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Nongenomic Effect of Estrogen on the MAPK Signaling Pathway and Calcium Influx in Endometrial Carcinoma Cells

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ABSTRACT

17β-Estradiol (E2) is well known to interact with intracellular receptors that act as nuclear transcription factors. However, abundant evidence now indicates that E2 can also rapidly induce several nongenomic effects through signaling pathways related to cell growth, preservation, and differentiation. We studied the nongenomic effects of E2 in two human endometrial carcinoma cell lines, Ishikawa (estrogen receptor (ER) positive) and Hec-1A (ER negative or low) by cultivating them with either E2 or its membrane-impermeable conjugate, E2–BSA. We found that phosphorylation of Erk1/2 could be induced by either E2 or E2–BSA in Ishikawa cells. In Hec-1A cells, only E2 was able to induce Erk1/2 phosphorylation. Although the existence of a nongenomic component to the response was indicated by the finding that it could not be completely inhibited by the ER antagonist ICI182780,and it can also be inhibited by calcium inhibitor Nifedipine partly. Phosphorylation of Akt could not be induced, either by E2 or E2–BSA, in either cell line. Both E2 and E2–BSA elicited calcium influx in Ishikawa cells. In contrast to these nongenomic effects, only E2 was able to stimulate expression of the anti-apoptotic-protein Bcl-2. Taken together, these data indicate that nongenomic effects such as Erk1/2 phosphorylation and calcium influx can be initiated from the membrane in Ishikawa cell, and calcium can activate Erk1/2 phosphorylation. Except for ER, there must be other binding location of estrogen in endometrial cancer cells, and the nongenomic effects of estrogen initiated from plasma membrane by E2–BSA cannot lead to transcriptional effect of Bcl-2 expression. J. Cell. Biochem. 106: 553–562, 2009.

KEY WORDS: NONGENOMIC EFFECT; ENDOMETRIAL CARCINOMA; Erk1/2; Akt; CALCIUM; BCL-2

The steroid hormone 17β-estradiol (E2) is classically understood to traverse the plasma membrane of target cells to interact with receptors in the cytosol, which then migrate to the nucleus to act as DNA-binding transcription factors. However, a number of studies over the past 15 years have convincingly demonstrated the existence, in several cell types, of rapid signaling responses to estrogen that are independent of nuclear localization and transcriptional effects. These nongenomic effects appear to be initiated at the cell surface, where estrogen binds to membrane receptors (mER). The mER family includes the well-characterized nuclear steroid receptors and other members that are less well understood, including the receptors ER46, ERx, and GPR30 [Toran-Allerand et al., 2002; Li et al., 2003; Thomas et al., 2005].

Although the mechanism by which estrogen exerts its nuclear effects is well documented, our understanding of its membrane-initiated nongenomic effects is only beginning to emerge, deriving from a collection of observations in benign and malignant cells of various origins. Many of these studies have employed a membrane-impermeable conjugate of estrogen and bovine serum albumin (BSA) (E2–BSA). Because E2–BSA is unable to traverse the plasma membrane, it is prevented from reaching the classical estrogen receptors in the cytosol.

The nongenomic effects of E2 have been reported in the following: Estrogens contribute to human testicular germ cell cancer proliferation by rapid activation of Erk1/2 and PKA through a membrane nonclassical ER [Bouskine et al., 2008]; The PI-3K/Akt pathway also plays a critical role in mediating the nongenomic, salutary effects of E2 on attenuation of shock-induced intestinal tissue damage [Yu et al., 2007]; Nongenomic salutary effect of E2 in reducing hepatic injury after trauma-hemorrhage is mediated

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through the PKA-dependent pathway [Hsieh et al., 2007]; E2 opens BK(Ca) channels in HCASMC by stimulating nNOS via a transduction sequence involving PI3-kinase and Akt [Han et al., 2007]; fluorescence spectroscopy analysis of Ca^{2+} from human umbilical vein endothelial cells showed through mER, estrogen induced a rapid rise of intracellular free Ca^{2+} concentration [Wang et al., 2006]; ERα can mediate the rapid effects of E2 on Shc, MAPK, Elk-1, and morphological changes in breast cancer cells [Migliaccio et al., 1996; Razandi et al., 2000; Song et al., 2002]. These observations collectively indicate that, in a variety of different cells, there exist several signaling pathways that can be influenced by the nongenomic effects of E2, via mER or perhaps other ligands. To begin to systematically define the nongenomic component of these pathways, we decided to compare the effects of E2 and E2–BSA on endometrial cancer cells, whose nongenomic response to estrogen has thus far received little study. We employed, first, the estrogen receptor (ER)-positive human endometrial Ishikawa cell line. In order to reveal nongenomic effects that may rely on other receptor types, we also included the ER-negative (or low expression) Hec-1A human endometrial cell line. We show here that E2–BSA is able to initiate a number of rapid, nongenomic effects in these cells that are distinct from estrogen's nuclear effects.

MATERIALS AND METHODS

MATERIALS

The following reagents were purchased from Sigma–Aldrich Corp. (St. Louis, MO): 17β-estradiol (Soluble Complexes); β-estradiol-6-(*O*-carboxymethyl)oxime: BSA (E2–BSA); β-estradiol-6-(*O*-carboxymethyl)oxime: BSA fluorescein isothiocyanate conjugate (E2–BSA–FITC); ICI182780, Nifedipine, Fluo-3AM; Bcl-2; and Bax. Phospho-p44/42 MAPK, p44/42 MAPK, phospho-Akt, and Akt were purchased from Cell Signaling Technology (CST, Beverly, MA). DID [DiIC18(5)] was purchased from Biotium (USA). E2–BSA was filtered through a 3-kDa cut-off filter (Millipore) to remove free E2 before use.

CELL CULTURE

Ishikawa and Hec-1A human endometrial adenocarcinoma cells were from stored laboratory stocks, and were maintained under 5% CO_2 at 37°C in DMEM (Gibco) with 10% fetal bovine serum (FBS; National Hyclone Bio-Engineering Co. Ltd., Lanzhou China). In order to avoid the potential interference of hormones derived from the media, the cells were cultured in phenol-red-free DMEM (Hyclone) containing 5% dextran-charcoal FBS (dcFBS; Biochrom AG, Germany) for 48 h before treatment.

WESTERN BLOT ANALYSIS

An Erk1/2 antibody that recognizes the dual phosphorylation state of MAPK on Thr202 and Tyr204 was used to detect activation of MAPK. Akt, Bcl-2, and Bax antibodies were also used in Western blot analysis. Ishikawa and Hec-1A cultures, either untreated or stimulated with E2 and E2–BSA for varying times, were harvested and lysed in buffer (50 mM Tris–HCl, pH 7.6; 250 mM NaCl; 5 mM EDTA; 50 mM NaF), followed by the addition of a protease inhibitor cocktail and NP-40 to 10%. After a 30-min incubation at 4°C, cells were centrifuged (3 min, 3,000 rpm) and the supernatant collected. Supernatant protein concentrations were determined using a bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL). Thirty micrograms of protein from each sample was resolved with 12% SDS–PAGE, followed by transfer to PVDF membranes. The membranes were blocked by incubation at room temperature for 2 h with 5% nonfat dry milk in TBS-T. Dually phosphorylated Erk1/2 or phospho-Akt was detected by incubating membranes overnight at 4°C with phospho-specific antibodies (1:1,000 dilution). After washing with TBS-T, membranes were incubated for 2 h at room temperature with horseradish-peroxidase-coupled secondary antibody (1:5,000) in TBS-T/5% dry milk. The proteins were detected with the enhanced chemiluninecence (ECL) system (CST). The membrane was sequentially exposed to Kodak X-Omat AR films and then processed. Band intensity was quantified using the Bio-Rad imaging system. After film exposure, membranes were stripped in stripping buffer (62.5 mM Tris–HCl, 100 mM β-mercaptoethanol and 2% SDS) at 50°C for 30 min, then re-probed with the primary antibody against Erk1/2 and Akt (1:1,000). Erk1/2 and Akt activation was measured as the ratio of band intensity of phosphorylated Erk1/2 and Akt in E2- or E2–BSA-stimulated cell lysates to control lysates obtained before stimulation. Identical methods were used for the Bcl-2 and Bax proteins, with the Bcl-2 and Bax primary antibodies used at 1:1,000 and 1:500 dilutions, respectively.

IMMUNOFLUORESCENT ANALYSIS

Cells were seeded on glass coverslips in 24-well plates and incubated in phenol-red-free DMEM containing 5% dcFBS for 48 h. After treatment with E2–BSA–FITC for 5 or 30 min, as well as DID for 30 min, cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 15 min. Specimens were blocked in 5% normal goat serum in PBS with 0.3% Triton (PBST) for 60 min, and then incubated with phospho-Erk primary antibody (rabbit anti-human IgG, 1:800) for 1 h at 37°C. Specimens were washed three times in PBS, then incubated with a TRITC-conjugated goat-anti-rabbit IgG secondary antibody (1:100 in PBST) for 1 h in the dark at room temperature. Excess antibody was removed by washing in PBS, and coverslips were mounted on microscope slides. Cells were visualized using a laser scanning confocal microscope (Leica TCS SP2). The absorption and emission wavelengths, respectively, for the fluorophores we used were: FITC, 488 and 515–545 nm; TRITC, 543 and 565–650 nm; and DID, 644 and 663 nm.

CALCIUM MEASUREMENT

Cells were seeded onto glass-bottom fluorescent measurement dishes (Corning), and the medium replaced with phenol-red-free DMEM containing 5% dcFBS for 48 h. Before treatment with E2 and E2–BSA, the cells were loaded with 10 μM Fluo-3AM for 30 min at 37°C, then washed and maintained for the remainder of the experiment in either Tyrode's salt solution (5 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.9 mM $CaCl_2$, 5.6 mM glucose, pH 7.4) or phenol-red-free DMEM with 5% dcFBS. The cells were

then bathed in this medium until treatment with E2 dissolved in such medium (Fig. 5B–G). Cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]i$) was measured using a Leica TCS SP2 laser scanning confocal microscope system. Absorption and emission wavelengths used were 488 and 515–545 nm, respectively. The resulting fluorescence values were recorded by the TCS System and transferred to Microsoft Excel for analysis.

STATISTICAL ANALYSIS

The gel quantities of Erk1/2 and Akt were quantified after measurement of the optical density of the protein bands. Data were presented as means ± standard deviation (SD), calculated from three independent experiments. Statistical comparisons were made using one-way ANOVA. *P*-Values less than 0.05 were considered significant.

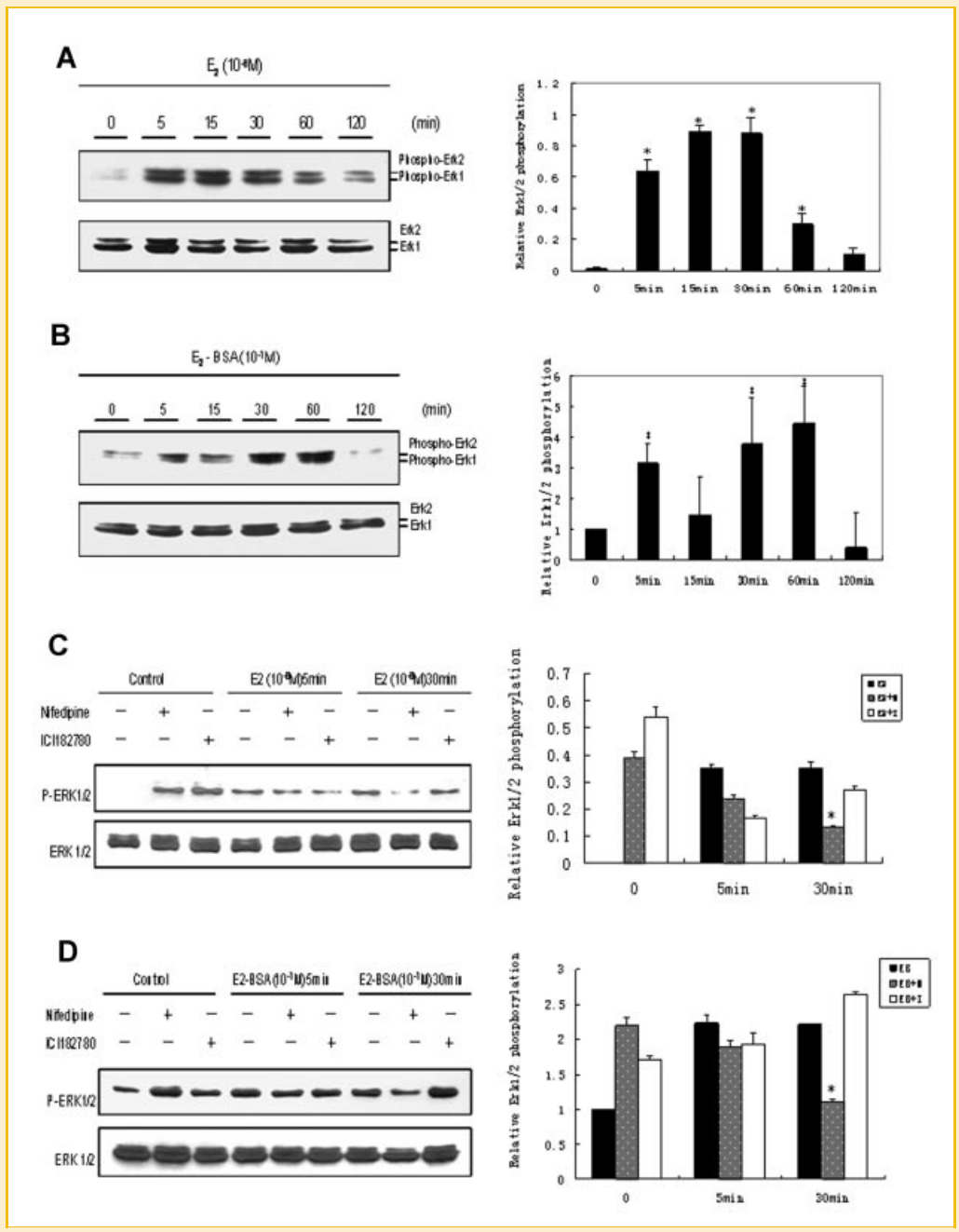

Fig. 1. Ishikawa cells treated with E2, E2–BSA (EB), Nifedipine (N), ICI182780 (I): Western blot analysis of whole cell lysates for expression of phosphorylated and nonphosphorylated Erk1/2. Cells were treated for the indicated times with 10 nM E2 (A), 100 nM E2–BSA (B), 10 nM E2 with 4 h pretreatment by 1 μM ICI182780 and 15 min pretreatment by Nifedipine (C), and 100 nM E2–BSA with 4 h pretreatment by 1 μM ICI182780 and 15 min pretreatment by Nifedipine (D). At right, the intensity of each band from treated cell extracts is normalized to the intensity of the band corresponding to untreated cells, which was arbitrarily assigned a value of 1 OD unit (mean ± SD of three independent immunoblots; asterisk (*) indicates $P < 0.05$).

RESULTS

E2 AND E2–BSA RAPIDLY INDUCED MAPK ACTIVATION IN ISHIKAWA CELLS

We first compared MAPK activation by E2 and E2–BSA in Ishikawa human endometrial carcinoma cells, which are ER positive (Fig. 1). Cells treated with E2 (Fig. 1A) showed a strong response in the phosphorylation of Erk1/2 within 5 min of exposure; this response decayed gradually over approximately 120 min. E2–BSA (Fig. 1B) elicited a similarly rapid, but initially lower, level of Erk1/2 phosphorylation compared to ER, but the peak was reached at 30 min, persisted to 60 min, and then decayed sharply between 60 and 120 min. These results showed that both E2 and E2–BSA were able to rapidly activate Erk1/2 in Ishikawa cells. To reveal the potential contribution of any nonclassical ERs in this response, we repeated stimulation by E2 and E2–BSA in cells that had been pretreated with the ER antagonist ICI182780 or Nifedipine (Fig. 1C,D), the increase in Erk1/2 phosphorylation cannot be blocked by ICI182780, but the phosphorylation was inhibited by Nifedipine after 30 min treatment either by E2 or by E2–BSA.

E2, BUT NOT E2–BSA, INDUCES Erk1/2 ACTIVATION IN HEC-1A CELLS

We next examined MAPK activation by E2 and E2–BSA in Hec-1A cells. The reported levels of ER expression in these cells ranges from low to absent [Castro-Rivera and Safe, 1998; Horne et al., 2006; Koster et al., 2006]. When treated with E2–BSA (Fig. 2A), no significant increase in the phosphorylation of Erk1/2 was observed. In contrast, E2 (Fig. 2B) triggered strong Erk1/2 phosphorylation within 30 min, which persisted for more than 120 min. To reveal the potential contribution of any nonclassical ERs in this response, we repeated stimulation by E2 in cells that had been pretreated with the ER antagonist ICI182780 (Fig. 2C). Although phosphorylation of

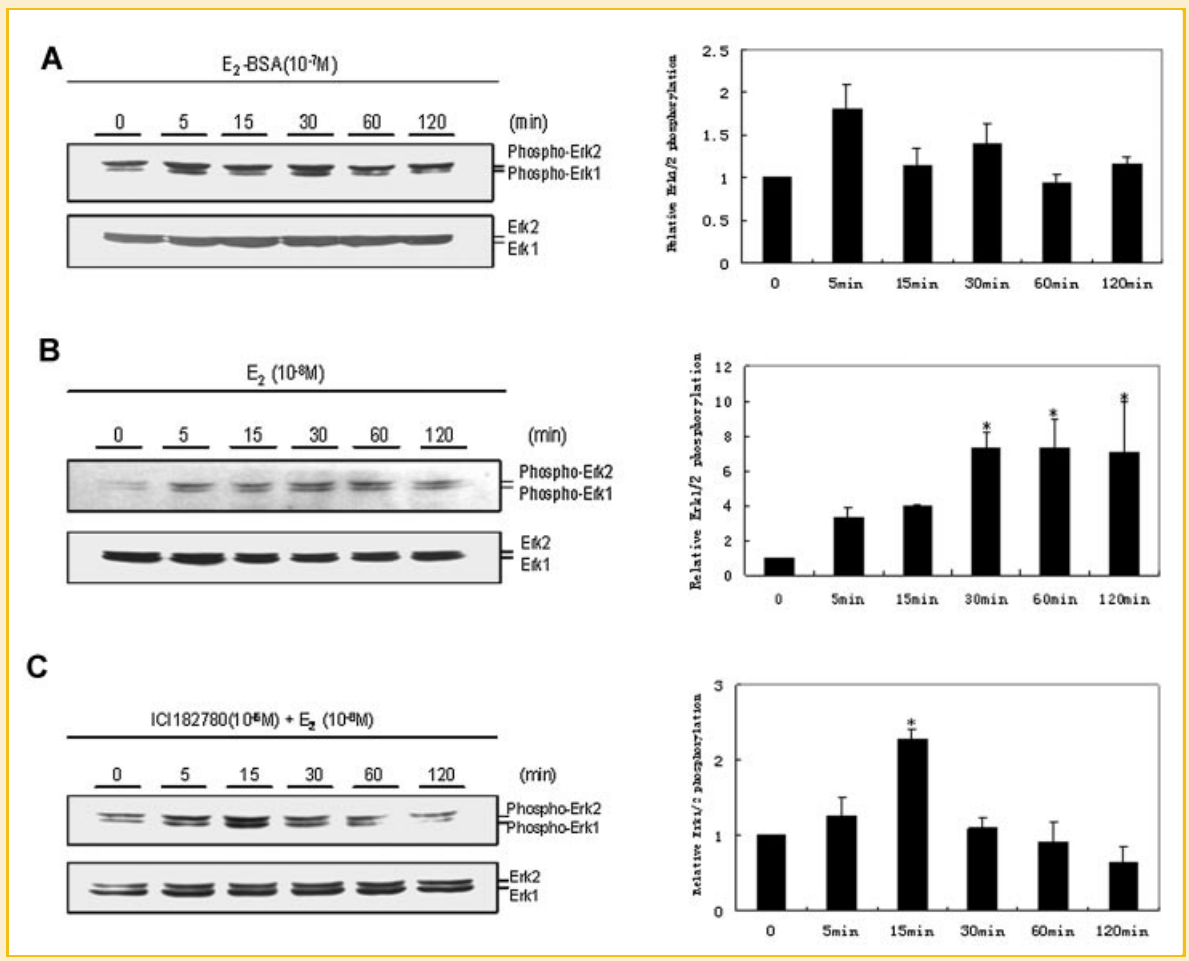

Fig. 2. Hec-1A cells treated with E2 or E2–BSA: Western blot analysis of whole cell lysates for expression of phosphorylated and nonphosphorylated Erk1/2. Cells were treated for the indicated times with 100 nM E2–BSA (A), 10 nM E2 (B), or 10 nM E2 with 4 h pretreatment with 1 μM ICI182780 (C). At right, the intensity of each band from treated cell extracts is normalized to the intensity of the band corresponding to untreated cells, which was arbitrarily assigned a value of 1 OD unit. (mean ± SD of three independent immunoblots; asterisk (*) indicates $P < 0.05$). The control bar in (C) represents cells treated with ICI182780 alone.

Erk1/2 was observed, it persisted for only 15 min and was only twofold higher than control values, compared to the sixfold increase seen in the absence of this antagonist.

E2 AND E2–BSA DO NOT INDUCE Akt ACTIVATION IN ISHIKAWA AND HEC-1A CELLS

Figure 3 shows the effect of E2 and E2–BSA on Akt activation in Ishikawa and Hec-1A cell lines. No activation of Akt could be seen in either of the two cell lines. In Ishikawa cells (Fig. 3A,B), there were no significant changes in Akt phosphorylation after treatments with E2 and E2–BSA, although a baseline level of phosphorylation was detectable. Similarly, in Hec-1A cells, neither E2 nor E2–BSA could activate the phosphorylation of Akt. Phosphorylation levels in Hec-1A cells were significantly lower than in Ishikawa cells: only after a long period chemiluminescence exposure could phosphorylated Akt be weakly detected (Fig. 3C). The results of treatment of Hec-1A cells with E2–BSA were similar to those with E2 treatment (data not shown).

CONFOCAL ANALYSIS OF E2–BSA-INDUCED Erk1/2 PHOSPHORYLATION INITIATED FROM THE PLASMA MEMBRANE IN ISHIKAWA CELLS

We next used immunofluorescence techniques to confirm that E2–BSA was able to promote Erk1/2 signaling without traversing the plasma membrane. Cells treated with E2–BSA that had been conjugated to FITC were fixed and immunocytochemically labeled for P-Erk1/2 using a TRITC label, so that the subcellular distribution of these two ligands could be compared. Figure 4A shows that E2–BSA was distributed around and within the plasma membrane, but did not reach the nuclear membrane. We compared cells fixed 5 and 30 min after E2–BSA–FITC treatment (Fig. 4B). At the 5 min time point, labeled E2–BSA had combined with the membrane, and the P-Erk1/2 signal was detectable but weak. By 30 min, most of the cells were activated, with a strong P-Erk1/2 signal, while the E2–BSA signal remained confined to the plasma membrane. We conclude from these findings that intracellular Erk1/2 signaling can be promoted by E2–BSA from the plasma membrane.

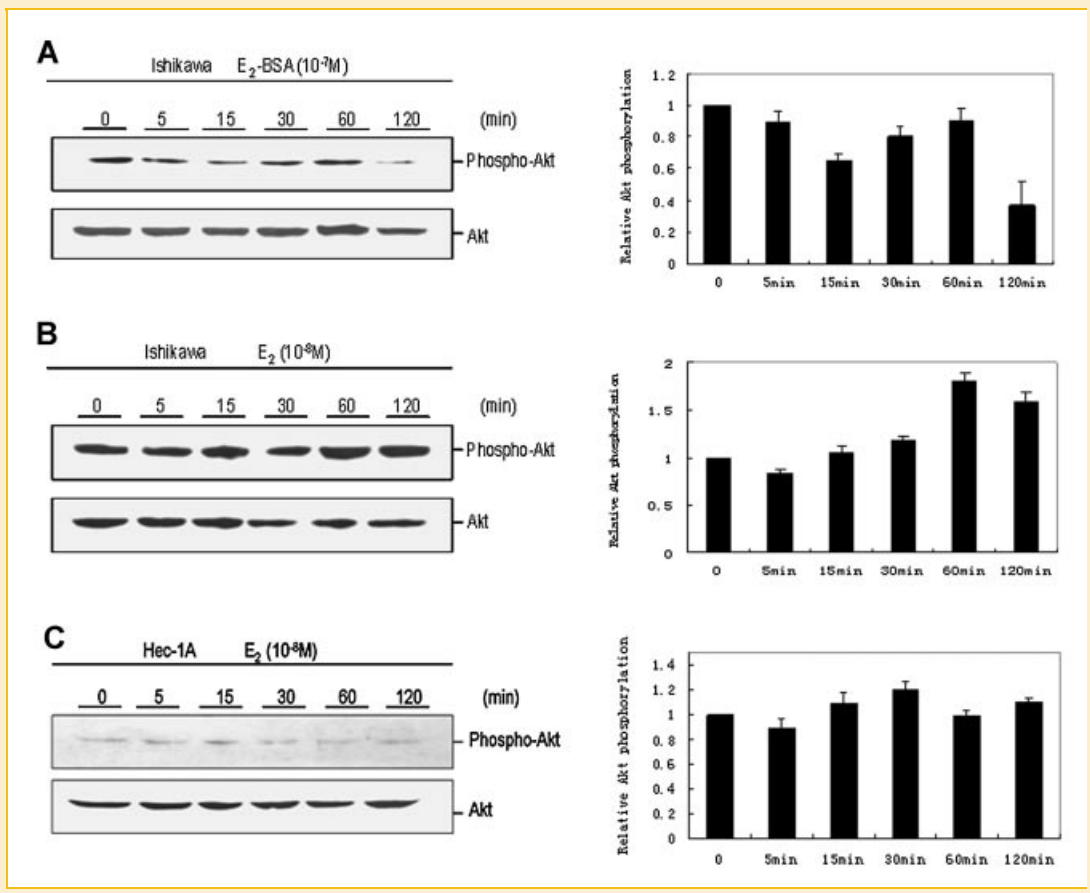

Fig. 3. Ishikawa and Hec-1A cells treated with E2 or E2–BSA: Western blot analysis of whole cell lysates for expression of phosphorylated and nonphosphorylated Akt. Ishikawa (A,B) or Hec-1A (C) cells were treated for the indicated times with 100 nM E2–BSA (A) or 10 nM E2 (B,C). At right, the intensity of each band from treated cell extracts is normalized to the intensity of the band corresponding to untreated cells, which was arbitrarily assigned a value of 1 OD unit (mean ± SD of three independent immunoblots; only significant differences in relative band intensity are shown).

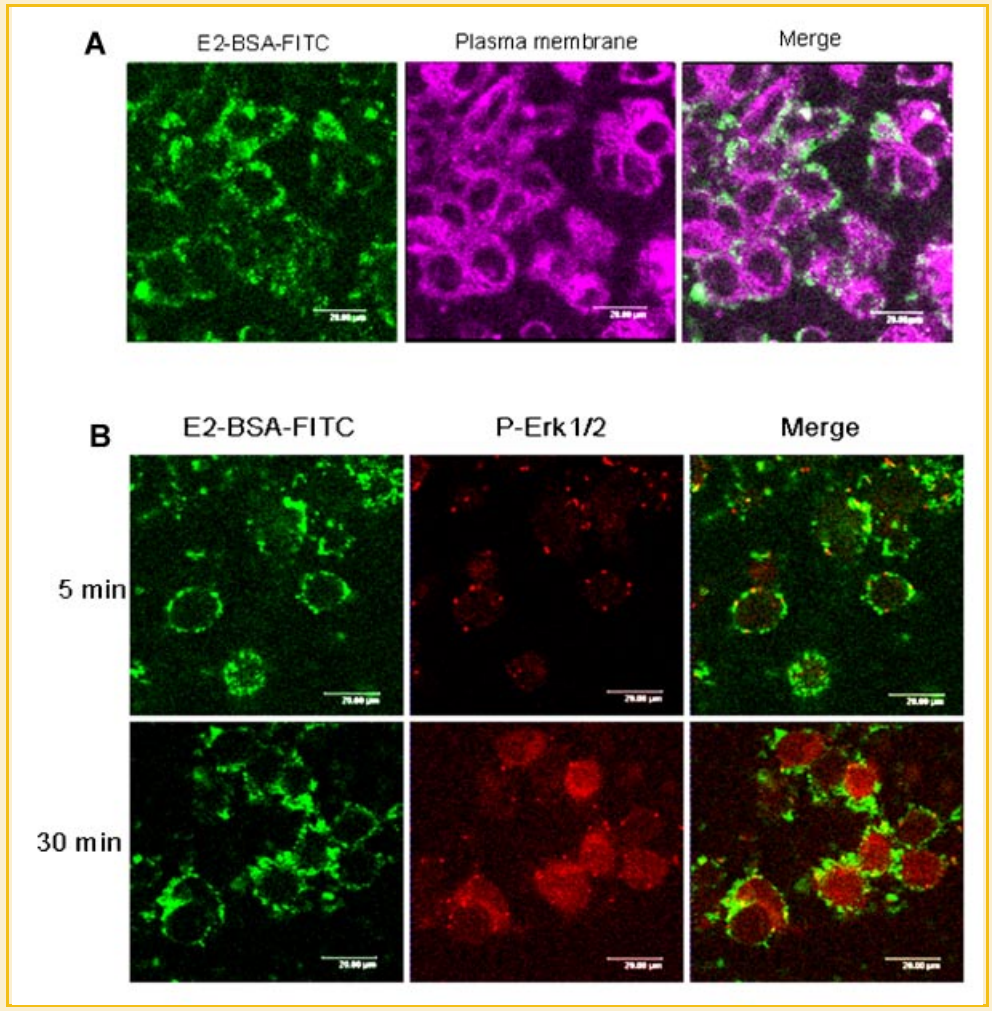

Fig. 4. Confocal whole-cell immunofluorescence analysis of membrane-induced Erk1/2 phosphorylation in Ishikawa cells. Three color-merged images are shown. Green indicates FITC-conjugated E2–BSA. Red represents phosphorylated Erk1/2 (detected using a TRITC-conjugated secondary antibody). Purple represents plasma membrane staining by DID. A: Comparison of E2–BSA distribution (left) with plasma membrane labeling (middle) in cells treated with E2–BSA for 30 min; the merged image (right) indicates that E2–BSA fails to penetrate the cell membrane. B: Cells were treated with E2–BSA for 5 (top) or 30 (bottom) min. Activation of phosphorylated Erk1/2 (middle) was clearly observed, particularly after the 30 min time point. The merged image at right indicates that Erk1/2 phosphorylation was stronger at 30 min compared with 5 min. Scale bars, 20 μm.

RAPID NONGENOMIC EFFECTS OF E2 AND E2–BSA ON $[CA^{2+}]I$ IN ISHIKAWA CELLS

Estrogen is known to induce calcium mobilization in receptive cells. In order to determine whether there is a nongenomic component to this response, we used confocal microscopy to compare the effect of E2 and E2–BSA on $[Ca^{2+}]i$. Ishikawa cells were loaded with Fluo-3AM to reveal relative calcium mobilization following stimulation by estrogen. To investigate the potential involvement of a membrane-associated receptor, Fluo-3AM-loaded cells were stimulated with varying concentrations of either E2 or E2–BSA. For each condition, three or four cells were chosen and their $[Ca^{2+}]i$ was followed over a series of time points following stimulation (Fig. 5A–D). Cells stimulated by E2 showed a rapid rise in $[Ca^{2+}]i$, which peaked at approximately 300 s and persisted for approximately 600 s. Cell stimulated by E2–BSA showed a similar rate of and persistence of $[Ca^{2+}]i$, but the response had two peaks. This phenomenon was apparent in both high (100 nM) or low (10 nM) E2–BSA conditions.

EXPRESSION OF BCL-2 INCREASED AFTER TREATMENT WITH E2 BUT NOT THAT WITH E2–BSA IN ISHIKAWA CELL LINE

The expression of the apoptosis-related proteins Bcl-2 and Bax are known to be regulated by ER receptors via the classical pathway. E2 activates rapid Ca(2+) influx in hippocampal neurons, which would lead to activation of the Src/Erk signaling cascade and up-regulation of Bcl-2 protein expression [Wu et al., 2005]. To explore if the nongenomic effects we had seen represented a distinct signaling mechanism, we investigate the effect of E2 and E2–BSA on the expression of these proteins (Fig. 6). The expression of Bcl-2

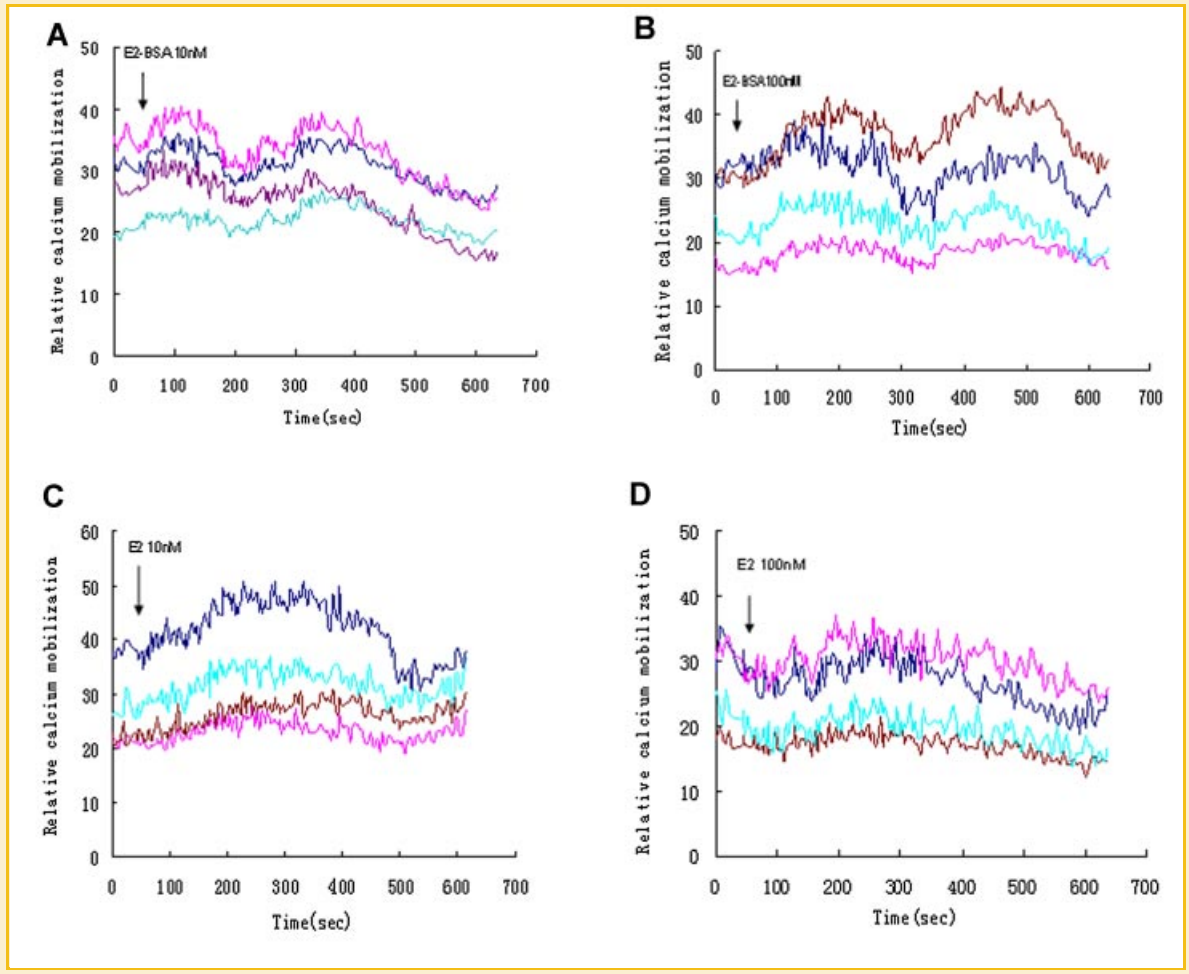

Fig. 5. Estrogen-mediated calcium mobilization in Ishikawa cells. Cells were loaded with Fluo-3AM, bathed in phenol red-free DMEM containing either 5% dcFBS (A–D), then stimulated with the indicated concentrations of E2 or E2–BSA or BSA alone. Fluorescence microscopy shows that cells loaded with Fluo-3AM appeared brighter after stimulation by E2, indicating an increase in the concentration of free calcium. For each dose of E2 or E2–BSA, three or four cells were chosen for analysis and their fluorescence intensity followed for 600 s (A–D). Each colored curve indicates the change in $[Ca^{2+}]i$ in a single isolated cell. Note the differing time course of $[Ca^{2+}]i$ change after stimulation by 10 nM E2–BSA (A) compared to that that of cells stimulated with 10 nM E2–BSA (C).

increased significantly after treatment with E2 for 8h, reaching normal levels after 24 h. In contrast, in Ishikawa cells, the expression of Bax was unaffected by treatment with E2 for up to 24 h (Fig. 6A). The expression of Bcl-2 and Bax were unaffected when either cell line was treated with E2–BSA (Fig. 6B).

DISCUSSION

E2 CAN RAPIDLY ACTIVATE Erk1/2 IN ISHIKAWA AND HEC-1A CELLS; E2–BSA CAN ALSO ACTIVATE Erk1/2 IN ISHIKAWA CELLS BUT NOT IN HEC-1A CELLS

Nongenomic activation of Erk by estrogen in endometrial cancer cells had been demonstrated earlier [Singleton et al., 2003]. In our present study, E2 and E2–BSA could both rapidly activate Erk1/2 in Ishikawa cells. However, the response to the two ligands differed in their time of onset, intensity, and time course, with E2–BSA generally yielding a less intense response than E2. The most likely explanation for this finding is that the E2 response stimulated both genomic and nongenomic pathways, with E2–BSA only stimulating the latter.

Ca^{2+} influx can activate Erk in many researches .In cultured rat astrocytes, the absence of extracellular Ca^{2+} completely abolished Erk activation [Schliess et al., 1996], regulation of cellular responses by Ca^{2+}-dependent pathways in VSM cells may be mediated in part by CaM kinase II-dependent activation of Erk1/2 [Abraham et al., 1997], and pretreatment of the cells with the Ca^{2+} chelators resulted in a partial decrease of the Erk response [Melien et al., 2002]. Estrogen has been shown to activate L-type voltage-gated calcium channels [Sarkar et al., 2008]. In this study, L-type Ca^{2+} channel inhibitor Nifedipine was used to detect the connection of estrogen and Erk signal transduction. The phosphorylation of Erk can be inhibited by both E2 and E2–BSA. But ER inhibitor ICI182780 had no effects.

In Hec-1A cells, whose ER expression is low, Erk1/2 could not be activated by E2–BSA. In contrast, the activation was rapid and apparent in the ER-positive Ishikawa cells. This suggests that ERs in

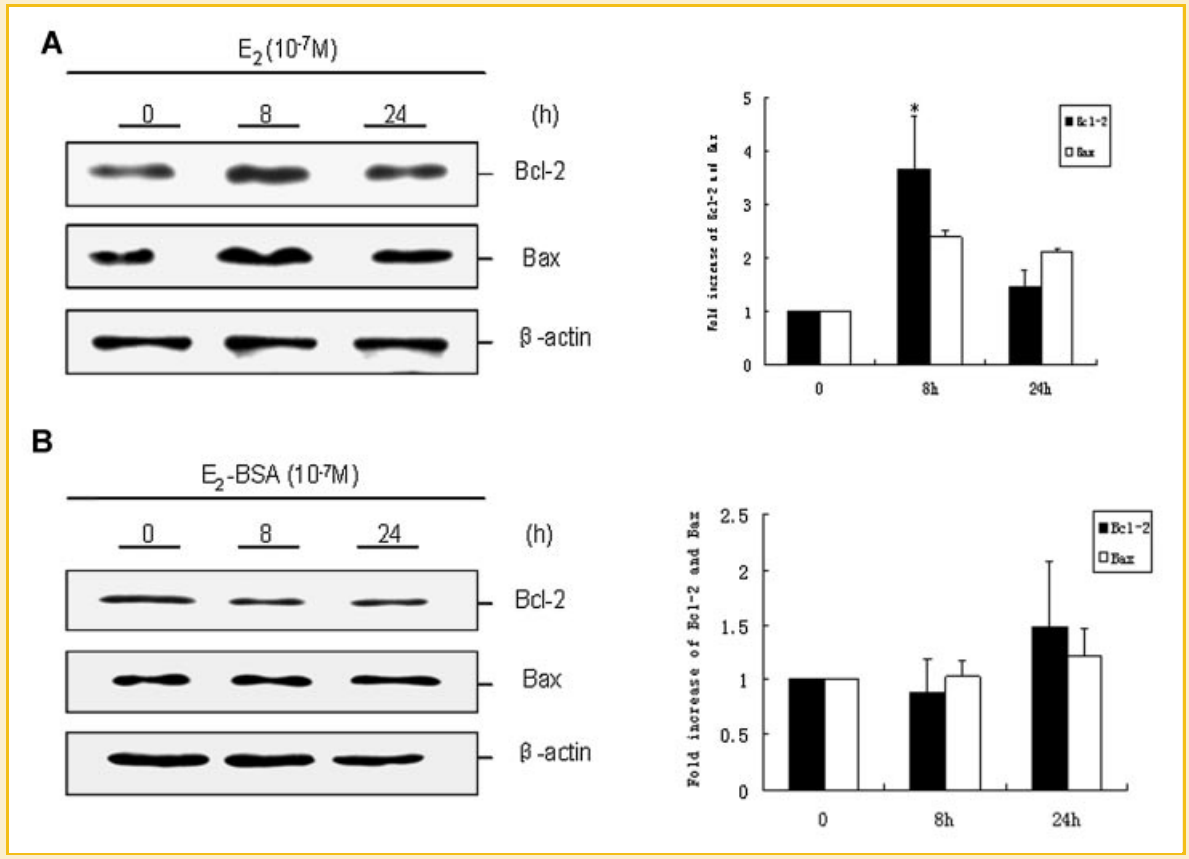

Fig. 6. Expression of Bcl-2 and Bax after treatment with E2 and E2–BSA: Western blot analysis of whole cell lysates. Blots were probed with anti-β-actin as a control. A: Expression of Bcl-2 and Bax after treatment with 100 nM E2 for the indicated time periods. Relative intensity of Bcl-2 and Bax bands at each time period are shown at right. B: Expression of Bcl-2 and Bax after the treatment of 100 nM E2–BSA for the indicated time periods. Relative intensity of Bcl-2 and Bax bands at each time period are shown at right. (mean ± SD of three independent immunoblots; a value of 1 OD unit was assigned to the untreated point; asterisk (*) indicates $P < 0.05$).

the plasma membrane are required for nongenomic effects in endometrial carcinoma cells. However, other ligands of E2 must also be involved in its nongenomic effects, because when the pure ER antagonist ICI182780 was used in Hec-1A cells, Erk1/2 phosphorylation was not inhibited completely.

Measurement of Erk1/2 activation by confocal microscopy provided further evidence of the nongenomic effects of E2 in Ishikawa cells. We found that the localization of E2–BSA to the cell surface was coincident with the appearance of Erk1/2 phosphorylation. The rapidity of this localization (within 5 min of stimulation), strongly suggests that there are estrogen-binding molecules estrogen in the plasma membrane; these may represent mER or other estrogen ligands.

E2–BSA AND E2 ARE UNABLE TO ACTIVATE AKT IN ISHIKAWA CELLS OR HEC-1A CELLS

In this study, E2 was unable to induce phosphorylation of Akt in either Ishikawa or Hec-1A cells. In Hec-1A cells, the phosphorylation of Akt was very weak even when treated with estrogen. This suggests that, in Hec-1A cells, direct stimulation of the Akt signaling pathway by estrogen is unlikely to play a significant physiological role.

A non-nuclear estrogen-signaling pathway involving the direct interaction of ERα with PI3K has been defined in human vascular cells [Simoncini et al., 2000]. In the endometrial carcinoma cells we studied, baseline levels of Akt phosphorylation were higher in the ER-positive Ishikawa cells than in Hec-1A cells. However, in neither line was E2 or E2–BSA able to effect on Akt phosphorylation.

It is not surprising that E2 can activate multiple signaling pathways in different cell lines, considering the fact that it can exert nongenomic effects on PKA, PKC, and MAPK. Estradiol may exert part of its proliferative and anti-apoptotic effects in a nongenomic manner through the Akt signaling pathway in endometrial cells [Guzeloglu Kayisli et al., 2004; Gielen et al., 2007]. Activation of the PI3K pathway appears to be a common requirement for the expression of estrogen-induced genes [Kazi and Koos, 2007]. Akt pathway also plays a role in breast cancer cell, estrogen acutely increases PI3-kinase and Akt activities in MCF-7 cells [Castoria et al., 2001].

E2–BSA AND E2 CAN ACTIVATE FAST CALCIUM FLUX IN ISHIKAWA CELLS

E2 rapidly activates signaling cascades that regulate important physiological processes, including transport of ions such as Ca^{2+}

across membranes to induce a rapid increase in $[Ca^{2+}]i$; this free $[Ca^{2+}]i$ may be released from either outside the plasma membrane or from the endoplasmic reticulum [Thomas et al., 2006]. In female rat distal colonic crypts, a rapid increase of $[Ca^{2+}]i$ has been shown to be elicited by both E2 and E2–BSA, through a G-protein-coupled membrane associated receptor distinct from the classical ER [Doolan and Harvey, 2003]. A similar increase of $[Ca^{2+}]i$ has also been observed in PC3 human prostate cancer cells [Huang and Jan, 2001], hippocampal neurons [Huang et al., 2004], human sperm cells [Luconi et al., 1999], and human endometrial cells [Perret et al., 2001].

In the present study, we observed a rapid increase in $[Ca^{2+}]i$ following stimulation by either E2 or E2–BSA, appearing within 1 min of stimulation and lasting for 8 min. However, in contrast to the single peak in $[Ca^{2+}]i$ triggered by E2, E2–BSA treatment elicited a dual peak: at approximately 3–6 min, the $[Ca^{2+}]i$ diminished and then increased again rapidly. The first wave may represent calcium influx from opening calcium channels in the plasma membrane, with the second wave representing the releasing of stored calcium. The fact that E2–BSA is concentrated at the plasma membrane, unlike E2, may allow it to stimulate G proteins at that locus, activating the PLC/IP3 signaling pathway to induce the release of stored calcium.

THE EXPRESSION OF APOPTOSIS-RELATED PROTEINS OF BCL-2 AND BAX IN ISHIKAWA CELLS

Bcl-2 expression may play a role in the inhibition of apoptosis in endometrial carcinoma, and its expression also seems to be associated with tumor differentiation and cell proliferation. E2 activates rapid Ca^{2+} influx, which can lead to activation of the Erk signaling cascade, and up-regulation of Bcl-2 protein expression, in THP-1 macrophages [Subramanian and Shaha, 2007] and hippocampal neurons [Wu et al., 2005]. Administration E2–BSA or E2 after trauma-hemorrhage has been shown to induce the up-regulation of ERα and GPR30; this is accompanied by increases in Bcl-2 expression [Hsieh et al., 2007]. The Bax gene is a member of the bcl-2 gene family and a pro-apoptotic protein. Bax can promote cell death and also heterodimerize with Bcl-2 to neutralize the anti-apoptotic function of Bcl-2. The role of Bax in endometrial carcinogenesis is largely unknown [Sakuragi et al., 2002].

In our study, the expression of Bcl-2 increased significantly after treatment with E2 for 8 h, but no change was seen in Bax expression. E2–BSA was unable to induce either Bcl-2 expression. This may indicate that E2 can induce anti-apoptotic activity via classic genomic effect, but that the nongenomic effect initiated from the plasma membrane by E2–BSA was not sufficient to lead to the genomically mediated anti-apoptotic mechanism.

In summary, we have characterized the effect of E2 and E2–BSA on stimulation of the Erk1/2 signal pathway and on calcium influx in human endometrial carcinoma cells. The release of Ca^{2+} from intracellular stores as well as influx of extracellular Ca^{2+} is of importance for the hormone-induced activation of Erk1/2. Calcium transients, as a result of entry through L-type Ca^{2+} channels, are known to activate MAPK [Dolmetsch et al., 2001], PI3K/AKT [Vaillant et al., 1999], and adenylyl cyclase signaling pathways [Chan et al., 2005]. E2 and E2–BSA can stimulate Erk1/2 signal pathway, but the inhibitor of L-type voltage-gated calcium channels decreased the stimulation of Erk 1/2.So the stimulation of Erk1/2 signal pathway by estrogen is through Ca^{2+} channels. In our present study, the relative contributions of the classical and nongenomic effects varied in the different cell lines we studied. In contrast, we found no nongenomic effect in the expression of the anti-apoptotic-protein Bcl-2, highlighting the range of influence of the nongenomic pathway on different downstream effectors. This study provides new insights into the diversity and variability of nongenomic effect elicited by E2 and E2–BSA. Future investigation is needed to more thoroughly explore both the possible interactions between the genomic and nongenomic and to identify and characterize the plasma membrane ERs involved in the nongenomic response.

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