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Biochemical and Biophysical Research Communications 300 (2003) 209–215
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17α-Estradiol-induced VEGF-A expression in rat pituitary tumor cells is mediated through ER independent but PI3K-Akt dependent signaling pathway☆,☆☆

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Received 16 November 2002

Abstract

17α-E_2, a weak estrogen exhibited both agonistic and antagonistic effects, and caused a time- and dose-dependent induction of VEGF-A mRNA expression in GH3 rat pituitary tumor cells. This effect was unaffected by the presence of the pure estrogen receptor antagonist ICI 182,780 but was specifically blocked by a protein synthesis inhibitor puromycin. Inhibition of phosphatidylinositol-3 kinase (PI3K) activity by wortmannin decreased the effect of 17α-E_2 on VEGF-A mRNA expression. This inhibitor also blocked the increase in phosphorylation of Akt induced by exposure to 17α-E_2. In contrast, exposure to the MAP kinase inhibitor, U0126, had no impact on 17α-E_2-induced VEGF-A mRNA expression. Taken together, these studies indicate that like potent estrogens 17α-E_2 up-regulates VEGF-A mRNA expression in estrogen responsive GH3 rat pituitary tumor cells, but this induction is not mediated through a classical estrogen receptor pathway. PI3K-Akt signaling pathway is required for the induction of VEGF-A mRNA in GH3 cells by 17α-E_2.

Keywords: Estrogen; Tumor cells; PI3K-Akt-signaling; MAP kinase; VEGF-A

17α-Estradiol (17α-E_2) is a weak or impeded, short-acting estrogen with a time-dependent, mixed agonistic–antagonistic effect [1]. It is produced in small quantities in ovarian follicular cells in the non-pregnant female [2] and converted into estrone and subsequent reduction of the 17 ketone to 17β-E_2 [3]. However, unlike other estrogens, including 17β-E_2, estrone, and synthetic estrogen such as ethinyl estradiol, 17α-E_2 is incapable of inducing uterine growth. When treated concomitantly with estrone or 17β-E_2, it efficiently reduced the uterine growth induced by estrone or 17β-E_2 [1,4]. Interestingly, 17α-E_2 would manifest full estrogenic capability if given continuously to animals [5] or treated breast tumor derived estrogen receptor positive MCF-7 cells [6]. However, the mode of action of 17α-E_2 is unclear. It is anticipated that 17α-E_2 is capable of acting as an estrogen, either alone or by conversion to naturally occurring female sex steroid, 17β-estradiol (17β-E_2) biologically derived from cholesterol [7]. This assumption, however, has yet to be elucidated and remains controversial whether 17α-E_2 is an innocuous hormone or an effective estrogen.

If 17α-E_2 is a proficient and effective estrogen as 17β-E_2, it would represent analogous effects on the expression of various genes including vascular endothelial growth factor-A (VEGF-A, formerly known as VEGF), which is a 17β-E_2 responsive gene and up-regulated in a variety of estrogen responsive neoplastic human and animal cells [8–14]. The purpose of the current study was to explore the effect of 17α-E_2 on VEGF expression in rat pituitary derived GH3 tumor cells and compare it

☆ This work was supported by NIHNCI CA 87680, V.A. Merit Review grant, Research Service Funds, Veterans Affairs Medical Center, Kansas City, MO, and the Midwest Biomedical Research Foundation, Kansas City, MO.

☆☆ *Abbreviations:* VEGF-A, vascular endothelial growth factor-A; 17β-E_2, 17β-estradiol, 17α-E_2, 17α-estradiol; DIG, digoxigenin, PI3K, phosphatidylinositol-3-kinase.

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PII: S0006-291X(02)02830-9

with the reported impact of a potent estrogen such as 17β-E_2 in estrogen responsive cells and tissues. In this context, our investigations aimed to investigate mechanism, if any, responsible for the action of 17α-E_2 on VEGF-A.

Materials and methods

Reagents. The DIG high prime DNA labeling and detection kit was obtained from Roche Diagnostics GmbH (Indianapolis, IN). Aprotinin, PMSF, leupeptin, 17β-E_2, 17α-E_2, and wortmannin were obtained from Sigma Chemical (St. Louis, MO). Fetal bovine serum and estrogen-free charcoal–dextran treated stripped fetal bovine serum were purchased from HyClone (Road Logan, UT). Anti-estrogen (ICI 182,780) was purchased from Tocris (Ellsville, MO). U0126 was obtained from Promega. DNA molecular weight marker was purchased from Bio-Rad (Richmond, CA). Anti-phospho-Akt antibody was purchased from Cell Signaling Technology (Beverly, MA). All other reagents, unless indicated otherwise, were obtained from Sigma Chemical.

Cell line, culture conditions, and treatment. Rat pituitary tumor derived GH3 cell line was obtained from American type culture collection (ATCC, Manassas, VA). For experiments, cells were grown in phenol red-free Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Rockville, MD) supplemented with 2.5% fetal bovine serum (HyClone, Road Logan, UT), 15% equine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin in a 37 °C, 5% CO_2 atmosphere. For experiments, the culture conditions and treatments were the same as described previously [15]. Briefly, GH3 cells at a concentration of 5×10^5/25 mm T-flask were grown in phenol red-free DMEM with 2.5% fetal bovine serum (FBS) and 15% equine serum. After reaching 70–80% confluent growth, the cells were grown in 5% charcoal-stripped FBS for three days to create an estrogen-free environment or complete serum-free medium overnight for serum starvation. GH3 cells were then exposed to different chemicals including 17α-E_2, 17β-E_2, pure anti-estrogen, puromycin, PI3K inhibitor, wortmannin, and MEK inhibitor U0126 as per the requirement of the experiments. DMSO or ethanol vehicle treatment was considered as untreated control.

RNA extraction, cDNA synthesis, and probe preparation. Total cellular RNA was extracted from GH3 cells using Trizol RNA isolation kit (Life Technologies, Grand Island, NY) following manufacturer's recommendations.

cDNA synthesis and probe preparation were performed according to the method described by Zoubine et al. [16].

Northern blot analysis. The Northern blot analysis was previously described [16]. Briefly, 10 μg of total RNA was fractionated by electrophoresis in 1.0% agarose gels containing formaldehyde and transferred to super charge nylon membrane (Schleicher and Schuell, Keene, NH). Membranes were hybridized with non-radioactive PCR generated-DIG-labeled VEGF or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene specific probe and washed according to protocols provided by the manufacturer of DIG high prime DNA labeling and detection kit. Relative expressions of VEGF mRNAs were calculated by densitometric analyses using the Gelexpert software program (NeucleoTech, CA). The signal intensity of VEGF bands was normalized to those obtained with the GAPDH bands.

Immunoblot assay for phospho-Akt. Whole cell lysates from GH3 cells treated with 17α-E_2 in presence or absence of wortmannin were prepared as previously described [15,16]. Fifty micrograms of protein from each sample was subjected to SDS–PAGE and immunoblot analysis as previously described [15,16]. After 1 h incubation in a blocking solution consisting of 5% dried milk in 20 mM Tris (pH 7.5) with 150 mM NaCl (TBS) plus 0.5% Tween 20 (TBST), the blots were exposed to a 1:500 dilution of anti-phospho-Akt (Ser473) overnight at 4 °C. After washing in 1× TBST, the blots were exposed to a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz, CA) for 1 h at room temperature. The membranes were washed five times in TBST and visualized using enhanced chemiluminescence (ECL, Amersham Life Sciences, Cleveland, OH).

Statistical analysis. All data are expressed as the means ± SD. Statistical significance was analyzed using non-paired Student's two-tailed t test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Up-regulation of VEGF-A mRNA by 17α-E_2 and 17β-E_2 in GH3 rat pituitary tumor cells is time- and dose-dependent

To determine the effect of 17α-E_2 on VEGF-A mRNA expression, GH3 cells were grown for three days in phenol red-free medium containing charcoal–dextran-stripped serum to eliminate estrogenic sources. Subsequently cells were exposed to either 10 or 100 nM 17α-E_2 for different times and their VEGF-A mRNA expression was determined using non-radioactive Northern blot analyses. As shown in Fig. 1, VEGF-A mRNA was constitutively expressed in GH3 rat pituitary tumor cells. The mRNA levels were significantly up-regulated by 2.5- and 2.0-fold when cells were exposed to 10 nM 17α-E_2 for 4 and 6 h, respectively. The VEGF-A mRNA

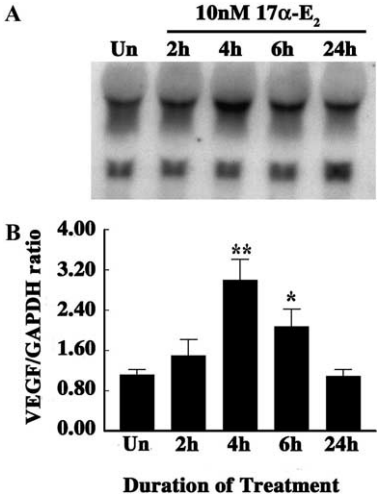

Fig. 1. Time course effects of 17α-E_2 on VEGF-A mRNA expression in GH3 rat pituitary tumor cells. Tumor cells were grown in estrogen-depleted medium (phenol red-free DMEM with 5% charcoal stripped serum) for three days. Cells were then exposed to 10 nM 17α-E_2 for indicated time and total RNA was analyzed by Northern blotting using non-radioactive DIG-labeled probe. (A) Single representative blot showing VEGF-A mRNA expression in 17α-E_2-treated and untreated cells, Un—untreated. (B) The normalized values indicate the relative abundance of VEGF-A mRNA in untreated and 17α-E_2-treated GH3 cells. Results are displayed as means ± SD from three separate experiments. p value was determined by Student's t test. *$p < 0.05$ versus control; **$p < 0.01$ versus control.

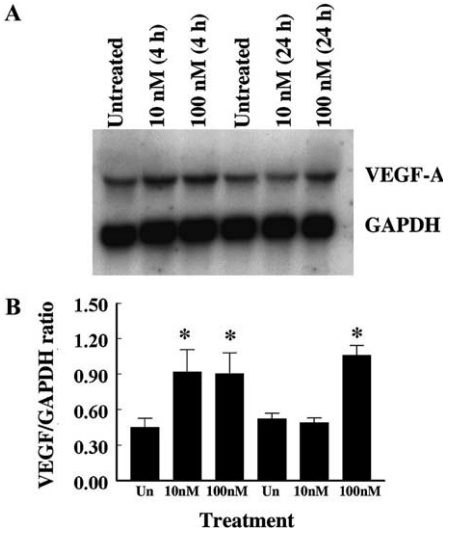

Fig. 2. Dose-dependent differential effects of 17α-E_2 on VEGF-A mRNA expression. GH3 cells were grown in estrogen-depleted medium for three days followed by the treatment of two different doses (i.e., 10 and 100 nM) of 17α-E_2 for two different indicated times. Total RNA was extracted and analyzed by Northern blotting using non-radioactive DIG-labeled probe. (A) Single representative blot showing VEGF-A mRNA expression in 17α-E_2-treated and untreated cells. (B) The normalized values indicate the relative abundance of VEGF-A mRNA in GH3 cells before and after treatment. Results are displayed as means ± SD from three separate experiments. $^{**}p < 0.01$ versus control.

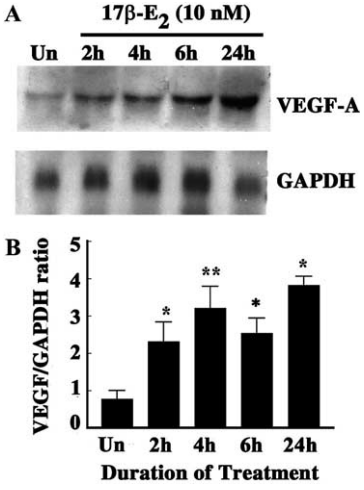

Fig. 3. Time course effects of 17β-E_2 on VEGF-A mRNA expression in GH3 rat pituitary tumor cells. Starved GH3 cells were exposed to 10 nM 17β-E_2 for indicated time and total RNA was analyzed by Northern blotting using non-radioactive DIG-labeled VEGF-A specific probe. (A) Single representative blot showing VEGF-A mRNA expression in 17β-E_2-treated and untreated cells, Un—untreated. (B) The normalized values indicate the relative abundance of VEGF-A mRNA in untreated and 17β-E2-treated GH3 cells. Results are displayed as means ± SD from three separate experiments. p value was determined by Student's t test. $^{*}p < 0.009$ versus control; $^{**}p < 0.001$ versus control.

levels were reduced to basal control level or declined to below the basal level at 24 h of 17α-E_2 (Figs. 1 and 2). In contrast, when cells were exposed to 100 nM 17α-E_2 for 4 or 24 h, VEGF-A mRNA levels were significantly up-regulated by 2.3- and 2.5-fold, respectively, i.e., mRNA did not return to basal level within 24 h (Fig. 2).

To compare the effects of a short-acting weak estrogen with those of the long-acting strong estrogen, 17β-E_2, starved cells were exposed to 10 nM of 17β-E_2 for different times and the level of VEGF-A mRNA was determined using Northern blots. As shown in Fig. 3, expression was up-regulated after exposure for 1 h and it steadily increased up to 5.0-fold by 24 h. Together, these studies show that VEGF-A mRNA expression can be induced in GH3 rat pituitary tumor cells by either short- or long-acting estrogens, but the effect of the former is transient.

Determination of the involvement of estrogen receptor and de novo protein synthesis in regulation of VEGF expression by 17α-E_2

To determine whether the effects of 17α-E_2 on VEGF-A mRNA expression in GH3 cells are mediated through an estrogen receptor, cells were grown under complete hormone depleted conditions for three days. Cells were then exposed to 10 nM of 17α-E_2 for 4 h in the presence or absence of 1 μM ICI 182,780 (a pure estrogen receptor antagonist totally lacking agonist activity) or 10 μg/ml puromycin (a protein synthesis inhibitor) and VEGF-A mRNA levels were evaluated. As seen in previous figures and shown here, 10 nM of

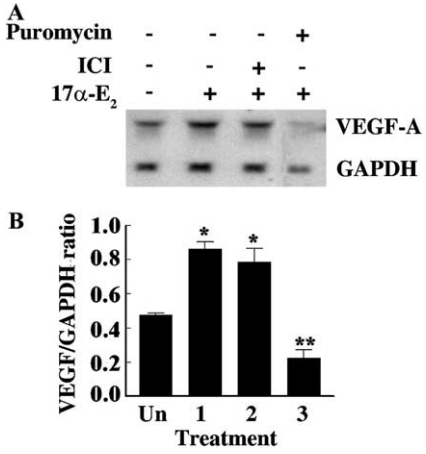

Fig. 4. Effects of pure anti-estrogen ICI 182,780 and puromycin on 17α-E_2-induced VEGF-A mRNA expression in GH3 rat pituitary tumor cells. GH3 cells were grown in estrogen-depleted medium for three days. Cells were then exposed to 17α-E_2 for 4 h in the presence or absence of ICI 182,780 or pre-exposed to puromycin for 30 min prior to the treatment of 17α-E_2 along with puromycin. (A) Single representative blot showing the impact of pure anti-estrogen and puromycin on 17α-E2-induced VEGF-A mRNA expression in GH3 cells. (B) The normalized values indicate the relative abundance of VEGF-A mRNA in GH3 cells before and after treatment. Results are displayed as means ± SD from three separate experiments. $^{*}p < 0.01$ versus control; $^{**}p < 0.001$ versus control.

17α-E_2 increased the VEGF-A mRNA level after 4 h of exposure (Fig. 4). ICI 182,780 was unable to suppress this 17α-E_2-stimulated increase in VEGF-A mRNA (Fig. 4). When cells were exposed to puromycin 20 min before the addition of 17α-E_2, puromycin suppressed 17α-E_2-induced VEGF-A mRNA expression (Fig. 4). These data suggest that 17α-E_2-induced up-regulation of VEGF-A mRNA in GH3 cells is accomplished by an estrogen receptor independent pathway, but that intermediate proteins are required for the stimulatory effect.

In comparison to the effect of 17α-E_2, 17β-E_2-induced up-regulation of VEGF-A mRNA expression was significantly decreased by concomitant exposure to 1 μM ICI 182,780 (Figs. 5A and B). The level after exposure to the anti-estrogen was only slightly higher than the basal levels (Fig. 5). The results are consistent with those of previous reports [6,15] and indicate that 17β-E_2-induced up-regulation of VEGF-A mRNA is mediated via an ER regulated pathway.

Involvement of the PI3K but not mitogen-activated protein kinase in 17α-E_2-induced VEGF-A mRNA expression

Phosphatidylinositol-3-kinase (PI3K), a family of enzymes with complex multifunctional roles, controls the activity of multiple downstream effectors including Akt [18,19]. The activation of PI3K-Akt signaling mediates several downstream cellular effects including vascular endothelial growth factor [20]. Furthermore, two independent studies have shown that PI3K-Akt signaling can be activated by estrogen via an estrogen receptor dependent [21] or independent pathway [22]. Since we found that 17α-E_2 stimulates VEGF-A mRNA expression in GH3 cells via non-estrogen receptor pathways (Fig. 4), a study was undertaken to determine whether PI3K-Akt-signaling was possibly involved in this process. Cells were grown in an estrogen-free environment for three days and then exposed to PI3K inhibitor, wortmannin, followed by treatment of 17α-E_2 for 4 h. Total RNA was extracted for the determination of VEGF-A mRNA expression. As shown in Figs. 6A and B wortmannin was able to inhibit significantly 17α-E_2-induced VEGF-A mRNA expression, but had no effect upon basal levels of VEGF-A mRNA. This result suggests that PI3K activity was required for the induction of VEGF-A expression by 17α-E_2.

In addition to regulating PI3K-Akt signaling, estrogen also augments mitogen-activated protein kinases (MAPK) activity in different cell types through ER-dependent or independent pathways [23–26]. Therefore, we are assuming that MAPK activity may also be involved in 17α-E_2-induced VEGF-A mRNA expression. To investigate if MAPK activity was required for VEGF-A expression induced by 17α-E_2, cells were exposed to 10 μM U0126, an inhibitor of MAP kinase kinase (MEK) for 30 min prior to the exposure of 10 nM of

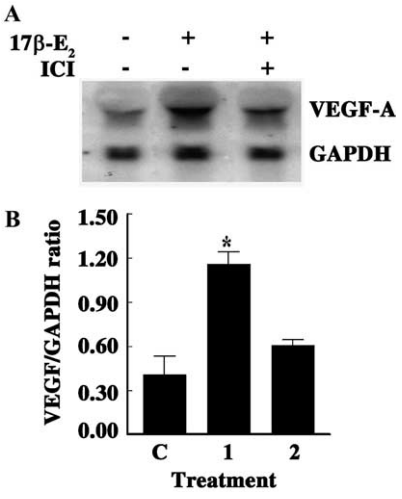

Fig. 5. Effects of pure anti-estrogen ICI 182,780 on 17β-E_2-induced VEGF-A mRNA expression in GH3 rat pituitary tumor cells. Starved GH3 cells were exposed to 10 nM 17β-E_2 for 2 h in the presence or absence of ICI 182,780 and then mRNA levels were evaluated using Northern blot analyses. Un—untreated control. (A) Single representative blot showing the impact of pure anti-estrogen and puromycin on 17β-E_2-induced VEGF-A mRNA expression in GH3 cells. (B) The normalized values indicate the relative abundance of VEGF-A mRNA in GH3 cells before and after treatment. Results are displayed as means ± SD from three separate experiments. $*p < 0.001$ versus control; $**p < 0.001$ versus control.

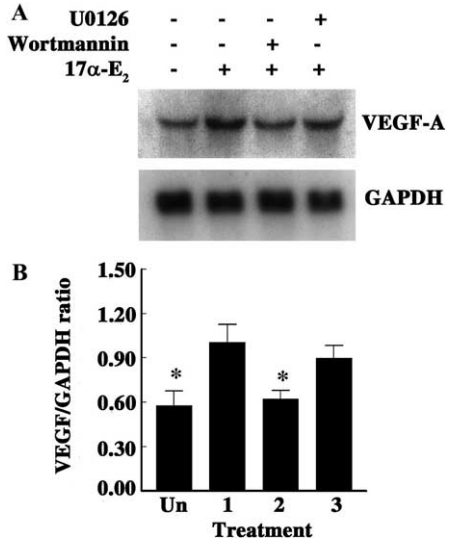

Fig. 6. Effects of PI3K and MEK inhibitor on 17α-E_2-induced VEGF-A mRNA expression. GH3 rat pituitary tumor cells were grown in serum-free medium for 3 days. Cells were exposed to 17α-E_2 for 4 h in the presence or absence of wortmannin or U0126. Total RNA was extracted and analyzed by Northern blotting using non-radioactive DIG-labeled probe. (A) Single representative blot showing the impact of wortmannin and U0126 on 17α-E2-induced VEGF-A mRNA expression in GH3 cells. (B) The normalized values indicate the relative abundance of VEGF-A mRNA in GH3 cells before and after treatment. Values shown are means ± SD of triplicate determinations. $*p < 0.01$ compared to 17α-E_2-treated samples.

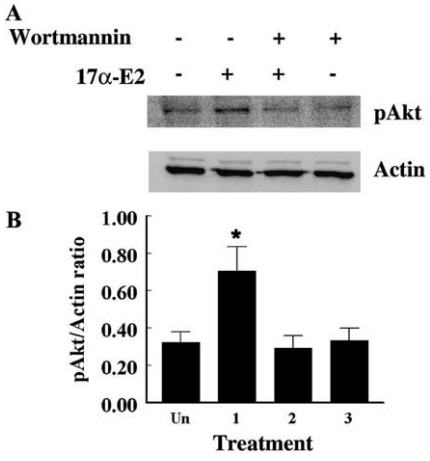

Fig. 7. 17α-E_2 up-regulates Akt phosphorylation in GH3 rat pituitary tumor cells. GH3 cells were grown in serum-free environment for 24 h and then cells were exposed to 10 nM of 17α-E_2 for 4 h in the presence or absence of wortmannin (100 nM). Cell lysates were subjected to SDS–PAGE, followed by Western blot analysis using an anti-phosopho-Akt antibody. (A) Single representative immunoblot showing the impact of 17α-E_2 on phosphorylation of Akt in GH3 cells. (B) The normalized values indicate the relative abundance of phosphorylation in Akt in GH3 cells before and after treatment. Values shown are means ± SD of triplicate determinations. *$p < 0.009$ compared to untreated control or wortmannin treated.

17α-E_2 for 4 h. As shown in Figs. 6A and B, U0126 did not alter significantly the expression and suggests that MAPK activity may not be required for 17α-E_2-induced VEGF-A mRNA expression.

17α-E_2-induced PI3K activity increases Akt phosphorylation in GH3 cells

To investigate whether 17α-E_2-induced PI3K activity in GH3 cells induces phosphorylation of Akt at Ser473, cells were grown in serum-free environment for 24 h followed by exposure to 10 nM 17α-E_2 for 4 h with or without pre-exposure to wortmannin for 30 min. The cells were washed with 1× phosphate-buffered saline (PBS) and total proteins extracted were used for Akt phosphorylation using immunoblot assay. As shown in Figs. 7A and B, 17α-E_2 increases phosphorylation of Akt at Ser-473 by 2.5-fold as compared to untreated cells. This induction of Akt phosphorylation was blocked by wortmannin, the PI3K inhibitor. The result of this study is consistent with the view that PI3K-Akt signaling is required for the induction of VEGF-A mRNA levels by 17α-E_2 in GH3 rat pituitary tumor cells.

Discussion

Vascular endothelial growth factor-A (VEGF-A) is an essential growth factor required for both physiological and pathophysiological angiogenesis [27–29]. The distributions of VEGF-A mRNA and proteins have been detected in a variety of estrogen responsive neoplastic tissues in human and animals [8–14]. Previous studies from our laboratory [12], and others [30], have demonstrated that 17β-estradiol (17β-E_2), a naturally occurring female sex steroid (estrogen) biologically derived from cholesterol, up-regulated VEGF-A expression during estradiol-induced tumorigenesis in rat pituitaries. Subsequently, transcriptional regulation of the VEGF-A gene by 17β-E_2 was demonstrated in different kinds of cancer cells [17,31] and in differentiated human monocyte cell lines [32], and suggested that 17β-E_2 induces new VEGF-A mRNA synthesis and transcriptional activation is ER-dependent [17]. Our recent unpublished observations using human breast tumor derived MCF-7 tumor cells, or normal mammary epithelial cells transfected with ER-α are consistent with other observations and indicated that up-regulation of VEGF-A mRNA expression by 17β-E_2 is ER-α dependent (Sengupta et al., unpublished data).

The objective of the current study was to determine whether VEGF-A in GH3 rat pituitary tumor cells can be modulated by 17α-E_2, a weak, short-acting and non-carcinogenic estrogen, and as such displays both agonistic and antagonistic properties [1], similar to those ligands, which are now identified as selective estrogen receptor modulators (SERMs) [33]. The results obtained from this study reveal that like other long-acting, carcinogenic estrogens such as 17β-E_2, 17α-E_2 was able to induce VEGF-A mRNA expression in a dose- and time-dependent manner (Figs. 1–3). The effect of 10 nM 17α-E_2 was transient and decreased to the basal level within 24 h, whereas the effect of 100 nM dose on VEGF-A mRNA expression lasted for >24 h. This study reveals a biphasic role of 17α-E_2 on VEGF-A expression that is dose-dependent. However, we were unable to discern a clear reason of dose-dependent biphasic effects of 17α-E_2.

Short-acting estrogen, such as estriol and 17α-E_2, binds to the estrogen receptor with an affinity similar to, or weaker than, that of 17β-E_2 or EE [1,7,34,35]. Therefore, one might anticipate that induction of VEGF-A expression by 17α-E_2 could be mediated via a receptor generated pathway similar to that of the classical one [17]. However, this does not appear to be the case, since ICI 182,780 (a pure estrogen receptor antagonist totally lacking agonist activity), which blocked the 17β-E_2-induced VEGF-A mRNA expression, was unable to block the action of 17α-E_2 on expression of VEGF-A. Conversely, inhibition of protein synthesis by puromycin effectively diminished the effect of 17α-E_2 on this action. Together, these studies indicate an alternative mechanism, which was possibly not involving the estrogen receptor. In support of this interpretation our findings showed that 17α-E_2-induced VEGF-A expres-

sion could be potentiated through a PI3K-Akt pathway because its inhibitor, wortmannin, suppressed the enforced VEGF-A mRNA expression. Further, 10 nM of 17α-E_2, which successfully up-regulated VEGF-A mRNA expression, was able to increase the phosphorylation of Akt at Ser-473 in the GH3 cells, and this was also blocked by wortmannin. Importantly, the MEK inhibitor, U0126, did not exhibit any effect on VEGF-A expression, suggesting that a mitogen-activated protein kinase pathway is probably not involved in the action of 17α-E_2.

In conclusion, these studies establish, for the first time, a novel mechanism by which 17α-E_2 induces increased expression of VEGF-A in GH3 rat pituitary tumor cells. Specifically, 17α-E_2 regulates this expression by activating a PI3K-Akt signaling pathway. Further, the up-regulation of VEGF-A mRNA expression by 17α-E_2 is not inhibited by the presence of a potent and specific inhibitor of estradiol action (ICI 182,780). This would appear to preclude the action of either of the transcription factors ER-α or ER-β, and therefore a genotropic mechanism, which is required by a long-acting estrogen such as 17β-E_2 for the induction of VEGF mRNA expression in GH3 rat pituitary tumor cells. These studies, therefore, suggest that strong and weak estrogens use at least two different signaling pathways for regulating VEGF gene in GH3 rat pituitary tumor cells. However, it remains uncertain whether these properties of 17α-E_2 are different from those of 17β-E_2 in regulation of physiological and patho-physiological functions. Further studies are warranted.

Acknowledgment

We thank Dr. Donald C. Johnson for fruitful collaborations in the reviewing of the manuscript.

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