Role of HIF-1α and VEGF in human mesenchymal stem cell proliferation by 17β-estradiol: involvement of PKC, PI3K/Akt, and MAPKs

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Yun SP, Lee MY, Ryu JM, Song CH, Han HJ. Role of HIF-1α and VEGF in human mesenchymal stem cell proliferation by 17β-estradiol: involvement of PKC, PI3K/Akt, and MAPKs. Am J Physiol Cell Physiol 296: C317–C326, 2009. First published November 5, 2008; doi:10.1152/ajpcell.00415.2008.—17β-Estradiol (E2) is a steroid hormone well known for its roles in the regulation of various cell functions. However, the precise role that E2 plays in the proliferation of human mesenchymal stem cells (hMSCs) has not been completely elucidated. In the present study, we examined the effects of E2 on cell proliferation and the related signaling pathways using hMSCs. We showed that E2, at ≥10−8 M, significantly increased [3H]thymidine incorporation after 24 h of incubation, and E2 also increased [3H]thymidine incorporation at >6 h. Also, E2 significantly increased the percentage of the cell population in the S phase based on FACS analysis. Moreover, E2 increased estrogen receptor (ER), PKC, phosphatidylinositol 3-kinase (PI3K/Akt), and MAPK phosphorylation. Subsequently, these signaling molecules were involved in an E2-induced increase of HRE binding and VEGF protein levels. These levels of protein expression were inhibited by ICI-182,780 (10−6 M, an ER antagonist), staurosporine and bisindolylmaleimide I (10−6 M, a PKC inhibitor), LY-294002 (10−6 M, a PI3K inhibitor), Akt inhibitor (10−5 M), SP-600125 (10−6 M, a SAMS/JNK inhibitor), and PD-98059 (10−6 M, a p44/42 MAPKs inhibitor). In addition, HIF-1α small interfering (si)RNA and ICI-182,780 inhibited E2-induced VEGF expression and cell proliferation. VEGF siRNA also significantly inhibited E2-induced cell proliferation. In conclusion, E2 partially stimulated hMSC proliferation via HIF-1α activation and VEGF expression through PKC, PI3K/Akt, and MAPK pathways.

mitogen-activated protein kinases; hypoxia-inducible factor-1α; vascular endothelial growth factor; protein kinase C; phosphatidylinositol 3-kinase

since recent studies (6, 13, 14) have reported the presence of estrogen receptors (ERs) on stem cells, suggesting that estrogen may modify the function of those cells, a greater understanding of estrogen regulation on diverse stem cell types from a cell biology perspective is required to improve their ultimate clinical efficacy. To achieve this goal, the role of estrogen on stem cell function must be elucidated. Mesenchymal stem cells are known as a population of multipotential cells able to proliferate and differentiate into multiple mesodermal tissues, including bone, cartilage, muscle, ligament, tendon, fat, and stroma (23). A study (4) has shown that estrogen [17β-estradiol (E2)], via the ER, affects the proliferation and differentiation of neural stem cells, probably in conjunction with other factors governing the development of neural stem cells, but not human mesenchymal stem cells (hMSCs). The classic mechanism of action of E2 on target tissues involves the binding of E2 to ERα and/or ERß. These ligand-activated nuclear receptors then act as transcription factors to stimulate mRNA synthesis over a time course of hours to days (34). Steroid hormones, including E2, are also known to exert rapid, nongenomic effects on target tissues. These effects involve the rapid (<30 min) activation of intracellular signaling pathways; the similarity of these effects to those induced by growth factors supports the existence of putative membrane receptors for E2 within the plasma membrane (18, 29). Rapid, nongenomic effects of E2 have been identified in mammalian colonic tissue (11). These include the activation of signal transduction pathways, such as PKC, intracellular calcium, intracellular pH, phosphatidylinositol 3-kinase (PI3K/Akt), and MAPKs (11, 31).

In addition to this, a number of studies have confirmed the role of phosphorylation by PI3K/Akt and MAPK pathways in the regulation of hypoxia inducible factor (HIF)-1α expression and/or stabilization and of HIF-1α transactivity (18). On the basis of these observations, we hypothesized that E2 is involved in the regulation of HIF-1α in hMSCs, a role not heretofore investigated. Similarly, the effect of E2 on VEGF production has been restricted to studies on mesodermal cell lines but not stem cells. Also, HIF-1α has recently been shown to mediate the induction of VEGF expression in a variety of cell types by a number of other hormones, growth factors, and cytokines under nonhypoxic conditions (7). When activated, HIF-1α binds to the DNA sequence 5′-ATACGTGGG-3′ [the hypoxia-responsive element (HRE)] of target genes, and a number of putative target genes have been detected on the basis of the presence of this HRE (21). These genes have several functions, including the promotion of cell survival and proliferation. Thus, in the present study, we investigated the potential activation of HIF-1α and expression of VEGF by E2 and identified the signaling pathways involved in the proliferation of hMSCs.

MATERIALS AND METHODS

Materials. hMSCs were obtained from JB Stem Cell Institute. FBS was obtained from BioWhittaker (Walkersville, MD). E2, ICI-182,780, LY-294002, Akt inhibitor, bisindolylmaleimide, staurosporine, PD-98059, and SP-600125 were obtained from Sigma Chemical (St. Louis, MO). [3H]thymidine was obtained from NEN [specific activity: 74 GBq/mmol (2.0 Ci/mmol), Amersham Biosciences, Buckinghamshire, UK]. Rabbit polyclonal anti-phospho-Akt (Thr308), anti-phos-

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pho-Akt (Ser473), anti-total Akt, anti-phospho-SAPK/JNK, anti-total SAPK/JNK, anti-pan PKC, anti-PKC-α, anti-PKC-ε, anti-PKC-ζ, anti-PKC-ρ, anti-VEGF receptor (Flk-1), anti-ERα, and anti-ERβ antibodies as well as mouse polyclonal anti-VEGF antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A SMARTpool of small interfering (si)RNAs specific for HIF-1α (100 nmol/l) and a nontargeting siRNA were obtained from Dharmacon (Lafayette, CO). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA). Rabbit monoclonal anti-phospho-p44/42 and anti-p44/42 MAPK antibodies were obtained from New England Biolabs (Herts, UK). Goat anti-rabbit IgG antibody was purchased from Jackson Immunoresearch (West Grove, PA). Liquiscint was obtained from National Diagnostics (Parsippany, NY).

Isolation of hMSCs. After the initial 31 days of primary culture, hMSCs adhered to a plastic surface and presented a small population of single cells with a spindle shape. On days 7–10 after the initial plating, cells had the appearance of long, spindle-shaped fibroblastic cells, began to form colonies, and became confluent. After being replated, the fibroblast-like cells were polygonal or spindly with a long process (Fig. 1, A–C). hMSC passages 4–12 were observed under a microscope. Cells appeared normal on the basis of typical morphology. hMSCs were positive for CD29, CD44, CD73, CD90, CD105, and HLA-ABC, but negative for CD14, CD34, CD45, CD74, CD106, and HLA-DR (Fig. 1, D and E). With osteogenic supplementation, differentiation was apparent after 1 wk of incubation. By the end of the second week, a portion of hMSCs became von Kossa positive (Fig. 2, A and B). Similarly, the portion of cells that was induced with adipogenic medium contained numerous oil-red-O-positive lipid droplets (Fig. 2, C and D). With neuronal supplementation, differentiation was apparent after 1 wk of incubation. After 1 wk, media were observed under a microscope to check for axon formation and enlargement of nuclei (Fig. 2, E and F).

hMSC culture. hMSCs were cultured without a feeder layer in phenol red-free DMEM (GIBCO-BRL, Gaithersburg, MD) supplemented with 3.7 g/l sodium bicarbonate, 1% penicillin and streptomycin, 1.7 mM l-glutamine, 0.1 mM β-mercaptoethanol, and 10% FBS. For each experiment, cells were grown on gelatinized six-well plates or 100-mm culture dishes in an incubator maintained at 37°C with 5% CO₂. The medium was replaced with serum-free DMEM for 24 h before experiments. After the incubation, cells were washed twice with PBS and then maintained in serum-free DMEM including all supplements and the indicated agents.

[^3H]thymidine incorporation and cell counting. [^3H]thymidine incorporation experiments were carried out using methods previously described by Brett et al. (4a). In this study, cells were cultured in a single well until they reached 70% confluence. They were then washed twice with PBS and maintained in serum-free DMEM including all supplements. After 24 h of incubation, cells were washed twice with PBS and incubated with fresh serum-free DMEM containing all supplements and the indicated agents. After the indicated incubation period, 1 µCi of [methyl-[^3H]]thymidine was added to the cultures. Incubation with [^3H]thymidine continued for 4 h at 37°C. Cells were...
washed twice with PBS, fixed in 10% trichloroacetic acid (TCA) at 23°C for 30 min, and then washed twice with 5% TCA. The acid-insoluble material was dissolved in 2 N NaOH for 12 h at 23°C. Aliquots were removed to measure the radioactivity using a liquid scintillation counter. All values are reported as means ± SE of triplicate experiments. Values were converted from absolute counts to percentages of the control to allow a comparison between experiments.

Trypan blue exclusion assay. Cells were incubated with E2 for 24 h as described above and washed twice with PBS. Cells were then detached from the culture dishes using 0.05% trypsin and 0.5 mM EDTA solution, and the action was quenched with soybean trypsin inhibitor (0.05 mg/ml). Subsequently, 0.4% (wt/vol) trypan blue solution (500 μl) was added to the cell suspension, and cells were counted on a hemocytometer under optical microscopy while a separate count of the blue cells was kept. Cells failing to exclude the dye were considered to be nonviable; data are expressed as percentages of viable cells.

HIF-1α and VEGF siRNA transfection. Cells were grown to 75% confluence in each dish and were transfected for 24 h with either a SMARTpool of siRNAs specific for HIF-1α and VEGF (100 nmol/l) or a nontargeting siRNA as a negative control (100 nmol/l, Dharmacon) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Western blot analysis. Cells were harvested, washed twice with PBS, and lysed with a buffer [20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mg/ml aprotinin, and 1 mM PMSF] for 30 min on ice. Lysates were then cleared by centrifugation (15,000 rpm at 4°C for 10 min). The protein concentration was determined by the Bradford procedure (3). Equal amounts of protein (20 μg) were resolved by electrophoresis by 10% SDS-PAGE and transferred to a polyvinylidene fluoride transfer membrane. After the blots had been washed with Tris-buffered saline-Tween 20 [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.05% Tween 20], membranes were blocked with 5% skimmed milk for 1 h and incubated with an appropriate primary antibody at dilutions recommended by the supplier. The membrane was then washed, primary antibodies were detected with horseradish peroxidase-conjugated secondary antibody, and bands were then visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

FACS analysis. Cells were incubated under E2-treated conditions for 12 h. They were then dissociated in trypsin-EDTA, pelleted by centrifugation, and resuspended at ~106 cells/ml in PBS containing 0.1% BSA. Cells were then fixed in 70% ice-cold ethanol followed by an incubation in a freshly prepared nuclei staining buffer [250 μg/ml propidium iodide (PI) and 100 μg/ml RNase] for 30 min at 37°C. Cell cycle histograms were generated after analyzing PI-stained cells by flow cytometry (Beckman Coulter, Fullerton, CA). At least 104 events were recorded for each sample. Samples were analyzed using CXP software (Beckman Coulter).

Statistical analysis. Results are expressed as means ± SE. All experiments were analyzed by ANOVA, followed in some experiments by a comparison of treatment means with the control using the Bonferroni-Dunn test. Differences were considered statistically significant when P < 0.05.

RESULTS

Effect of E2 on cell proliferation. To examine the effect of E2 on cell proliferation, hMSCs were incubated with 10−9 M E2 for various periods of time (0–24 h) or for 24 h with various concentrations (10−9–10−5 M) of E2. Figure 3A shows that E2, at ≥10−9 M, significantly increased the level of [3H]thymidine incorporation in a 24-h incubation period. As shown in Fig. 3B, a significant increase in [3H]thymidine incorporation was initially observed 6 h after incubation with 10−9 M E2 (22% increase vs. control, P < 0.05). Figure 3C shows that E2 increased the percentage of the cell population in the S phase to 49.7% compared with the control (9.27%). In addition, there was a significant increase in cell number with E2 (10−9 M) after a 12-h incubation (Fig. 3D). We first hypothesized that E2 induced cell proliferation via ER (i.e., ERα and ERβ) expression in hMSCs. As shown in Fig. 4A, ERα and ERβ expression were increased by E2 treatment in a time-dependent manner.
Fig. 3. Effects of 17β-estradiol (E2) on cell proliferation. A: hMSCs were incubated for 24 h with various concentrations of E2 (10^{-10}–10^{-5} M) and pulsed with 1 μCi of [3H]thymidine for 1 h. B: hMSCs were incubated in the presence of E2 (10^{-9} M) for varying periods of time (0–24 h) and subsequently pulsed with 1 μCi of [3H]thymidine for 1 h before being counted. In A and B, values are means ± SE of 3 independent experiments with triplicate dishes. *P < 0.05 vs. control. C: FACS analysis. Propidium iodide-positive cells in response to different concentrations of E2 (10^{-9} M) for 12 h are shown. Cells were washed with PBS, fixed, stained, and analyzed by flow cytometry. The gates were configured manually to determine percentages of cells in the G1, S, and G2 phases based on DNA content. In C, values are means ± SE of 3 independent experiments. D: hMSCs were treated by E2 (10^{-9} M) for 12 h, and cells were then counted on a hemocytometer. In D, values are means ± SE of 5 independent experiments. *P < 0.05.

Fig. 4. Effects of E2 on estrogen receptor (ER) expression. A: hMSCs were incubated in the presence of E2 (10^{-9} M) for varying periods of time (0–24 h) and with 10% serum (positive control) and then harvested. Top, total protein was extracted and blotted with antibody against ERα (left) and ERβ (right). Each of the examples shown is representative of 5 independent experiments. Bottom, values are means ± SE of 5 independent experiments for each condition determined from densitometry relative to β-actin. *P < 0.05 vs. control. B: cells were pretreated with 10^{-6} M ICI-182,780 (an ER antagonist) for 30 min before E2 treatment for 12 h and then with 1μCi of [3H]thymidine for 1 h before being counted. Values are means ± SE of 5 independent experiments with triplicate dishes. *P < 0.05 vs. control; **P < 0.05 vs. E2 alone. C: FACS data for hMSCs pretreated with 10^{-6} M ICI-182,780 (an ER antagonist) before 12-h E2 treatment. Cells were washed with PBS, fixed, stained, and analyzed by flow cytometry. The gates were configured manually to determine percentages of cells in the G1, S, and G2 phases based on DNA content. Values are means ± SE of 3 independent experiments.
The maximum expression of ERs appeared 12 h after E2 treatment. To examine the involvement of ERs on the E2-induced increase in \( ^{3}H \)thymidine incorporation, hMSCs were pretreated with ICI-182,780 (10\(^{-6}\) M, an ER inhibitor) before E2 treatment. As shown in Fig. 4, B and C, ICI-182,780 reduced the E2-induced increase in \( ^{3}H \)thymidine incorporation and percentage of the cell population in the S phase.

**Involvement of PKC, PI3K/Akt, and MAPK pathways in the E2-induced increase in \( ^{3}H \)thymidine incorporation.** To examine the role of PKC, PI3K/Akt, and MAPK pathways in E2-induced cell proliferation, we first determined whether E2 induced the phosphorylation of pan PKC in hMSCs. As shown in Fig. 5A, Western blot analysis showed that there was an increase in phosphorylated pan PKC in hMSCs. As shown in Fig. 5, C and D, bisindolylmaleimide I and staurosporine reduced the E2-induced increase in \( ^{3}H \)thymidine incorporation and percentage of the cell population in the S phase. In addition, to assess the PI3K/Akt pathway, Western blot analysis was carried out. As shown in Fig. 6A, Western blot analysis showed that there was an increase in phosphorylated Akt (Thr\(^{308}\)) and phosphorylated Akt (Ser\(^{473}\)) 15 min after E2 treatment. To further elucidate the involvement of PI3K/Akt in E2-induced cell proliferation, we examined the effects of the PI3K/Akt pathway on the E2-induced increase in \( ^{3}H \)thymidine incorporation. hMSCs were pretreated with LY-294002 (10\(^{-6}\) M, a PI3K inhibitor) and Akt inhibitor (10\(^{-5}\) M) before E2 treatment. As shown in Fig. 6, B and C, LY-294002 and Akt inhibitor reduced the E2-induced increase in \( ^{3}H \)thymidine incorporation and percentage of the cell population in the S phase. Finally, to examine the role of the MAPK pathway in E2-induced cell proliferation, Western blot analysis was carried out. As shown in Fig. 7A, the phosphorylation of p44/42 MAPK and SAPK/JNK occurred as a time-dependent response to E2. The maximum phosphorylation of p44/42 MAPK and SAPK/JNK appeared 30 and 90 min after E2 treatment. To further elucidate the involvement of MAPKs in E2-induced cell proliferation, we observed the upstream signal pathway of p44/42 MAPK and SAPK/JNK. E2-induced phosphorylation of p44/42 MAPK was attenuated by pretreatment of the cells with bisindolylmaleimide I and staurosporine as well as Akt inhibitor (Fig. 7C). However, E2-induced phosphorylation of SAPK/JNK was not attenuated by pretreatment of the cells.

**Fig. 5. Effects of E2 on PKC expression.** A: hMSCs were incubated with E2 (10\(^{-9}\) M) for 0–120 min and with 10% serum (positive control) and then harvested. Top, total protein was extracted and blotted with antibody against phosphorylated pan PKC. Western blot analysis showed that there was an increase in phosphorylated pan PKC 90 min after E2 treatment. Each of the examples shown is representative of 3 independent experiments. Bottom, values are means ± SE of 3 independent experiments. B: hMSCs were stimulated with E2 (10\(^{-9}\) M) for 90 min, and extracted cytosolic and membrane proteins were then detected with PKC translocation of PKC isoforms. Western blot analysis showed that only PKC-ε translated after E2 treatment. C: cells were pretreated with 10\(^{-6}\) M bisindolylmaleimide I and staurosporine (PKC inhibitors) for 30 min before E2 treatment for 12 h and then with 1 \( \mu \)Ci of \( ^{3}H \)thymidine for 1 h before being counted. Values are means ± SE of 3 independent experiments with triplicate dishes. *P < 0.05 vs. control; **P < 0.05 vs. E2 alone. D: FACS data for hMSCs pretreated with 10\(^{-6}\) M bisindolylmaleimide I and staurosporine before 12-h E2 treatment. Cells were washed with PBS, fixed, stained, and analyzed by flow cytometry. The gates were configured manually to determine percentages of cells in the G1, S, and G2 phases based on DNA content. Values are means ± SE of 3 independent experiments.
with bisindolylmaleimide I and staurosporine (Fig. 7B). To examine the involvement of the MAPK pathway on the E2-induced increase in \([^{3}H]\)thymidine incorporation, hMSCs were pretreated with PD-98059 (10^{-5} M, a p44/42 MAPK inhibitor) and SP-600125 (10^{-6} M, a JNK/SAPK inhibitor) before E2 treatment. As shown in Fig. 7, D and E, PD-98059 and SP-600125 reduced the E2-induced increase in \([^{3}H]\)thymidine incorporation and percentage of the cell population in the S phase.

**Involvement of HIF-1α and VEGF expression in the E2-induced increase in \([^{3}H]\)thymidine incorporation.** The effect of E2 on the levels of expression of HIF-1α proteins, which are believed to be essential factors in VEGF expression, were examined to confirm the effect of E2 on the proliferation of hMSCs. HIF-1α levels increased significantly in response to E2 for 6 h (Fig. 8A). The increased level of HIF-1α expression was inhibited by SP-600125 and PD-98059 (Fig. 8B). We also determined the efficiency of HIF-1α-specific siRNA transfection using HIF-1α Western blot analysis (Fig. 8C). To further elucidate the involvement of HIF-1α in E2-induced cell proliferation, hMSCs were transfected with a pool of HIF-1α-specific siRNA (100 nmol/l) or nontargeting siRNA (100 nmol/l) before E2 treatment. As shown in Fig. 8, D and E, HIF-1α-specific siRNA reduced the E2-induced increase in \([^{3}H]\)thymidine incorporation and percentage of the cell population in the S phase. The effect of E2 on the levels of expression of VEGF proteins was examined to confirm the effect of E2 on the proliferation of hMSCs. VEGF receptor (Flk-1) and VEGF levels increased significantly in response to E2 for 12 h (Fig. 9, A and B). To further define the signaling molecules involved in E2-induced VEGF expression, we investigated whether the E2-dependent activation of ERs influenced VEGF expression in hMSCs. As shown in Fig. 9C, ICI-182,780 (10^{-6} M, an ER inhibitor) inhibited the levels of VEGF expression. This E2-induced VEGF expression was completely prevented by ICI-182,780. In addition, cells transfected with a pool of HIF-1α-specific siRNA (100 nmol/l) inhibited the level of VEGF expression (Fig. 9D). We determined the efficiency of VEGF-specific siRNA transfection using VEGF Western blot analysis (Fig. 9E). To further elucidate the involvement of VEGF in E2-induced cell proliferation, hMSCs were transfected with a pool of VEGF-specific siRNA (100 nmol/l) or nontargeting siRNA (100 nmol/l) before E2 treatment. As shown in Fig. 9, F and G, HIF-1α-specific siRNA reduced the E2-induced increase in \([^{3}H]\)thymidine incorporation and percentage of the cell population in the S phase.

**DISCUSSION**

In this study, E2 significantly increased the proliferation of hMSCs via HIF-1α activation and VEGF expression through PKC, PI3K/Akt, and MAPK pathways. The effect of E2 was measured at concentrations of 10^{-10}–10^{-7} M, representing physiological conditions, although the normal serum concentration of E2 depends to some extent on the species (9). In this study, E2 at \(\geq 10^{-9} M\) significantly increased the level of \([^{3}H]\)thymidine incorporation. In addition, E2 at 10^{-9} M increased \([^{3}H]\)thymidine incorporation, cell number, and percentage cells in the S phase during a 12-h incubation period. These findings strongly suggest that E2 plays a pivotal role in stimulating cell proliferation. As a general rule, the primary action of E2 is mediated by ERs. In addition, conventional ERs are transcription factors regulating gene expression, and these mechanisms may be mediated by a complex system of interacting signal molecules in various cell types (10, 15, 19, 20, 30). Moreover, current evidence indicates that the small population of EROx and ERβ is localized within the plasma mem-
brane (1, 5, 25). Therefore, we first examined the correlation between the effect of E2 and ERs. In this study, the pure ER antagonist ICI-182,780 blocked E2-induced hMSC proliferation. In addition, E2 treatment in hMSCs increased the expression of ERs. It is believed that genomic and nongenomic effects of steroid hormones regulate cell processes. Therefore, we think that ERs might be important for the E2 effect in hMSCs.

Various reports have suggested that E2 interacts with cell surface binding sites and induces intracellular signal molecules (26, 33). The present study suggested that E2 is linked to the activation of the PKC pathway in hMSCs. It was observed that PKC translocates from the cytosol to the membrane fraction. We also identified that PKC-β1 and -ε were activated in response to E2. Furthermore, bisindolylmaleimide I and staurosporine (a PKC inhibitor) decreased [3H]thymidine incorporation in hMSCs. These results suggest that PKC activation plays an important role in E2-induced hMSC proliferation. This is the first report showing that PKC activation is responsible for E2-induced proliferation in hMSCs. E2 also increased the
phosphorylation of Akt. The most striking example was the direct association between ERs and PI3K in cells stimulated by E2. Expression of PI3K by this mechanism results in the activation of Akt and downstream antiapoptotic signaling (8). In our study, the activation of Akt by E2 was a relatively fast response. To determine whether the activation of Akt by E2 was dependent on PI3K activity, we observed the effect of the PI3K specific inhibitor LY-294002 on the activation of Akt by E2. The induction of Akt by E2 was blocked by the PI3K inhibitor, indicating that E2 regulated Akt activity through the PI3K pathway. Taken together, these experiments suggest that PI3K inhibitors have regulating potential in self-renewal of hMSCs. Our results of blockade of Akt activation by PI3K inhibitors in hMSCs offer new hints for alternative strategies in the regulation of self-renewal, suggesting the use of compounds targeting the PI3K/Akt signaling cascade.

Many studies using somatic cells have demonstrated that PKC, PI3K/Akt, and MAPKs are essential for mediating mitogen-induced growth responses (2, 18, 36). For that reason, we hypothesized that PKC and PI3K/Akt are linked to the phosphorylation of the MAPK pathway and proliferation in hMSCs. Several groups have reported that E2 induces a rapid activation of MAPKs in breast cancer cells, putting into doubt the initial consensus that the mitogenic effects of E2 rely on transcriptional mechanisms (24, 39). Various studies have shown that phosphorylation of PI3K and the activation of p44/42 MAPK and JNK/SAPK are related to E2-mediated proliferation of several cell types (19, 22, 30, 37, 38). Also, p44/42 MAPK activation appeared to be downstream of PKC (18). Therefore, to determine whether the activation of p44/42 MAPK and JNK/SAPK by E2 was dependent on PI3K/Akt and PKC activity, we observed the effect of Akt inhibitor and bisindolylmaleimide I and staurosporine (PKC inhibitors) on the activation of p44/42 MAPK and JNK/SAPK by E2. In our study, the activation of p44/42 MAPK and JNK/SAPK by E2 was related to PI3K/Akt and PKC. PI3K/Akt and PKC are upstream mediators of p44/42 MAPK or JNK/SAPK. The induction of p44/42 MAPK by E2 was blocked by Akt inhibitor and PKC inhibitors, indicating that E2 regulates p44/42 MAPK activity through PI3K/Akt and PKC pathways. However, the induction of JNK/SAPK by E2 was only blocked by Akt inhibitor. These results suggest that MAPK activation via PKC...
and PI3K/Akt plays an important role in the proliferation of hMSCs.

Our results have established, for the first time, the relationship between the effect of E2 on the activation of HIF-1α, the involvement of PKC, PI3K/Akt, and MAPK signaling pathways in this activation, and the concomitant increase in VEGF production related to cell proliferation in hMSCs. We analyzed the effect of E2 on PKC, phosphorylated Akt, and HIF-1α expression and demonstrated the regulatory role of PKC and PI3K/Akt pathways in the regulation of HIF-1α expression. Thus, our results provide additional evidence that PKC and PI3K/Akt are required for E2-induced HIF-1α expression. However, our results provide additional evidence that PKC and PI3K/Akt signaling are a prerequisite for the activation of HIF-1α by E2. A number of different signaling pathways, including PKC/Akt or MAPK pathways, have been reported to be involved in VEGF gene induction by way of converging in the activation of HIF-1α (7, 17, 37). Indeed, our data also revealed that the activation of PKC, PI3K/Akt, and p44/42 MAPK pathways are required for the induction of HIF-1α, which induces VEGF expression. Many researchers have reported that VEGF expression is induced by HIF-1α activation by various extracellular stimuli, including E2 (12, 16, 28, 35), and these findings support the results of our experiments.

In conclusion, E2 in part stimulates hMSC proliferation via HIF-1α activation and VEGF expression through PKC, PI3K/Akt, and MAPK pathways. These observations of the effect of estrogen on hMSCs will allow investigators and clinicians to directly modulate the functions of these cells with the ultimate goal of generating more potent mesenchymal stem cell applications for the treatment of human disease.
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