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Difficulty demonstrating estradiol-mediated Erk1/2 phosphorylation in MCF-7 cells

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Abstract

While some studies report that estradiol (E2) activates extracellular-signal regulated kinase (Erk1/2) in MCF-7 breast cancer cells, others report E2 does not activate this signaling pathway. This study attempted to resolve the conflicting reports by investigating experimental variables that could impact Erk1/2 activation using a high through-put assay that quantitatively assessed Erk1/2 phosphorylation. Variables tested included: cell staging and dosing regimes with and without charcoal-stripped serum, different MCF-7 cell sublines and culture densities and several E2 formulations and solvents. Levels of phosphorylated Erk1/2 were normalized to cellular protein rather than to total Erk1/2 protein because an antibody purported to recognize total Erk1/2 preferentially reacted with non-phosphorylated Erk1/2, potentially exaggerating the apparent level of Erk1/2 activation. Dosing MCF-7 cells with E2 containing small amounts of stripped serum induced Erk1/2 phosphorylation; however, this induction was largely attributed to serum factors. E2 administered in serum-free medium did not significantly alter Erk1/2 phosphorylation under any condition tested; immunocytochemistry corroborated this conclusion. While phosphatase inhibitors generally increased Erk1/2 phosphorylation, they did not impact E2-altered Erk1/2 phosphorylation. It remains important to resolve the basis of conflicting reports regarding E2-induced Erk1/2 activation due to the potential importance of this pathway on breast cancer and other processes.

Keywords: MCF-7 cells; Estradiol; Erk/MAPK

1. Introduction

Many effects of estradiol (E2) are attributed to the steroid binding the estrogen receptor (ER) subtypes ERα and/or ERβ, activating ERs as transcription factors that increase the expression of specific genes [1–3]. E2 has also been reported to have "non-genomic" effects, including activation of extracellular-signal regulated kinase (Erk1/2) in the mitogen-activated protein kinase (MAPK) signal transduction pathway. However, there is controversy over the extent to which E2 may influence Erk1/2 activation, as well as the potential mechanism by which E2 could influence cross-talk between different signaling pathways (reviewed in [4]).

E2 has been reported to activate Erk1/2 in MCF-7 human breast cancer cells that express high levels of ERα, as well as in several other cell types, including neural cells and osteoblasts. The majority of studies reporting E2 activation of Erk1/2 in MCF-7 cells show transient increases in the kinase activity, or phosphorylation state, within 2–15 min [5–10]; however, one study describes E2-induced elevations in Erk1/2 activity only after 2 h of estradiol treatment with persistent activation for up to 24 h [11]. Upstream members of the Erk/MAPK pathway reportedly activated by E2 include: c-src non-receptor tyrosine kinase, p21ras, Shc, p190 [5], Raf-1 [12], as well as Egr-1 [12]. However, another study reports E2-induced Erk/MAPK activation that is mediated

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through a G protein-coupled receptor, GPR30, and involves EGF receptor activation as a result of the release of pro-heparan bound EGF from cell membranes [8]. In rat cerebral cortical explants [13,14] and SK-N-SH human neuroblastoma cells [15], levels of phosphorylated Erk1/2 reportedly rise ∼15 min after treatment with E2 and persist for up to 2 h. However, in rat osteoblast ROS 17/2.8 cells, Erk1/2 activity was reportedly increased within 5 min of treatment with E2 then rapidly returned to basal levels [16].

In studies reporting E2-induced Erk1/2 activation, there are conflicting reports regarding the role of ER in mediating this effect. Some studies report the pure antiestrogen ICI 182,780 abolishes E2-induced elevations in Erk1/2 activity in MCF-7 cells [5,7,9], suggesting E2 binding ER mediates Erk1/2 activation. While this idea is supported by reports that E2 does not activate Erk1/2 in cells lacking ER [7], a different study indicates that ICI 182,780 alone activates Erk1/2 to the same extent as E2 [8]. Furthermore, Erk1/2 activation was not elicited by treatment with ERα and ERβ selective ligands (16α-iodo-17β-estradiol and genistein, respectively) [13,14].

In distinct contrast, other studies report E2 does not significantly activate Erk1/2 in the same breast cancer and neuroblastoma cell lines reported to exhibit E2-induced Erk1/2 activation. In MCF-7 and MDA-MB-231, breast cancer cells E2 was reported to not alter Erk1/2 phosphorylation, while EGF induced robust Erk1/2 activation [6,17–21]. In addition, in SK-N-SH neuroblastoma cells E2 was reported to not induce Erk1/2 activation by any concentration of E2 tested (1–100 nM), relative to time-matched control cells [22].

The basis of the conflicting reports regarding the extent to which E2 activates Erk1/2 is not clear; however, different experimental variables may impact Erk1/2 activation and interpretation of results. Lobenhofer and Marks [20] report administering E2 in a small volume of phenol red-free, serum-free medium to MCF-7 cells did not activate Erk1/2; however, when all of the medium was changed, Erk1/2 phosphorylation was induced, possibly via stress-signaling pathways. Another study reports low levels of ethanol (E2 solvent) alone induce Erk1/2 activation [18]. Thus, conclusions from studies investigating E2-induced Erk1/2 activation that did not include solvent- and time-matched control cells may be compromised. Furthermore, different conditions for maintaining cells, staging cells in E2-free medium, treating cells with E2, lysing cells and assessing Erk1/2 activation may contribute to the conflicting reports regarding E2-induction of Erk1/2 phosphorylation. A recent review also suggested inherent differences in MCF-7 cell sublines may be responsible for these conflicting reports [4].

The goal of the present study was to develop a high through-put, quantitative assay for assessing Erk1/2 phosphorylation and use this assay to examine potential variables that could impact the conflicting reports regarding the extent of E2 activation of Erk1/2 in MCF-7 cells. Studies were restricted to MCF-7 cells because most controversy in the literature regarding E2-activation of Erk1/2 centers on these cells. The impact of different: culture media composition (including DMEM versus DMEM:F-12 and media with charcoal-stripped serum versus serum-free media), dosing methods, E2 preparations, cell densities, lysis solutions, MCF-7 cell sublines, as well as the influence of phosphatase inhibitors, on E2-mediated Erk1/2 phosphorylation were investigated. While some variables that can influence the apparent degree of Erk1/2 phosphorylation in MCF-7 cells were identified, these studies were not able to demonstrate significant E2 induction of Erk1/2 phosphorylation nor resolve all of the conflicting reports regarding E2 activation of Erk1/2 in MCF-7 cells.

2. Materials and methods

2.1. Reagents

Polyclonal phospho-p44/42 MAP Kinase (Thr202/Tyr204), polyclonal p44/42 MAP Kinase, phospho-estrogen receptor α (Ser118) monoclonal antibody 16J4, goat anti-rabbit IgG horseradish perxidase (HRP)-conjugated and goat anti-mouse IgG HRP-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA). Estrogen receptor α antibody (H-184) was purchased from Santa Cruz (Santa Cruz, CA). FITC-conjugated donkey anti-rabbit antibody was obtained from Chemicon (Temecula, CA). PVDF and nitrocellulose membranes were obtained from Bio-Rad Laboratories (Hercules, CA). Phenol red-free (PRF) DMEM, L-glutamine and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). DMEM was obtained from Bio-Whittaker (Walkersville, MD). Protease inhibitor cocktail tablets (Complete) were purchased from Roche Molecular Biochemicals (Indianapolis, IN). 17-β-Estradiol (E8875), EGF (E9644), water-soluble 17-β-estradiol (E4389), 2-hydroxypropyl-β-cyclodextrin, phosphatase inhibitors, SDS, acrylamide and all other electrophoretic reagents of the highest grade were purchased from Sigma (St. Louis, MO).

2.2. Cell culture

MCF-7 cells used in most studies were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 0.1% gentamycin. A different stock of MCF-7 cells, reported to exhibit E2-induced Erk1/2 phosphorylation [8] was kindly donated by Dr. Edward J. Filardo (Rhode Island Hospital and Brown University, Providence, RI); these cells were maintained in phenol red-free DMEM/F12, 10% FBS and 0.01% gentamycin [8]. All cells were kept at 37 °C with 10% CO_2.

2.3. Cellular staging and protein harvesting

MCF-7 cells were plated into 6-well culture dishes at 3×10^5 cells/well, unless indicated otherwise. In some

studies cells were depleted of E2 and staged to reduce basal Erk1/2 phosphorylation by maintaining them in PRF-DMEM, 2% charcoal-dextran stripped FBS [23] for 4 days with one medium change on day 2. In other studies cells were depleted of E2 and staged by maintaining them in serum-free medium, as described in figures. Cells were treated with appropriate mediators by removing and replacing all medium or by addition of 1/10 (v/v) PRF-DMEM, with or without 2% stripped serum, as indicated; triplicate cultures for each treatment and time point were analyzed. At indicated times, medium was aspirated from cell monolayers, cells were quickly rinsed with ice-cold phosphate buffered saline (PBS) and lysates were prepared by adding 150 μL boiling harvesting buffer (1% SDS, 10 mM Tris, pH 7.4) followed by sonication. When indicated, phosphatase inhibitors (3.5 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM sodium pyrophosphate) were added prior to sonication. In designated experiments cells were lysed by adding 150 μL RIPA buffer (150 mM NaCl, 100 mM Tris, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 2 mM PMSF, 1× Complete protease inhibitor cocktail), in the presence [8] or absence of phosphatase inhibitors (3.5 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM sodium pyrophosphate). All cell lysates were assayed for total cellular protein (BCA Protein Assay, Pierce).

2.4. DNA assay

To assess cell proliferation, MCF-7 cultures were plated in 6-well culture dishes at 1×10^5 cells/well and maintained in PRF-DMEM containing 2% charcoal-dextran stripped FBS [23] for 4 days with one medium change on day 2. For 6 consecutive days cells were completely re-fed PRF-DMEM, 2% stripped FBS and no additions (control), 3 nM E2 or 100 ng/mL EGF. Medium was aspirated from cell monolayers, cells were rinsed once with ice-cold PBS, and DNA was precipitated by adding 10% trichloroacetic acid (TCA). After TCA removal, DNA was dissolved in 0.1N NaOH and re-precipitated with 20% TCA. After centrifugation, precipitated DNA was assayed according to Burton [24]. Known concentrations of DNA were used to generate a standard curve from which the DNA content of cellular samples was interpolated. Results are expressed as microgram DNA per centimetre square surface culture dish.

2.5. Immunoblotting

ER phosphorylation was investigated by Western blotting using two different antibodies. ER phosphorylation results in retarded migration (i.e. up-shift) of the ERα band in Western blotting [19,25,26] using anti-ERα antibody H-184 (Santa Cruz), whereas monoclonal antibody 16J4 (Cell Signaling Technology) specifically reacts with ERα phosphorylated at Ser118. Phospho-specific Erk1/2 antibodies (Cell Signaling Cat. No. 9101) were used to evaluate Erk1/2 phosphorylation at residues T202/183 and Y204/185 (phospho-p44/42 MAPK), and antibodies purported to react with total Erk1/2 protein (p44/42 MAPK) (Cell Signaling Cat. No. 9102) were used to evaluate levels of phosphorylated + non-phosphorylated forms of Erk1/2.

For Western blotting, 20 μg cell lysate protein were heated at 95 °C for 3 min then separated by electrophoresis in 7.5% SDS-polyacrylamide gels (PAGE) and electroblotted to PVDF membranes. Membranes were blocked in Tris-buffered saline (TBS), 5% non-fat milk prior to incubation with the primary antibodies (1:1000) diluted in 5% bovine serum albumin (BSA) overnight at 4 °C. An HRP-conjugated secondary antibody (1:1000) diluted in TBS, 5% milk, in conjunction with a luminol substrate (LumiGlo®; Cell Signaling Technology), was used for detection. For Slot blotting, 60 μg cellular protein were heated as above then serially diluted in TBS, with 250 μL final volume in each dilution. Phosphorylated and non-phosphorylated MAPK Control Proteins (Cell Signaling Technology) were also serially diluted into TBS. Dilutions of lysates and control protein was directly applied to nitrocellulose membranes using the Bio-Dot SF Microfiltration Apparatus (Bio-Rad). Membranes were then incubated in TBS containing 0.1% Tween-20 (TBST) before blocking in TBS, 5% milk. Blots were processed as described for Western blotting. The optical densities of resultant bands were analyzed in the WVU Image Analysis Facility with Optimas 6.2 software; known amounts of control phosphorylated Erk1/2 proteins were used to generate a standard curve from which the amount of phosphorylated Erk1/2 in cell lysates was interpolated.

2.6. Immunocytochemistry

MCF-7 cells were plated in eight-chambered slides at 1×10^4 cells/well. Twenty-four hours after plating, cultures were maintained in serum-free PRF-DMEM for 3 days, with medium changes every 24 h. Cells were treated with the indicated mediators in 1/10 (v/v) serum-free medium. After aspirating medium, cell monolayers were quickly rinsed with ice-cold PBS, fixed with 4% paraformaldehyde (freshly prepared) at 4 °C for 10 min, and permeabilized with TBS, 0.2% Triton X-100 at room temperature for 5 min. Following several washes in TBS, 0.1% Triton X-100, fixed cell monolayers were blocked with TBS, 5.5% goat serum for 1 h and then incubated with primary antibody (1:300 in TBS, 3% BSA) overnight at 4 °C. Monolayers were thoroughly rinsed, incubated with FITC-conjugated secondary antibody (1:300 in TBS, 3% BSA) for 1.5 h at room temperature, thoroughly rinsed and mounted with Prolong anti-fade solution (Molecular Probes) under a coverslip. Cells were observed and imaged using an LSM 510 laser-scanning confocal microscope (Zeiss Inc., Thornwood, NY). An artificially assigned rainbow color scale was used to indicate intensity of FITC staining; blue and green colors represent areas of low intensity staining, with yellow, red and white representing areas of increasing staining intensity.

2.7. Statistical analysis

For all experiments, one-way ANOVA was performed, followed by two-population (independent) *t*-test; significant differences ($p < 0.05$) are indicated.

3. Results

Comparing published variables (cell culture media, staging and treating cells with medium containing charcoal-stripped serum versus serum-free medium, E2 preparations and concentrations, and procedures used to lyse cells and assess Erk1/2 activation) in studies reporting E2 did [5–11] or did not [17–21] activate Erk1/2 in MCF-7 cells did not provide insight to potential reasons for the conflicting conclusions. One to ten nanometre E2 was used most frequently in studies investigating the effects of E2 on Erk1/2 activation in MCF-7 cells [5–11,17–21]. Therefore, before investigating E2 effects on Erk1/2, 3 nM E2 (Sigma, E8875) was shown to induce MCF-7 cell proliferation better than 100 ng/mL EGF, as expected (Fig. 1A, a chronic treatment), as well as to induce ERα phosphorylation (Fig. 1B, an acute treatment), indicating these MCF-7 cells were responsive to this concentration of the E2 preparation used in these studies.

3.1. Effects of E2 on Erk1/2 phosphorylation in the presence of charcoal-stripped serum

An equal number of studies reporting E2-induced Erk1/2 activation staged and treated MCF-7 cells with medium containing 0.05–5.0% charcoal-stripped fetal calf serum [5,7,9] or with serum-free medium [6,8,11]; 1–10 nM E2 was used most frequently in PRF-DMEM. Initial studies assessed the effect of 3 nM E2 (Fig. 1) on Erk1/2 phosphorylation in MCF-7 cells staged and treated in PRF-DMEM, 2% charcoal-stripped fetal calf serum as described in Fig. 2. To minimize activation of stress-response pathways by completely replacing medium on cells [18], 1/10 (v/v) culture medium (PRF-DMEM, 2% charcoal-stripped fetal calf serum) with 30 nM E2 was added to cultures (3 nM final E2). Erk1/2 phosphorylation before adding E2 (“time 0”) and 15 min after adding E2 was assessed by Western blot (Fig. 2). In each sample the level of phosphorylated Erk1/2 was normalized to “total” Erk1/2, as described; comparing the normalized levels of phosphorylated Erk1/2 in “time 0” and E2-treated cells indicated

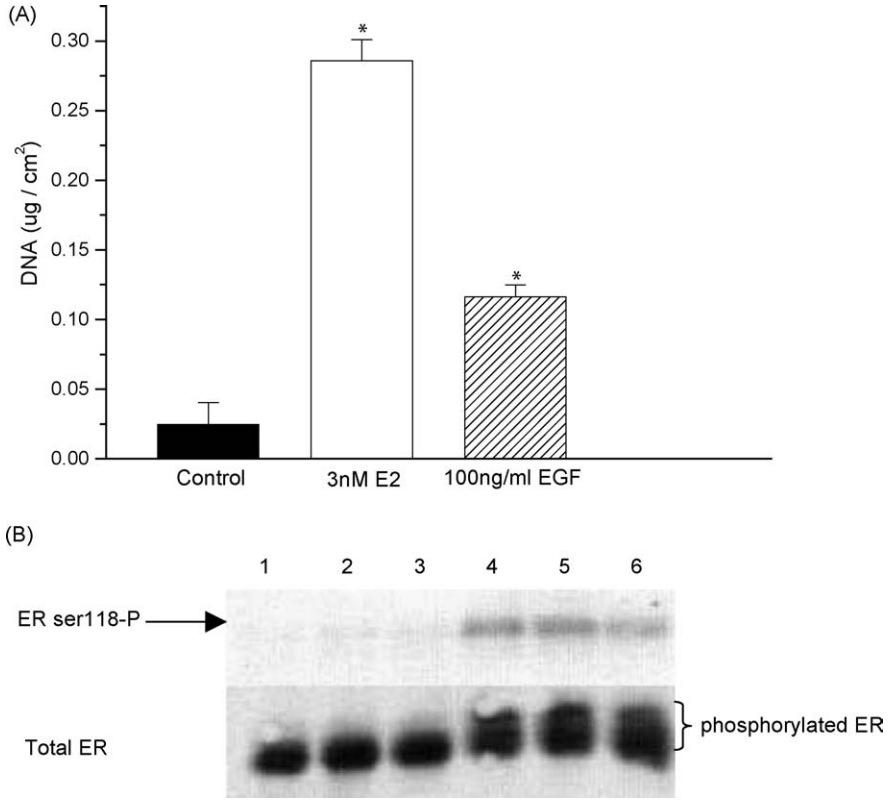

Fig. 1. Estradiol induces MCF-7 cell proliferation and estrogen receptor phosphorylation. MCF-7 cells were maintained in PRF-DMEM, 2% stripped serum for 4 days. (A) Cultures were administered PRF-DMEM, 2% charcoal-stripped FBS containing no mediator (control), 3 nM E2 or 100 ng/mL EGF every day for 6 consecutive days. DNA in triplicate cultures were quantified [24] and presented as microgram DNA per centimetre square surface area ± S.E. Significantly different from control ($^*p < 0.05$). (B) Cultures were treated with 1/10 (v/v) serum-free PRF-DMEM alone (control; lanes 1–3) or 3 nM E2 (final concentration) (lanes 4–6) for 30 min. Lysates were prepared as described and electrophoresed (20 μg protein each lysate) on a 10% SDS-PAGE at 4 °C and electroblotted to a PVDF membrane. *Top panel:* membrane was probed with an antibody that specifically recognizes ERα phosphorylated at Ser118 (ER Ser118-P). *Bottom panel:* membrane was stripped and probed with an antibody that recognizes ERα; ERα phosphorylation results in retarded migration indicated by the bracket.

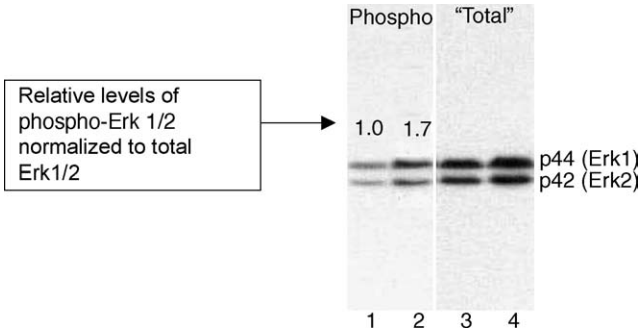

Fig. 2. Effects of E2 on phospho-Erk1/2 by treating MCF-7 cells with E2 in charcoal-stripped serum. MCF-7 cells were maintained in PRF-DMEM, 2% stripped serum for 4 days prior to treatment. Cell lysates were prepared at time 0 prior to any treatment (lanes 1 and 3), or 15 min after 1/10 (v/v) of PRF-DMEM, 2% charcoal-stripped FBS containing 30 nM E2 (3 nM final E2 concentration; lanes 2 and 4) was added to cultures. Twenty microgram of each cell lysate was subjected to Western blotting for phospho-Erk1/2 (lanes 1 and 2) and "total" Erk1/2 (lanes 3 and 4) protein. The optical densities of phospho-Erk1/2 bands were determined and normalized to those of "total" Erk1/2 bands from the same sample. By dividing the normalized value of phospho-Erk1/2 in E2-treated cells by the normalized value of phospho-Erk1/2 in time 0 cells, E2 treatment was calculated to increase Erk1/2 phosphorylation ∼1.7-fold. Similar results were seen in triplicate samples of each treatment.

Erk1/2 phosphorylation was ∼1.7-fold higher in E2-treated cells than in "time 0" cells.

Given the high degree of antibody specificities for recognizing phosphorylated or non-phosphorylated Erk1/2 in MCF-7 cell lysates, as well as the apparent modest E2-mediated increase (∼1.7-fold) in phospho-Erk1/2 (Fig. 2), a higher through-put, quantitative slot blot procedure was developed to better study Erk1/2-phosphorylation. Known amounts of phosphorylated- and non-phosphorylated-Erk1/2 control proteins were probed with phospho-Erk1/2 antibodies (Fig. 3A) and total Erk1/2 antibodies (Fig. 3B). Although the phospho-Erk1/2 antibody specifically recognized only phospho-Erk1/2 protein (Fig. 3A), the antibody purported to recognize total Erk1/2 exhibited a much stronger reaction with non-phospho-Erk1/2 protein than with phospho-Erk1/2 protein (Fig. 3B), indicating that these total Erk1/2 antibodies may not be appropriate to use in normalizing phospho-Erk1/2 levels in different samples. Therefore, for all further studies described herein known amounts of phospho-Erk1/2 protein were probed with phospho-Erk1/2 antibody (Fig. 3A), and the resultant optical densities were used to establish standard curves (Fig. 3C) from which the amounts of phosphorylated-Erk1/2 in MCF-7 cell lysates could be interpolated and normalized to total cellular protein.

The effects of 3 nM E2 and 100 ng/mL EGF (positive control for inducing Erk1/2 phosphorylation) on phospho-Erk1/2 in MCF-7 cells were determined over 2 h. Cells were staged in PRF-DMEM, 2% stripped serum for 4 days prior to treatment with 1/10 (v/v) of PRF-DMEM, 2% stripped serum alone (controls) or with E2 or EGF or with serum-free PRF-DMEM; cell lysates were prepared at indicated times and analyzed by Slot blotting. In both control and E2-treated cultures, 2 min after additions the level of phospho-Erk1/2 reproducibly declined, relative to the amount at time 0, while EGF-treated cultures consistently displayed increases in the level of phospho-Erk1/2 (Fig. 4A and C). However, compared to control cells at the 2 min time point, the levels of phospho-Erk1/2 were significantly elevated in both E2- and EGF-treated cultures (∼2.3- and ∼6-fold, respectively), this apparent elevation of phospho-Erk1/2 at 2 min in E2-treated cells relative to control cells is due to less reduction of phospho-Erk1/2 in E2-treated cells relative to control cells. Fifteen minute after additions all cultures displayed 2–3-fold increases in the levels of phospho-Erk1/2, relative to time 0; however, at none of the times between 5 and 120 min were the levels of phospho-Erk1/2 significantly increased by E2 or EGF, relative to time-matched control cultures (Fig. 4A and C). These time-dependent changes in phospho-Erk1/2 did not appear to be due to changes in Erk1/2 protein as assessed with the "total" Erk1/2 antibody described in Figs. 2 and 3 (not shown). Comparison of phospho-Erk1/2 levels in cultures treated with 1/10 (v/v) serum-free PRF-DMEM or PRF-DMEM containing 2% charcoal-stripped FBS demonstrated that both the transient reduction of phospho-Erk1/2 at the 2 min time and subsequent increase in phospho-Erk1/2 observed at the 15 min time were attributed to stripped serum (Fig. 4A and D). Cells receiving 1/10 volume serum-free medium maintained approximately constant levels of phospho-Erk1/2 for at least 15 min.

3.2. *Estradiol does not activate Erk1/2 in serum-free medium*

All further experiments examined MAPK activation by adding mediators in serum-free medium. When MCF-7 cells were "staged" in PRF medium containing 2% stripped serum prior to addition of mediators in 1/10 volume serum-free PRF medium, 100 ng/mL EGF induced an ∼6-fold rapid, transient increase in phospho-Erk1/2 2 min after addition, relative to control-treated and to "time 0" cells (not shown). However, phospho-Erk1/2 levels in cells treated with 1–100 nM E2 were not significantly elevated above those in control-treated cells over a 2 h time course or after 24 h (not shown). Additional variables that might impact Erk1/2 phosphorylation by E2 were then investigated.

Several studies reporting E2 activation of Erk1/2 maintained MCF-7 cells in serum-free medium 24 to 72 h prior to E2 treatment [6,8,11]. Staging MCF-7 cells in serum-free, PRF-DMEM for 48 h prior to addition of EGF in serum-free medium induced an ∼8-fold increase in phospho-Erk1/2 at 2 min that remained significantly elevated (∼2.5-fold) at 5 min, before returning to basal level by 15 min (Fig. 5A). However, phospho-Erk1/2 was essentially unchanged in E2-treated cells over 30 min (Fig. 5A).

To minimize potential autocrine effects of growth factors released into culture medium, MCF-7 cells were plated at a

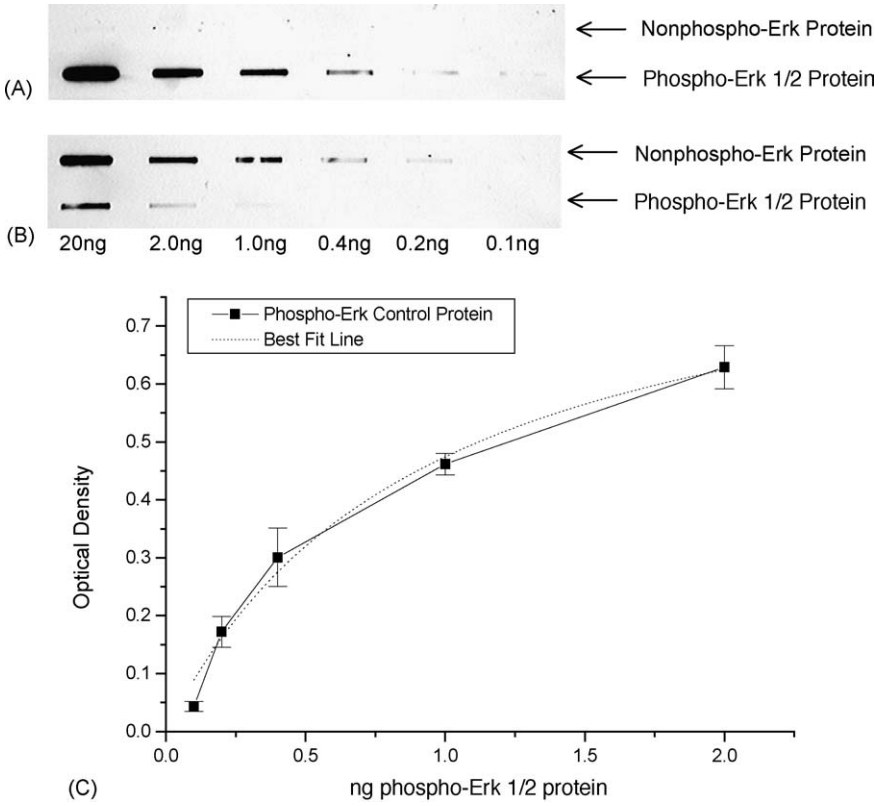

Fig. 3. Erk1/2 phosphorylation is quantitatively assessed by slot blotting. Indicated amounts of non-phosphorylated (upper rows) and phosphorylated (bottom rows) Erk1/2 control proteins were processed in slot blots and probed with phospho-Erk1/2 antibodies (A) or "total" Erk1/2 antibodies (B) as described in Section 2. The optical densities of phospho-Erk1/2 in three separate blots were determined and used to generate a standard curve (C) for interpolating the amount of phospho-Erk1/2 protein in cell lysates.

lower density, and serum-free medium was replaced every 24 h over a 72 h staging period [8]. Treatment with EGF resulted in a rapid increase in phospho-Erk1/2 at 2 min that decreased more gradually, remaining significantly elevated 3- and 2-fold at 5 and 15 min, respectively (Fig. 5B). In contrast, E2-treated cells maintained a constant, basal level of phopho-Erk1/2 (Fig. 5B).

To determine if the lack of E2-mediated Erk1/2 activation was due to a MCF-7 subline difference, Dr. Edward J. Filardo (Rhode Island Hospital and Brown University, Providence, RI) kindly donated a stock of MCF-7 cells that have displayed E2-mediated Erk1/2 phosphorylation [8] in serum-free medium. Those cells were maintained in phenol red-free DMEM:F12, plated at 1.25×10^5 cells/well of 6-well plates, staged and treated in serum-free, phenol red-free DMEM:F-12, precisely according to Dr. Filardo's published [8] and personal instructions. However, when the study detailed in Fig. 5B was repeated using Dr. Filardo's cell stock and procedures, the results were essentially identical to those depicted in Fig. 5B; phospho-Erk1/2 levels were not significantly altered in E2-treated cells, whereas EGF induced maximal Erk1/2 phosphorylation at 2 min which then gradually decreased (not shown). Additional studies with cells obtained from Dr. Filardo, assessed different components of the dosing and cell lysing protocols for their potential impact on E2-mediated Erk1/2 phosphorylation. A water-soluble preparation of E2 (Sigma E4389) [8] was tested in place of ethanol-soluble E2 (0.0003% total ethanol upon dosing, Figs. 1, 2, 4 and 5); and cells were lysed in either RIPA buffer without first rinsing cell monolayers with PBS [8] or in the SDS-based harvesting buffer used in previous experiments; both buffers were used with and without phosphatase inhibitors. One nanomolar water-soluble E2 was used for 5 min, duplicating conditions in Dr. Filardo's studies [8]. Neither E2 preparation induced Erk1/2 phosphorylation in any lysis buffer tested, while EGF induced robust Erk1/2 phosphorylation (Fig. 6 and not shown). Erk1/2 phosphorylation was reduced somewhat in both lysis buffers in the absence of phosphatase inhibitors. Additionally, neither ethanol-soluble E2, 0.0003% ethanol alone nor 2-hydroxypropyl-β-cyclodextrin alone (vehicle for water-soluble E2) altered EGF induced Erk1/2 phosphorylation (not shown), indicating an inhibitor of Erk1/2 phosphorylation was not present in any of these compounds.

Phospho-Erk1/2 was also assessed in situ via immunocytochemistry (Fig. 7). At 2 min, EGF induced significant phosphorylation of Erk1/2, as evidenced by a greater number

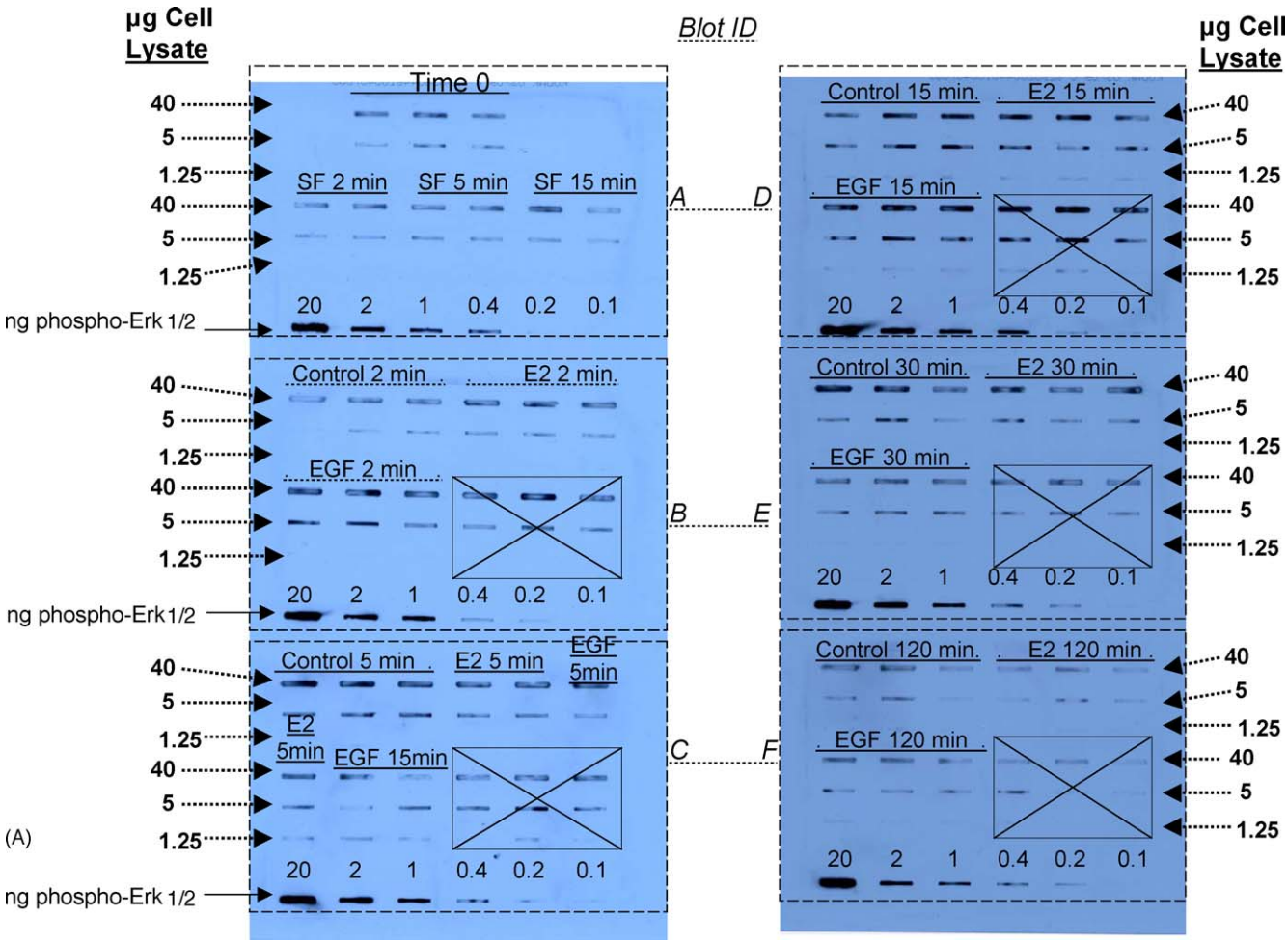

Fig. 4. Addition of charcoal-stripped serum complicates interpretation of effects of E2 or EGF on phospho-Erk1/2. MCF-7 cells were maintained in PRF-DMEM, 2% stripped serum for 4 days prior to treatment with 1/10 (v/v) PRF-DMEM, 2% stripped FBS alone ("control" in Panel A), with 1/10 (v/v) PRF-DMEM, 2% stripped FBS containing 30 nM E2 ("E2" in Panel A) or 1000 ng/mL EGF ("EGF" in Panel A), yielding final concentrations of 3 nM E2 or 100 ng/mL EGF, respectively, for 2, 5, 15, 30 or 120 min. Other cells were treated with 1/10 (v/v) serum-free, PRF-DMEM ("SF" in Panel A, Blot *A*) for 2, 5 or 15 min. Cells not subject to any dosing or medium change were harvested at time 0 ("time 0" in Panel A) to assess basal levels of phospho-Erk1/2. Triplicate cultures for each treatment were harvested at indicated times, except those receiving serum-free medium, where duplicate cultures were harvested at indicated times. Cell lysates were prepared from each culture as described and 40, 5 and 1.25 µg each lysate was applied to slot blots; each blot also contained 0.1, 0.2, 0.4, 1.0, 2.0 and 20 ng phospho-Erk1/2 standard proteins. (A) Slot blots (*A–F*) were processed as described and exposed to X-ray film (30 s). The approximate border of each blot is indicated by dashed lines; standard phospho-Erk1/2 proteins (0.1–20 ng) are across the bottom of each blot; the source of cell extracts (underlined within blots) and microgram of extract proteins loaded on to blots (dashed arrows on left side [blots *A–C*] and right side [blots *D–F*]) are indicated. Lysates unrelated to these studies are marked "X" with solid lines. (B) A composite standard curve of the mean optical density vs. nanogram of phospho-Erk1/2 protein (0.1–2.0 ng) from the six blots (*A–F*) in Panel A was constructed. Optical densities of cell lysates that fell within the optical density of phospho-Erk1/2 standards, especially within the 0.4–2.0 ng range of standards (Panel A) were used to estimate the amount of phospho-Erk1/2 protein in the different cell lysates; the insert shows the standard curve that includes 0.1–20 ng phospho-Erk1/2 protein. (C) The effects of treating MCF-7 cells with 1/10 volume PRF-DMEM, 2% stripped FBS with or without (control, closed square) 3 nM E2 (open circle) or 100 ng/mL EGF (open triangle) (final concentrations) on levels of phospho-Erk1/2. The amounts of phospho-Erk1/2 in cell lysates were determined from the blots shown in Panel A following interpolation from the standard curve depicted in Panel B and are expressed as means nanogram phospho-Erk1/2/mg cell lysate protein ± standard error (S.E.). Significantly different from time-matched control ($^{*}p < 0.05$). (D) The effects of treating MCF-7 cells with 1/10 volume PRF-DMEM, 2% charcoal-stripped serum (+2% sFBS, closed square) or with serum-free PRF-DMEM (open circle) for 15 min on levels of phospho-Erk1/2. Cell lysates were prepared, slot blotted and probed to detect phospho-Erk1/2 (Panel A); amounts of phospho-Erk1/2 were interpolated from a standard curve (Panel B) as described. Phospho-Erk1/2 levels are expressed as means nanogram phospho-Erk1/2/mg cell lysate protein ± S.E.

of cells expressing phospho-Erk1/2, as well as by an elevated level of phospho-Erk1/2 within cells (Fig. 7A and B). However, neither ethanol-soluble E2 (Fig. 7C) nor water-soluble E2 (Fig. 7D) displayed a pattern of phospho-Erk1/2 protein expression noticeably different from that seen in control cells (Fig. 7A).

4. Discussion

One objective of this study was to develop a quantitative, high through-put assay for Erk1/2 phosphorylation. The high specificity of the phospho-Erk1/2 antibody employed (Fig. 2) permitted immuno slot blotting, allowing processing

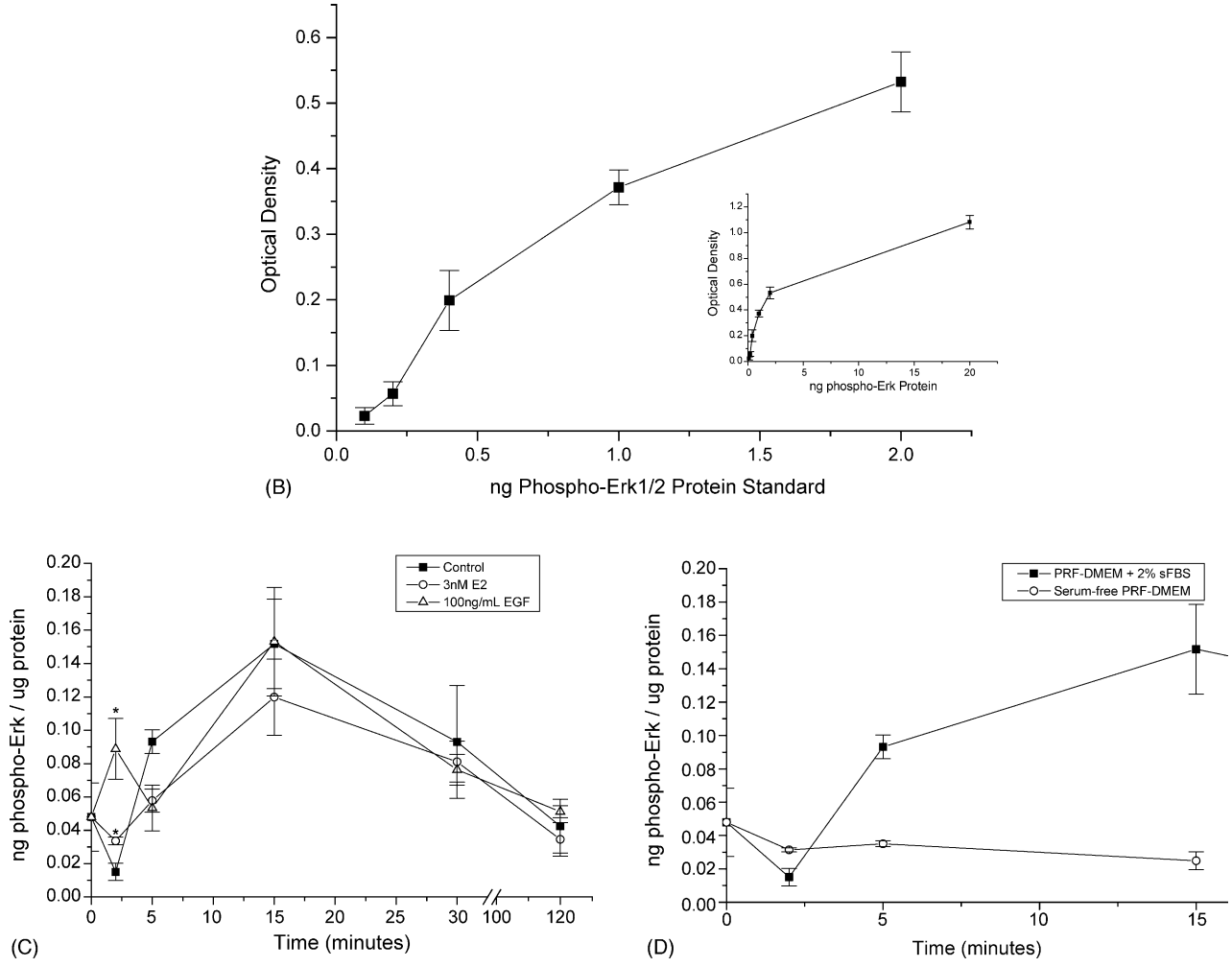

Fig. 4. (*Continued*).

of a greater number of samples in a shorter period of time than Western blotting, as well as quantitative analysis by generating a standard curve with known amounts of phosphorylated Erk1/2 protein on blots along with cell lysates (Fig. 3). A standard curve is essential for accurately assessing changes in the level of phospho-Erk1/2 in different samples because antibody-derived signals from the enhanced chemiluminescence detection method are often not proportional to the amount of antigen in a sample, as demonstrated in Figs. 3C and 4B. Although phospho-Erk1/2 is commonly normalized to total Erk1/2, the "total" Erk1/2 antibody used in this study did not efficiently recognize phospho-Erk1/2 (Fig. 3B), which could result in unequal normalization between samples with different ratios of phospho-Erk1/2 and non-phospho-Erk1/2. Therefore, levels of phospho-Erk1/2 in different treatments were normalized to total protein; different treatments were also assayed in triplicate and statistically compared.

Experimental variables that might contribute to conflicting literature reports regarding E2-induced Erk1/2 phosphorylation were then investigated. The addition to cells of mediators with even small amounts of charcoal-stripped serum can complicate interpretation of results (Fig. 4). When MCF-7 cells maintained in medium containing 2% stripped serum were dosed with 1/10 (v/v) PRF-DMEM, 2% stripped serum containing 30 nM E2, relative to time 0 there is a reproducible, transient reduction in basal phospho-Erk1/2 at 2 min followed by an apparent induction of phospho-Erk1/2 that peaks ∼15 min after E2 addition before returning to initial (time 0) levels (Fig. 4C). However, the apparent increase in phospho-Erk1/2 during the ∼15–30 min period is also seen in time-matched control cells and is attributed to added serum factors (Fig. 4). Although cells treated with E2 in 0.2% stripped serum for 2 min exhibited a reduction in phospho-Erk1/2, relative to time 0 cells, this reduction was significantly less than that observed in control cells receiving 0.2% serum alone and could be interpreted as induction of phospho-Erk1/2, relative to time-matched controls. In contrast, when cultures were dosed in the same manner with EGF, phospho-Erk1/2 was significantly elevated at 2 min, relative to both time-matched controls and the time 0 level; by the 15 min time point, there was no significant difference in the level of

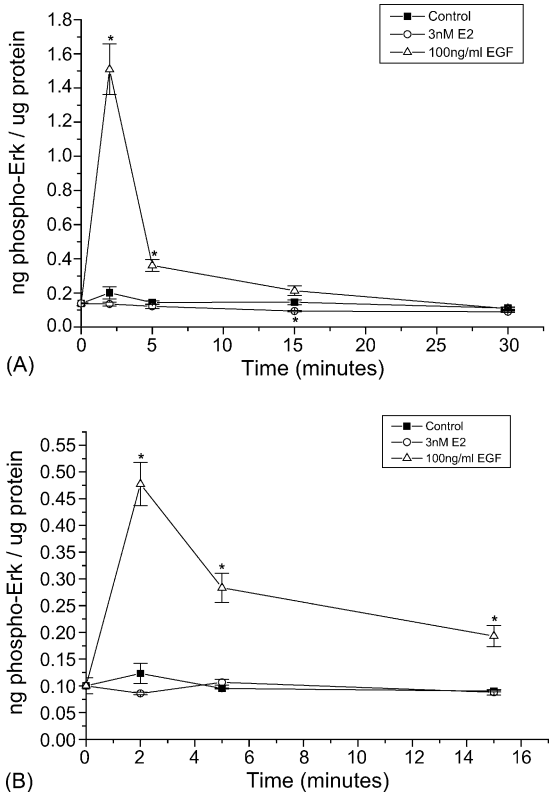

Fig. 5. E2 does not increase Erk1/2 phosphorylation in serum-free medium. (A) MCF-7 cells were plated at 3×10^5 cells/well in 6-well plates and maintained in serum-free, PRF-DMEM for 2 days prior to complete change of serum-free PRF-DMEM containing no mediator (control, closed square), 3 nM E2 (open circle) or 100 ng/mL EGF (open triangle) (final concentrations) for indicated times. Cells not subject to any dosing or medium changes were evaluated at time 0 to assess basal levels of phospho-Erk1/2. Cell lysates were prepared, and phospho-Erk1/2 was quantitated as described. A representative experiment is shown with each condition tested in triplicate. Values are means ± S.E. Significantly different from time-matched control ($^*p < 0.05$). (B) MCF-7 cells were plated at a lower density of 1.25×10^5 cells/well in 6-well plates and maintained in serum-free, PRF-DMEM for 3 days, with daily medium changes, prior to complete change of serum-free, PRF-DMEM containing no mediator (control, closed square), 3 nM E2 (open circle) or 100 ng/mL EGF (open triangle) (final concentrations) for indicated times. Cells not subject to any treatment were evaluated at time 0 to assess basal levels of phospho-Erk1/2. Cell lysates were prepared, and phospho-Erk1/2 was quantified as described. A representative experiment is shown, with each condition assayed in triplicate. Values are means ± S.E. Significantly different from time-matched control ($^*p < 0.05$).

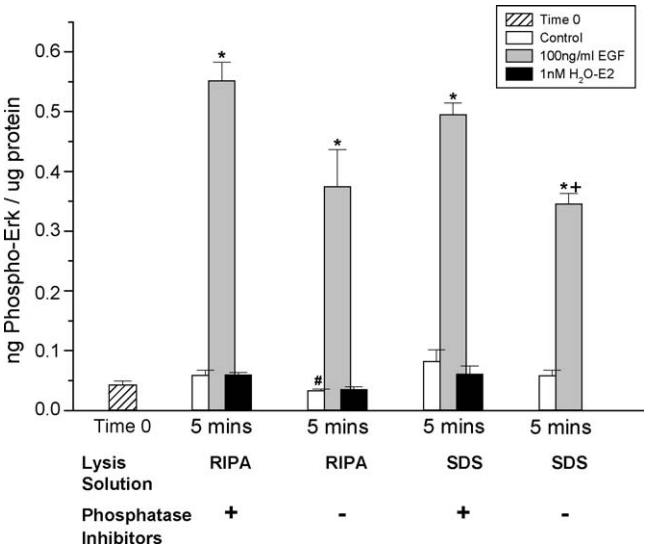

Fig. 6. E2 preparation and lysis buffer composition do not impact Erk1/2 phosphorylation in MCF-7 cells. MCF-7 cells were plated at 1.25×10^5 cells/well in 6-well plates and maintained in serum-free, PRF-DMEM for 3 days, with daily medium changes, prior to treatment via complete re-feeding with serum-free, PRF-DMEM containing no mediator (control), 100 ng/mL EGF or 1 nM water-soluble E2 (final concentrations) for 5 min. Cells not subject to any treatment were evaluated at time 0 to assess basal levels of phospho-Erk1/2. Cells were immediately lysed in RIPA buffer with or without phosphatase inhibitors, or in SDS harvesting buffer with or without phosphatase inhibitors, as indicated. Phospho-Erk1/2 protein in lysates was quantitated by slot blotting as described, and a representative experiment with each condition assayed in triplicate is shown. Values are means ± S.E. Water-soluble E2-treated cultures lysed with SDS buffer lacking phosphatase inhibitors were assayed in a different experiment. Significantly different from control cells lysed in same buffer ($^*p < 0.05$); significantly different from control-treated cultures lysed in same buffer but containing phosphatase inhibitors ($^\#p < 0.05$) and significantly different from EGF-treated cultures lysed in same buffer but containing phosphatase inhibitors ($^+p < 0.05$).

phospho-Erk1/2 between control-, E2- and EGF-treated cells (Fig. 4C). The importance of including both time-matched controls as well as a time 0 control when assessing Erk1/2 phosphorylation/activation is illustrated in Fig. 4; some reports of E2-induced Erk1/2 phosphorylation may be attributed to the absence of one of these controls.

However, other studies reporting E2-induced Erk1/2 phosphorylation avoided serum-mediated complications by maintaining and dosing MCF-7 cells in serum-free medium. In an attempt to reproduce those studies, the effects of serum-free staging and dosing conditions (Fig. 5A), low cell density (Fig. 5B), a water-soluble E2 preparation (Fig. 6), different cell lysis buffers (Fig. 6), different E2 concentrations (1–100 nM, not shown) and the addition of phosphatase inhibitors (Fig. 6) were all assessed for their impact on the ability of E2 to induce Erk1/2 phosphorylation. Despite duplicating as closely as possible experimental conditions described in studies reporting E2-induced Erk1/2 phosphorylation [6,8,11], we were unable to demonstrate E2-mediated Erk1/2 phosphorylation in MCF-7 cells, while EGF consistently induced robust activation (Figs. 5 and 6). Basal (time 0) levels of phospho-Erk1/2 were maintained whether E2 was applied in a small volume of serum-free medium or with a complete change of serum-free medium (Fig. 5). Similar results were obtained using immunocytochemistry to assess Erk1/2 phosphorylation, an alternative method that minimizes subjecting cells to potential stresses and artifacts of cell lysis (Fig. 7). Furthermore, Dr. Edward J. Filardo (Rhode Island Hospital and Brown University, Providence, RI) kindly donated an MCF-7 cell line reported to exhibit E2-induced Erk1/2 phosphorylation [8], as well as cell lysates prepared in his laboratory from that MCF-7 subline. When assayed for Erk1/2 phosphorylation via Western- and slot-blotting,

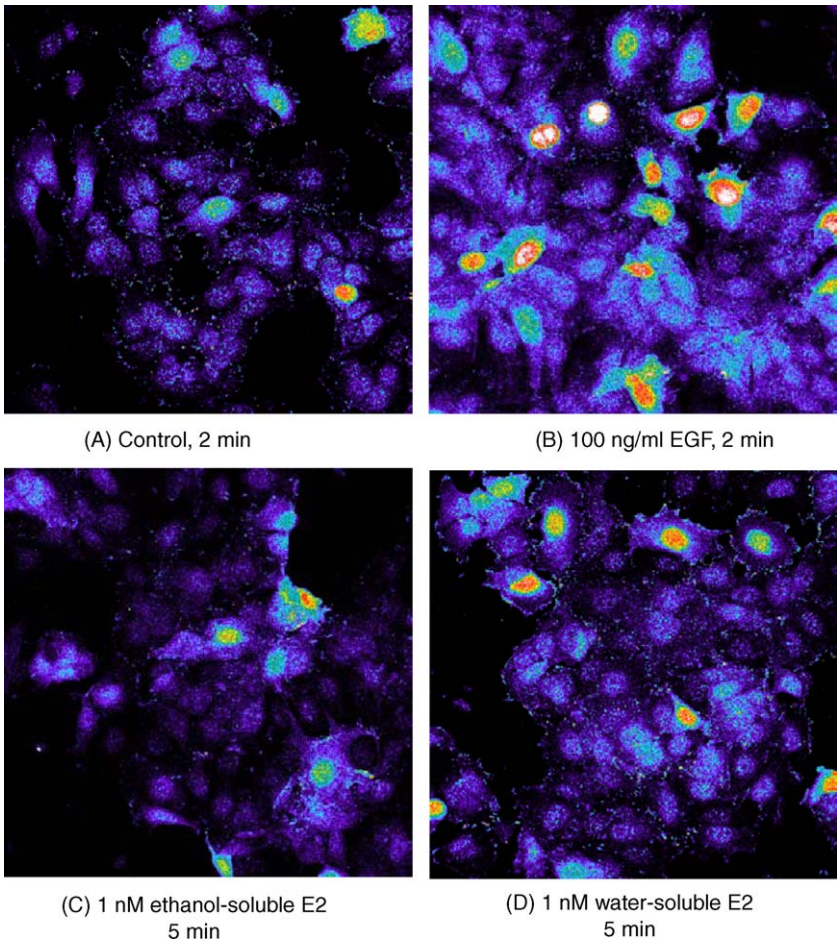

Fig. 7. Erk1/2 phosphorylation in situ reflects immunoblotting results. MCF-7 cells were seeded into 8-chambered glass slides at 1×10^4 cells/well and maintained in serum-free PRF-DMEM for 3 days, with daily medium changes. No mediator control (A), 100 ng/mL EGF (B), 1 nM ethanol-soluble E2 (C) or 1 nM water-soluble E2 (D) were added to cultures in 1/10 (v/v) serum-free medium for 2 min (control, EGF) or 5 min (both E2 preparations). Cells were fixed in paraformaldehyde, permeabilized with Triton X-100 and probed for phospho-Erk1/2, as described in Section 2. Images were captured via laser-scanning confocal microscopy and are displayed using a rainbow-colored scale to indicate intensity; low intensity labeling appears as blue, and increasingly higher intensity sites appear as green, yellow, red, and then white, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the lysates prepared in Dr. Filardo's laboratory from those E2-treated MCF-7 cells displayed a prominent increase (~4-fold) in phospho-Erk1/2, relative to controls (not shown), demonstrating that the antibodies and detection systems used in Western and Slot blotting were not the source of differential results between the two laboratories. However, in our hands we were unable to observe E2-induced Erk1/2 phosphorylation in that MCF-7 cell subline under any of the conditions described above. We specifically strove to duplicate Dr. Filardo's procedures for maintaining, staging, dosing and lysing that MCF-7 cell subline. Thus, it is not at all clear why some laboratories report E2-induced Erk1/2 phosphorylation/activation in MCF-7 cells [5–11] and others are not able to demonstrate this response [17–21]. Because the potential for E2 to mediate Erk1/2 activation has important implications for breast cancer and various other processes, it remains important to resolve this issue, perhaps requiring concerted efforts by multiple laboratories.

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