis. Finally, the roles of E_2 as a mitogen and

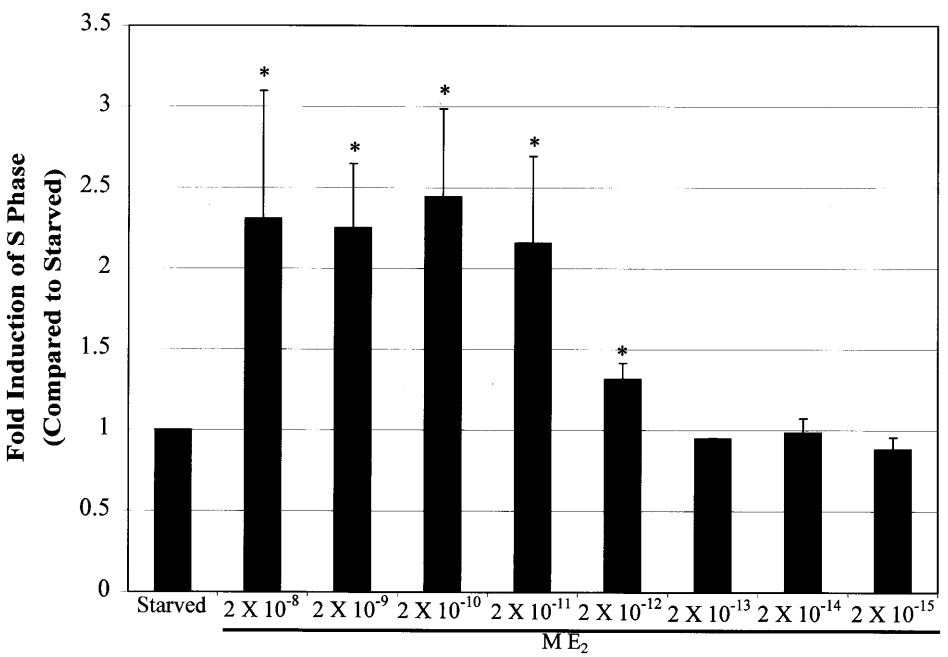
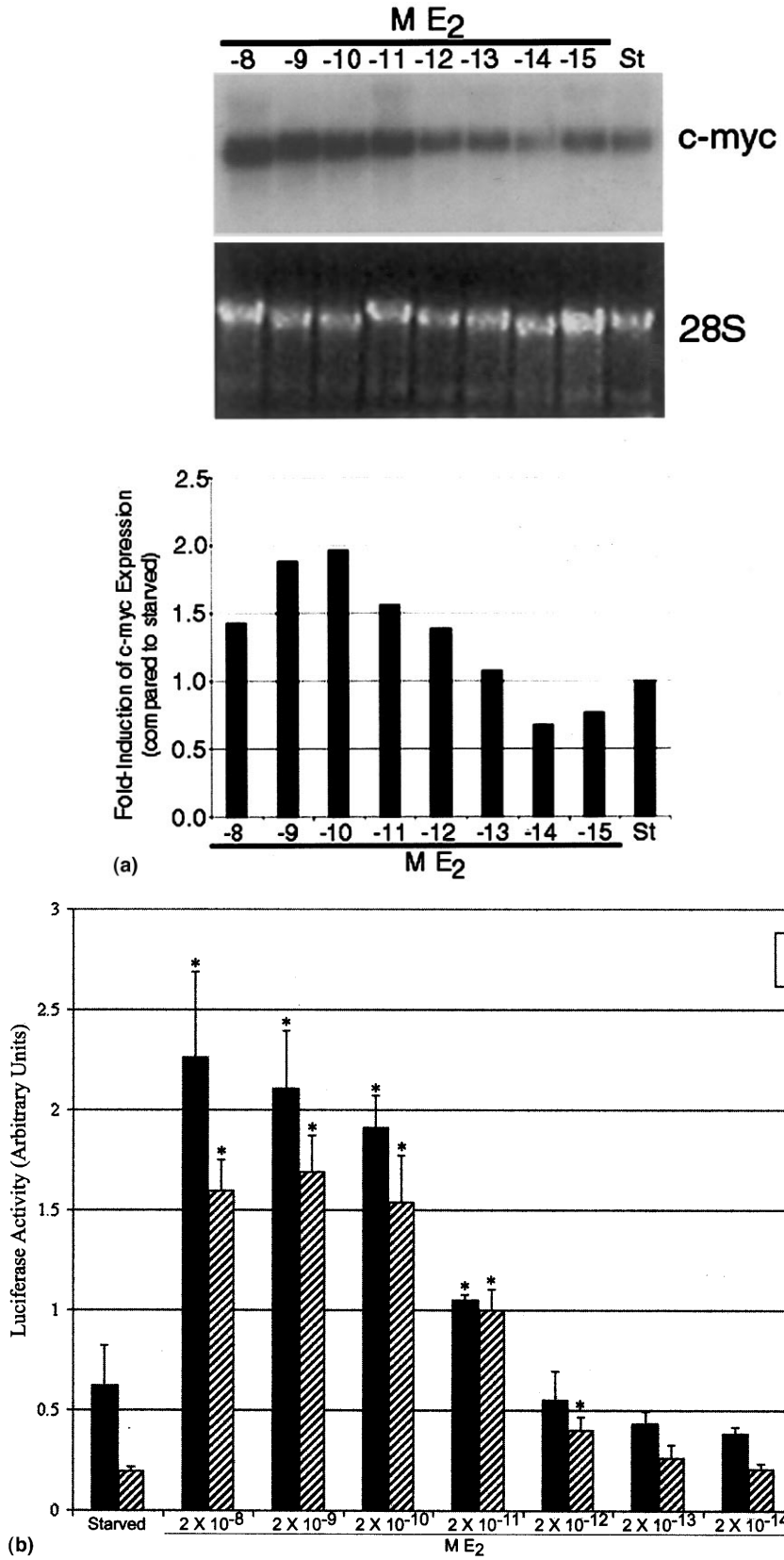


Fig. 5. Minimal concentration of E_2 able to induce mitogenesis. Serum-starved MCF-7 cells were stimulated with variable concentrations of E_2 (2×10^{-8}–2×10^{-15} M) for 24 h. Cells were harvested, fixed, and stained. The S-phase population was determined using flow cytometry. The data represent an average of results obtained from three experiments. Bars, S.D. Performing a Student's t-test revealed statistically significant increases in the percentage of cells in S-phase, compared with the serum-starved control. * Indicates a P-value < 0.05.


M E2
-8 -9 -10 -11 -12 -13 -14 -15 St
c-myc
28S
Fold-Induction of c-myc Expression (compared to starved)
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TK-ERE
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Luciferase Activity (Arbitrary Units)
3
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2 X 10-14
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M E2
(b)

Fig. 6.

transcription factor could not be uncoupled based on hormone concentration. Cumulatively these data indicate that MAPK activation is not an essential feature of E_2-stimulated proliferation and further support the central role of ER-mediated transcription in this process.

4. Materials and methods

4.1. Cell culture

MCF-7 cells were obtained from American Type Culture Collection and propagated in RPMI 1640 containing 10% (v/v) FBS (Life Technologies Inc.) at 37°C in 95% O_2/5% CO_2. ER content was measured using the ERICA kit (Abbott Labs. Inc.) and quantitated using the CAS 200 Image Analyzer (Cell Analysis Systems Inc.). MCF-7 cells express 170 fmol/mg of protein as compared with less than 10 fmol/mg of protein found in normal breast epithelial cells.

For experimental purposes, cells were seeded (except where otherwise noted) in six-well dishes at a density of 3×10^5 cells per well, 12-well dishes at 2×10^5 cells per well, or in 60-mm dishes at a density of 5×10^5 cells. 24 h after seeding, cells were washed once with 'starvation media' (phenol red-free RPMI 1640 without FBS, Life Technologies Inc.). Cells were then incubated for an additional 24 h in this medium to arrest a majority of the cells in G0/G1 and to down-regulate MAPK activity.

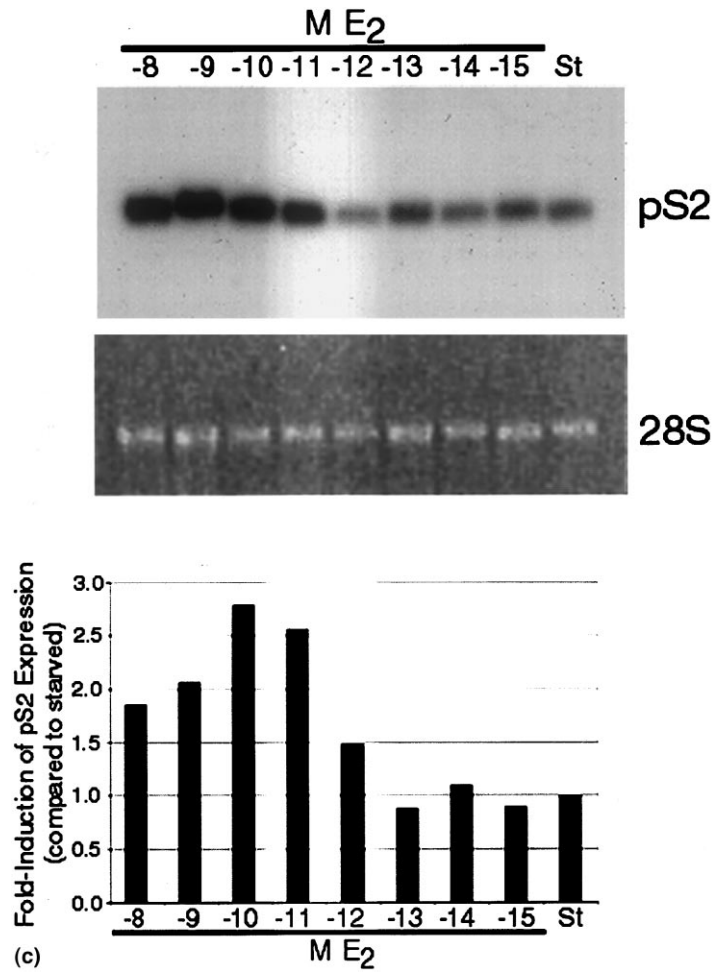

Fig. 6. Minimal concentration of E_2 able to induced transcription. (A) Representative Northern blot analysis of c-*myc* RNA expression in MCF-7 cells treated with different concentrations of E_2 (2×10^{-8}–2×10^{-15} M) for 1 h. The starved lane displays the amount of c-*myc* RNA present at time 0. Levels of the 28S ribosomal subunit were used as an internal control to correct for loading inconsistencies. (B) MCF-7 cells were transfected with a luciferase construct containing either an artificial (3X-ERE) or a naturally occurring (TK-ERE) E_2-responsive promoter. After 24 h of stimulation with varying concentrations of E_2 (2×10^{-8}–2×10^{-15} M), samples were harvested and the luciferase activity was measured. Corrections for differences in protein concentration and transfection efficiencies were made using detected levels of a cytomegalovirus-luciferase construct from a different species. The average of the normalized activity for three experiments is given. Bars, S.D. Statistically significant increases in luciferase activity, compared with the serum-starved control, were detected by performing a Student's *t*-test. * Indicates a *P*-value < 0.05. (C) Representative Northern blot of pS2 RNA expression in MCF-7 cells treated with different doses (2×10^{-8}–2×10^{-15} M) of E_2 for 24 h. Basal levels of pS2 RNA are represented in the starved lane (time 0). Loading discrepancies were corrected by measuring differences in the level of the 28S ribosomal RNA in each lane.

4.2. MAPK assay

In order to remove any factors in the conditioned medium that could affect the induction of MAPK activity, fresh starvation medium in the presence or absence of 50 μM PD098059 or 2 μM ICI 182 780 was replaced 2 h prior to stimulation with EGF or E_2. For 'Add-to' experiments, the requisite amount of EGF or E_2 necessary to achieve a final concentration of 10 μg/ml or 20 nM (respectively) was diluted in 100 μl of starvation medium. This solution was pipetted into the medium and the dish was gently swirled to disperse. For the 'Change' experiments, the starvation medium was aspirated and replaced with a fresh aliquot of starvation medium containing 10 ng/ml EGF or 2×10^{-8} M E_2. For the conditioned medium experiment, cells were stimulated for 3 h in the presence of hormone. This medium was transferred to a well of untreated cells. In all experiments, cells were stimulated for 5 min at room temperature, at which point the medium was aspirated. Cells were washed once in phosphate buffer saline (PBS) then lysed in 250 μl of 2 × sodium dodecyl sulfate (SDS) sample buffer (25 mM Tris–HCl (pH 6.8), 12.5% glycerol, 6% SDS, 0.045% bromophenol blue, 4% β-mercaptoethanol, 1 mM sodium orthovanadate). Lysates were sonicated briefly and boiled for 5 min prior to separation using 10% SDS-polyacrylamide gel electrophoresis (PAGE). The resulting gel was electrotransferred to nitrocellulose membranes (Schleicher and Schuell). Blots were blocked for 1 h at room temperature with PBS containing 0.01% Tween 20 (PBS-T) and 5% nonfat dry milk. Membranes were incubated with recommended dilutions of a phosphospecific MAPK antibody (Sigma Chemical Co. and Promega Co.) or a total MAPK antibody (Santa Cruz Biotechnology) in PBS-T milk for 2 h at room temperature. Antigen–antibody complexes were visualized by incubation with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories Inc.) for 1 h at room temperature, followed by ECL detection (DuPont NEN Life Science). Between successive probings, blots were stripped for 15 min at 54°C with 62.5 mM Tris–HCl (pH 6.7), 2% SDS, and 100 mM 2-mercaptoethanol.

4.3. Cell cycle analysis

After 24 h of stimulation with 2×10^{-8} M E_2, 10 ng/ml EGF, 30 ng/ml IGF-I, or 10 ng/ml FGF in the presence or absence of 10 μg/ml neutralizing antibodies, cells were trypsinized and pelleted. Cell pellets were washed once in PBS then fixed in 70% ethanol (in PBS) for 30 min on ice. Fixed cells were washed once in PBS followed by staining with 50 μg of propidium iodide (Roche Molecular Biochemicals) and 100 μg of RNaseA (Sigma Chemical Co.) in PBS. DNA content was determined on a per cell basis using flow cytometry. Performing a Student's *t*-test revealed statistically significant induction or repression of cell cycle progression.

4.4. Northern blotting

Total RNA was extracted from 5×10^5 cells using the Trizol reagent according to the manufacturer's instructions (Life Technologies Inc.). RNA (5 μg) was electrophoresed on a 1% agarose–2.2 M formaldehyde gel then transferred onto a nylon membrane (ICN Biomedicals Inc.). Blots were cross-linked using ultraviolet irradiation and hybridized with a ^{32}P-labeled probe generated by random priming (cDNA plasmid clones were purchased from American Type Culture Collection). Blots were washed for 10 min at 65°C with 2 × SSC (150 mM NaCl and 15 mM $Na_3C_6H_3O_7$, pH 7.0) containing 1% SDS and then with 0.1 × SSC/0.1% SDS prior to autoradiography at −80°C with intensifying screens. IMAGEQuant (Molecular Dynamics) was utilized to quantify the expression levels of the gene of interest and loading inconsistencies were corrected using detected levels of the 28S ribosomal RNA.

4.5. Transient transfections/luciferase assay

For liposome delivery, cells were washed with serum-free medium (Opti-Mem I; Life Technologies Inc.). The firefly luciferase gene (*Photinus pyralis*) under the control of an E_2-responsive promoter (artificial or from the human vitellogenin gene, kindly provided by Donald P. McDonnell) as well as a cytomegalovirus-driven luciferase construct (*Renilla reniformis*) were combined (2.5 μg/ml and 5 ng/ml, respectively) prior to transfection following the standard protocol for Lipofectin (Life Technologies Inc.). Cells were incubated with the liposome solution for 5 h, at which time the medium was aspirated and replaced with RPMI 1640 without phenol red supplemented with 10% (v/v) charcoal-stripped FBS (CS-FBS). Twenty-four hours later cells were treated with E_2 in fresh RPMI 1640 medium containing 10% CS-FBS. Cells were lysed after 24 h of stimulation and luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega Co.) and a 20/20 dual-channel luminometer (Turner Designs). Statistically significant increases in luciferase activity, compared with the serum-starved control, were determined by performing a Student's *t*-test.

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