Journal of Applied Biological Sciences
E-ISSN: 2146-0108 15(1): 113-125, 2021
Research Article

HUMAN ESTROGEN SULFOTRANSFERASE (EST) ENZYME MODULATES MAPK PATHWAY IN HUMAN BREAST CANCER CELL LINE MCF-7

Yavuz Cakir*

Department of Science, Technology, Health & Human Services, Voorhees College, Denmark, SC 29042

**Corresponding Author:*
E-mail: ycakir@voorhees.edu

(Received 08th December 2020; accepted 30th January 2021)

ABSTRACT. Estrogen sulfotransferase (EST) enzyme conjugates estrogens to protect the cells from estrogen-initiated carcinogenesis by inactivating biological actions of estrogens. In the breast tissue, sulfation reaction occurs in the target cell, and the sulfated estrogens do not bind to their nuclear receptor. The transfection of EST into MCF-7 cells (MCF-7/EST) decreased the response of these cells to estrogen stimulation leading reduced cell proliferation and growth. In this study, we investigated the role of EST in the regulation of MAPK signal transduction pathway in MCF-7/EST cells treated with estradiol (E_2). We demonstrate that MAPK was activated by E_2 in both MCF-7/EST and control cells (MCF-7/PCDNA) in a concentration dependent manner. The concentrations of E_2 to activate MAPK were much lower in MCF-7/EST cells than control cells. In both cell line, E_2 did not activate c-RAF-1 protein kinase activity. In MCF-7/EST cells, E_2 did not cause any significant change in the expression of nuclear estrogen receptor (nER). The specific ER antagonist ICI 182,780 significantly decreased MAPK activation caused by E2 at only 1 hour in the EST cells, and this activity was abolished by E_2. However, in MCF-7/PCDNA cells, ICI 182,780 significantly reduced the activation of MAPK at only 24 h, and E_2 abrogated the inhibitory effect of ICI 182,780 on the MAPK protein. Therefore, our data indicate that in both cell lines the MAPK is activated by other signaling pathways or mechanisms than classical Ras-Raf-MAPK-ERK pathway, and that stimulatory effects of E2 on the MAPK activation in MCF-7/ EST cells may be attributed to a novel membrane receptor that may regulate the signal transduction pathways involved in the aberrant cellular proliferation. In breast cancer cells, estrogen-stimulated MAPK phosphorylation within seconds or minutes indicates that estrogen by binding a putative mER might act to modulate MAPK pathway in the cytosol or membrane.

Keywords: *Estrogen sulfotransferase, EST, sulfation, MAPK, estrogen, breast cancer, MCF-7*

INTRODUCTION

Estrogen (E) plays an important role in the human breast development and in the normal physiological functioning of the adult breast tissue because it is required for normal ductal development and induction of progesterone expression [2, 13, 31]. The activity and the growth stimulatory effect of estrogens in the breast are generally attributed to their binding to estrogen receptor (ER) located within target nuclei [13,14]. Binding of β-estradiol (E_2) to nER (nuclear ER) causes a conformational change that allows ER+E complex to bind specific response element called estrogen response element

(ERE) on the target genes [14,30-32]. The genes having ERE on their regulatory regions are transcribed by E+ERE complex. Apart from this well-known established classical pathway of estrogen, a putative membrane estrogen receptor (mER), which may be activated by estrogen, has been proposed by many reports. Estrogen, in this pathway, may rapidly induce the activation of second messengers and a variety of signaling proteins including MAPK, PI3K, PKA, IP3, SRC kinase [27,28,32]. Estrogen, by initiating the cellular signaling pathways, may act a survival factor to induce cell growth and proliferation as well as block the programmed cell death [1,2,13,14].

Mitogen-activated protein kinase or extracellular-regulated kinase (MAPK/ERK) pathway transmits the extracellular signals to membrane receptors or intracellular targets by modulating several other signaling proteins or cascades in the mammalian cells [15, 17-19]. The unregulated activation of MAPK pathway leads to cancer formation and inhibit apoptosis [15,20]. There are three major MAPK pathways, but ERK 1/2 is the most activated MAPK in breast cancer [15,18,19,21]. MAPK pathway is activated in estrogen receptor positive human breast cancer cell line MCF-7, resulting with aberrant growth and proliferation [20,23,29]. The documented evidence indicates the activation of MAPK pathway by estradiol via membrane-associated receptors [15,22,28]. In ER (+) breast cancer cell lines, in addition to its classical well-known genomic pathway, estrogen induces cell proliferation with the activation of MAPK through in a rapid and non-genomic effects by either binding to its membrane receptor or increasing the growth factor production leading to stimulation of MAPK pathway. This may suggest a crucial early involvement of ER in the MAPK cascade as well as represent an important regulation of cellular proliferation of breast cancer cells by estrogens.

The cytosolic sulfotransferase (SULT) enzymes are phase II metabolic enzymes and play an important role in the detoxification of the several chemicals, drugs and many endogenous compounds, leading to a decrease in the biological activity of these compounds [3,4,10]. Estrogen sulfotransferase (EST), found in the human mammary epithelium, is the major form of human cytosolic sulfotransferases involved in the conjugation of estrogens, which may protect the cells from the estrogen-initiated carcinogenesis [7,12]. In the breast tissue, EST inactivates the biological action of estrogens by forming estrogen sulfates (ESs) [5-8]. All SULTs including EST use 3' phosphoadenosine 5'-phosphosulfate (PAPS) as the sulfate donor to an acceptor compound [3-6]. Sulfation reaction occurs in the target cell, and the sulfated estrogen may not traverse cell membranes due to their charge as well as they do not bind to their nuclear receptor.

The function of the EST in the breast cancer may modulate the effects of several anti-carcinogenic agents by controlling the mitogenic action of estrogens as well as changing microenvironment of the breast tissue with a possible inhibition in the paracrine or autocrine pathway of estrogen. Previously in our lab, the human ER (+) breast cancer cell line, MCF-7, has been shown not to express EST and to have estrogen sulfation pathway [12], even though the normal mammary epithelium has been shown to have endogenous EST activity [7,12]. Recently, we also have shown that in stably transfected MCF-7/EST cells the cell growth was inhibited in the presence of E_2, and the nER, PR and TGF-β1 mRNA expression level were not changed by E_2 treatment [16]. Qian et al also demonstrated the inhibition of estrogen-mediated DNA synthesis and cell proliferation in MCF cells expressing EST (7). It seems that the presence of the estrogen-metabolizing enzyme, EST, in the breast tumors, may decrease the response of these cells to estrogen,

and the absence of this enzyme may very well lead a sustained stimulation of ER by high amount of estrogen in this ER (+) breast cancer cells.

In this study, we investigated the relationship between MAPK and E_2-induced mitogenesis in MCF-7/EST cells. The dependence of breast cancer growth to estrogen and various cellular signaling pathways led us to investigate the role of estrogen sulfation in ER (+) breast cancer cell line, MCF-7. Due to importance of MAPK pathway in the estrogen-mediated breast cancer cells, we studied the effects of EST in MCF-cells on the activation of MAPK cascade by E_2. Rapid action of estrogen on the MAPK pathway was evaluated in MCF-7 cells, stably transfected with EST (MCF-7/EST). Because estrogen will be metabolized by EST inside the cells, we hypothesized that in MCF-7/EST cells estrogen will activate MAPK pathway through a membrane receptor in the non-genomic manner. We observed a significant activation of MAPK in both MCF-7 and MCF-7/EST cells with different responses to specific nER antagonist. In addition, we showed that E_2-induced MAPK pathway did not require Raf-1 kinase (c-RAF), the upstream ffector of MAPK pathway. At present, this is the first report demonstrating the effect of estrogen on the MAPK pathway in MCF-7 cells expressing EST.

MATERIALS AND METHODS

Chemicals

MCF-7 cells were purchased from the American Type Culture Collection (Rockville, MD). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and geneticin were obtained from Life technologies (Grand Island, NY). E_2 was purchased from Sigma Co. (St. Louis, MO). Anti-estrogen receptor antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz , CA). Anti-active-MAPK, anti-active-Raf-1, total MAPK antibodies and PD 98059 were purchased from Cell Signaling Technology (Beverly, MA). Super Signal West Pico kit and M-PER cell lysis buffer and were obtained from Pierce (Rockford, IL). ICI 182, 780 was purchased from Tocris (Ellisville, MO). [^{3}H]-E_2 (56 Ci/mmol) was obtained from Dupont-NEN (Boston, MA). PAPS was purchased from Dr. Sanford Singer (University of Dayton, OH).

Cell Culture

Human ER (+) breast cancer cell line, MCF-7, was maintained at 37 ^{0}C in T150 flasks in DMEM (PR +) with 7% FBS in a 95 % air / 5 % CO_2 atmosphere. Geneticin (400 mg/l) was added to MCF-7/EST cells. Medium has been changed every other day, and all cells were grown to 60-70 % confluence in 75 cm^2 cell culture flasks before experimental procedures. Before the treatments of the cells, the media was changed to DMEM (PR-) + Gen + 0.1 % sFBS (stripped of any endogenous estrogen) for 24 or 48 hours to slow the cell growth and down-regulate MAPK activity. New fresh media has been added (DMEM (PR-) + Gen + 0.1 % sFBS), and the cells were treated with the chemicals or steroids at specific time points (0/1/2/3/6/9/12/15/18/24h). The nER antagonist ICI 182,780 and MEK inhibitor PD 98059 was added to cells for 1 hour prior to estrogen adding.

Generation of Stably Transformed MCF-7 Cells

The stably transformed ER (+) human breast cancer cell line MCF-7 with either vector/control (pcDNA3) or EST (EST/pcDNA3) has been described previously [12].

EST Assay

Both control MCF-7/pcDNA and MCF-7/EST cells were grown on 100-mm tissue culture plates. Cell cytosols were prepared by washing the plates three times in phosphate-buffered saline (PBS), pH 7.4, and the cells were scraped off from the plates into 0.5 ml/plate of M-PER lysis buffer. After the cell disruption, the cell extracts were centrifuged at 100,000 x g for 30 min to obtain the cell cytosolic fractions. Protein concentration was quantified using Bradford protein assay kit. Estrogen sulfation activity was then assayed using 20 nM [^{3}H]-E_2 as a substrate described previously using alkaline-chloroform extraction procedure [10,12]. The sulfated E_2 was mixed with the scintillation fluid, and radioactivity in the aqueous phase was determined to quantify the amount of sulfated E_2 released. Each reaction also included 20 μM PAPS as the sulfate donor, 50 mM Tris-HCL, pH 7.4, and 7 mM MgCl2 in a final volume of 0.125 ml. Control reactions did not have substrate.

Western Blotting

Depending on the specific time intervals, cell were stopped and washed two times in the PBS to remove any chemicals, growth factors, or cytokines. The cell lysates were prepared by using 400- 500 μL of M-PER cell lysis buffer (Pierce). Lysates were centrifuged at 14,000g for 10 min, and the supernatant of the cells was stored at -80C for further analysis. Before immunoblotting, the protein concentration of the samples was determined using Bradford assay and enough protein (20-50 μg) mixed with 2X SB was loaded on the gel. Aliquots of the extracts were analyzed using 10 % SDS-PAGE with a 4 % stacking gel at 150V for 1 h at room temperature. Gel was transferred to nitrocellulose membrane in the transfer buffer at 100V for 1 - 1.5 hour. The membrane was immediately removed and washed in the transfer buffer. To make sure that the transfer process were carried out perfectly, the membrane was soaked in the Ponceau S solution to observe the protein bands.

The membrane, then, was washed in the PBS and washing buffer three times for 5 min, blocked in the 5 % non-fat milk for 1 hour at the room temp and washed three times in the washing buffer. The membrane was incubated with the primary antibody, the phospho-specific MAPK antibody, at the recommended concentration overnight. Then, membrane was washed three times, incubated with the HRP-linked secondary antibody (1: 20,000) for 1 hour at the room temp and washed again three times for 5 min. Membrane was incubated 5 min in Super Signal West Pico (PIERCE) chemiluminescent substrate for detecting HRP on the immunoblots. The specific bands were detected after the membrane was exposed to X-ray film, and quantified by densitometric scanning. Membranes were stripped and reprobed with anti-total MAPK antibody to show the equal loading.

Statistical analysis

GraphPad Prism software was used for the statistical analysis. Two-way ANOVA (analysis of variance) with Tukey post-test was used, and differences were considered significant if $p < 0.05$.

RESULTS

EST Activity

To show the EST activity in the stably transformed MCF-7/EST cells, we assayed the EST activity in the presence of 20 nM E_2. As it has been shown in Fig. 1, the induction of estrogen sulfation activity was significantly increased by estrogen in MCF-7/EST cells compared to control vector cells. Control cells almost did not have any endogenous EST activity. Human EST enzyme also showed high EST activity. This shows that EST activity is present in the transformed cells, and estrogen is metabolized by EST.

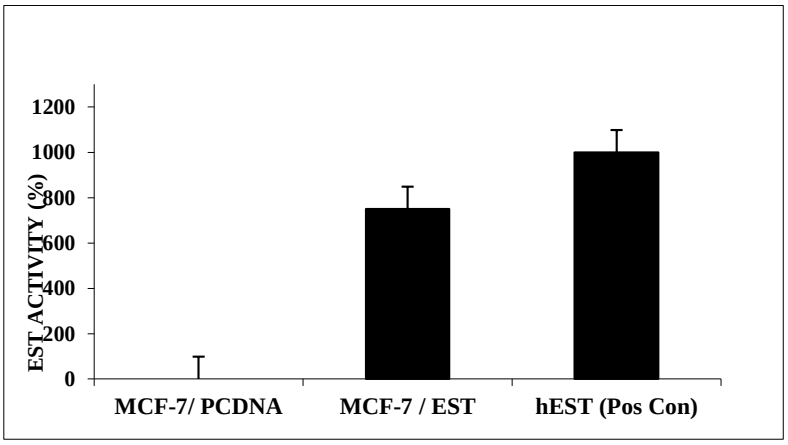

***Fig. 1**. Estrogen sulfation by EST in MCF-7/PCDNA and MCF-7/EST cells. Cells were plated in 100-mm plates and allowed to attach for 24 hour. Cytosols were prepared and EST activity was assayed with 20 nM [^{3}H]-E_2 using the alkaline-chloroform extraction protocol described in the text. Radioactivity was determined by scintillation spectroscopy. Results are the mean of two separate assays. Values are means ± SE.*
Two-way ANOVA (analysis of variance) with Tukey post-test was used, and differences were considered significant if $p < 0.05$.

Level and intensity of basal activated MAPK (P-MAPK)

The discrepancies in the onset of ERK activation might be attributable to the level of endogenous phosphorylated ERK 1,2 induced before E_2 stimulation in both cell types. At some time points, the level of activated ERK 1,2 differed depending on the cellular activities and endogenous compounds affecting MAPK level. The charcoal stripped FBS used in our experiments may have residual E_2 to activate MAPK levels.

Autocrine or paracrine pathways may also activate the basal MAPK levels. Estradiol may stimulate growth factors or their receptors to induce MAPK pathway, or another pathway that may also interact or cross-react with mitogenic MAPK pathway. The fact that the breast cancer cells (MCF-7/PCDNA and MCF-7/EST) have endogenous MAPK activation may be a requirement for the cellular growth and proliferation of these tumor cells.

Regulation of MAPK Activation by Estrogen

MAPK signaling pathway plays a crucial role in the growth and proliferation of breast cancer cells. It is over-expressed by several growth factors and estrogens [15]. To determine the MAPK signaling pathway in response to estrogen and its antagonist, the MAPK activation was assayed in both cells by immunoblotting. Threonine and tyrosine residues on the MAPKs have to be

phosphorylated for the activation of MAPKs [17]. This was analyzed by anti-active–MAPK antibody (anti-phospho-MAPK), which has the same phosphorylation sites with ERK1/2 MAPK proteins. The estrogen treated cells were incubated with this antibody. MAPK activity was analyzed at different concentrations of estrogens. In the first set of experiments, we used high estrogen concentrations to measure MAPK activity. At 1 nM concentration, E2 significantly increased the MAPK level in EST cells at 24 h (Fig. 2A).

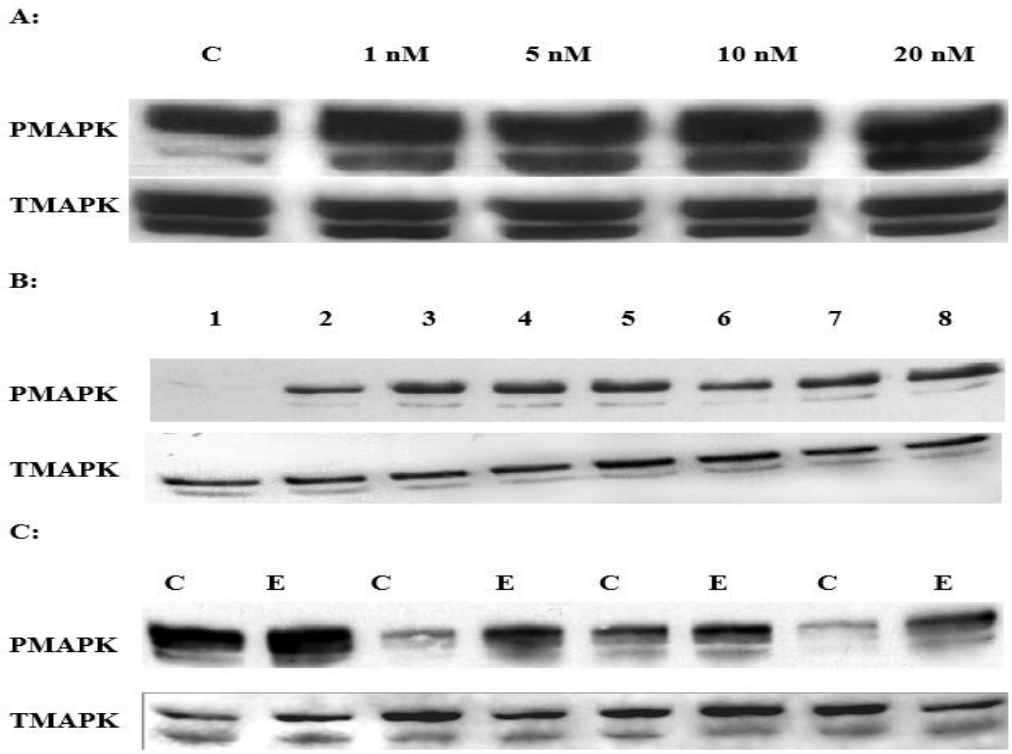

Fig. 2*. Western blot analysis of the effect of E_2 on MAPK activation in MCF-7/EST breast cancer cells. (A) Cells were exposed to increasing concentrations of E_2 for 24 h and proteins extracted. Proteins were resolved by 10 % SDS-PAGE as described in the "Materials and Methods" section. Lane 1 represents the cells treated with vehicle alone. Lanes 2,3,4, and 5 represent the cells treated with 1 nM, 5 nM, 10 nM, and 20 nM E_2, respectively. (B) Cells were exposed to increasing concentrations of E_2 for 24 h and proteins extracted. Lane 1 represents the cells treated with vehicle alone. Lanes 2,3,4,5,6,7 and 8 represent the cells treated with 100 pM, 250 pM ,500 pM, 750 pM and 1 nM E_2, respectively. (C) Cells were exposed to 50 pM E_2 for 0 h (Lanes 1 and 2), for 1 h (Lanes 3 and 4), for 2 h (Lanes 5 and 6), and 3 h (Lanes 7 and 8), and proteins extracted. Upper bands show the activation of MAPK visualized using anti-active MAPK rabbit polyclonal antibody and chemiluminescence. Lower bands show the total MAPK expression performed by stripping and reprobing the membrane. Significant difference exists among control and E_2 groups.*

The data showed that ERK1 protein activity was higher than ERK 2 protein activity. At higher concentrations of E2 (5, 10, 20 nM), the activated MAPK levels stayed same and did not change significantly (Fig. 2A). Densitometric analyses of the bands also show the activation of MAPK. The total MAPK (T-MAPK) expression was not affected by estrogens and they were same at every concentrations of estrogen (data not shown). Second set of experiments involved lower concentrations of estrogen. We wanted to find

the lowest concentration of estrogen to activate MAPK protein. Figure 2B demonstrates that the activity of MAPK was dramatically increased by increasing concentrations of E2 from 10 pM to 1 nM in a dose-dependent manner in MCF-7 EST cells at 24 h. Even the lowest concentration of E2 (10 pM) significantly activated MAPK in these cells (Fig. 2B). Again, the effect of estrogen seemed to be more pronounced in ERK 1 MAPK activity because the ERK 2 MAPK activity almost did not change in response to estrogen. We also analyzed the activation of MAPK at 1 and 3 hour in that MAPK activation was enhanced by 50 pM E2 in MCF-7 / EST cells (Fig. 2C). This data demonstrates that in MCF-7 / EST cells, estrogen activates MAPK protein activity and that ERK1 activity appears to be more sensitive to the effect of estrogen.

In MCF-7 / PCDNA cells, when we use high estradiol concentrations, the activation in the MAPK protein was observed at 5 nM E2 concentration, and this activation was significantly increased by 10 nM E2 at 24 hours (Fig. 3A). The MAPK protein activity was also elevated by increasing concentrations of E2 from 10 pM to 1nM in a dose-dependent manner at 24 hours (Fig. 3B). However, the lowest concentration of E2 to cause a significant increase in MAPK level was 50 and 100 pM. At the higher concentrations the activity level of MAPK did not change. The changes in the ERK 1 activity were much higher than that of ERK 2 activation. Total MAPK expression did not change by the effects of estradiol. The attempts to activate MAPK by estradiol at short time courses such as 0 min / 15 min / 30 min / 1 h / 6h and 9 h failed because MAPK activation was not changed by increasing concentration of E2 in MCF-7 / PCDNA cells at those times (Data not shown). This data shows that estrogen at different concentrations causes the activation of MAPK in the control MCF-7 cells.

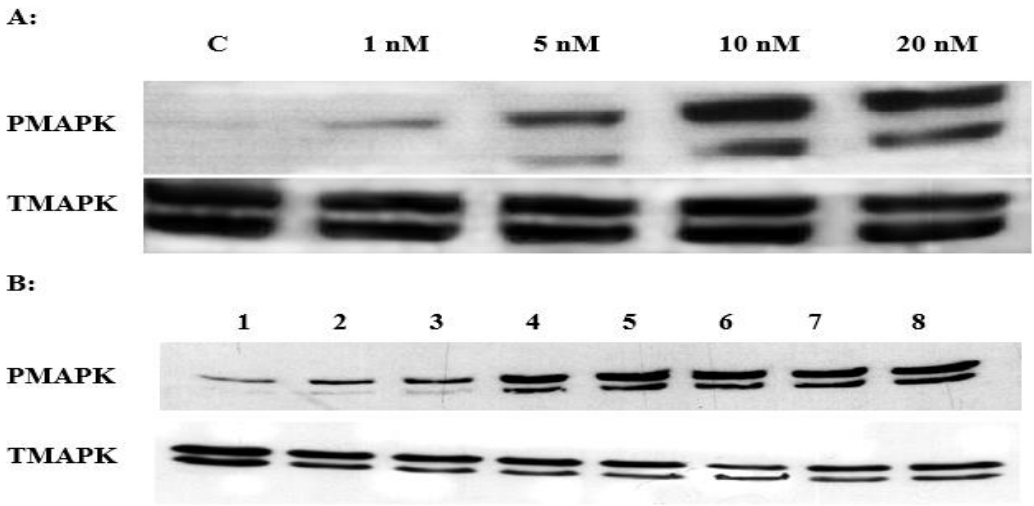

***Fig. 3**. Western blot analysis of the effect of E_2 on MAPK activation MCF-7/PCDNA breast cancer cells. (A) Cells were exposed to increasing concentrations of E_2 for 24 h and proteins extracted. Proteins were resolved by 10 % SDS-PAGE as described in the "Materials and Methods" section. Lane 1 represents the cells treated with vehicle alone. Lanes 2,3,4, and 5 represent the cells treated with 1 nM, 5 nM, 10 nM, and 20 nM E_2, respectively. (B) Cells were exposed to increasing concentrations of E_2 for 24 h and proteins extracted Lane 1 represents the cells treated with vehicle alone. Lanes 2,3,4,5,6,7 and 8 represent the cells treated with 100 pM, 250 pM, 500 pM, 750 pM and 1 nM E_2, respectively. Upper bands show the activation of MAPK visualized using anti-active MAPK rabbit polyclonal antibody and chemiluminescence. Lower bands show the total MAPK expression performed by stripping and reprobing the membrane. Significant difference exists among control and E_2 treated groups.*

Estrogen actions on the RAF-1 (c-Raf) activation

To further investigate the upstream effectors of MAPK pathway in response to estrogen, Raf-1 activation was measured by western blotting. RAF–1 is the initial MAP kinase downstream of Ras protein. It is activated by Ras, and in turn the activated Raf-1 phosphorylates MEK-1 that activates ERK-1, 2 MAPKs [17,21]. E2 neither at high nor low concentrations increased RAF protein kinase activity in both cells (Fig. 4). This data shows that in both cell lines the MAPK is activated by other signaling pathways or mechanisms than classical Ras-Raf-MAPK-Erk pathway.

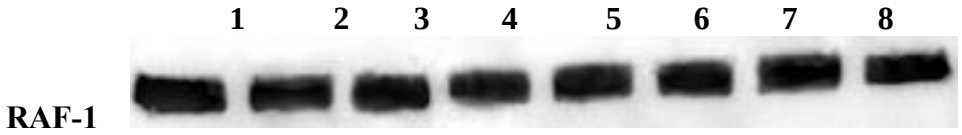

Fig. 4*. Western blot analysis of the effect of E_2 on RAF-1 activation in MCF-7/EST breast cancer cells. Cells were exposed to increasing concentrations of E_2 for 24 h and proteins extracted. Proteins were resolved by 10 % SDS-PAGE as described in the "Materials and Methods" section. Lane 1 represents the cells treated with vehicle alone. Lanes 2,3,4,5,6,7 and 8 represent the cells treated with 100 pM, 250 pM, 500 pM, 750 pM and 1 nM E_2, respectively. Bands show the activation of RAF-1 (c-RAF) visualized using anti-active RAF-1 rabbit polyclonal antibody and chemiluminescence. Significant difference exists among control and E_2 treated groups.*

The effect of nER antagonist ICI 182, 780 on the MAPK activation

ICI 182, 780 is a pure ER antagonist lacking agonist activity. It binds to both ER α and ERβ receptors and prevents them from E2 binding, thereby inhibits the actions of both receptors. It has been demonstrated that ICI 182, 780 also inhibits the binding of any ligand to AF-1 and AF-2 regulatory sites of ER receptors. AF-1 is required for the growth factor binding whereas AF-2 is for E2 binding. ICI 182,780 has been shown to inhibit MAPK activity in MCF-7 cells [20,26)].

The specific nER antagonist ICI 182,780 alone at 100 nM, 500 nM and 1 μM concentrations did not change the activity of MAPK caused by 10 pM E2 in MCF-7 / EST cells at 24 h (Fig. 5A). However, the pure nER antagonist ICI 182,780 alone at 100 nM, 500 nM and 1uM concentrations significantly reduced the activation of MAPK at 1 hour. (Fig. 5B). Estradiol at 10 pM concentration abrogated the inhibitory effect of ICI 182, 780 on the MAPK activation. Total MAPK expression did not change. This data demonstrates that pure nER antagonist do not change the activation of MAPK induced by E2 at 24 hour whereas it significantly causes the inhibition of estradiol-induced MAPK activity at only 1 hour.

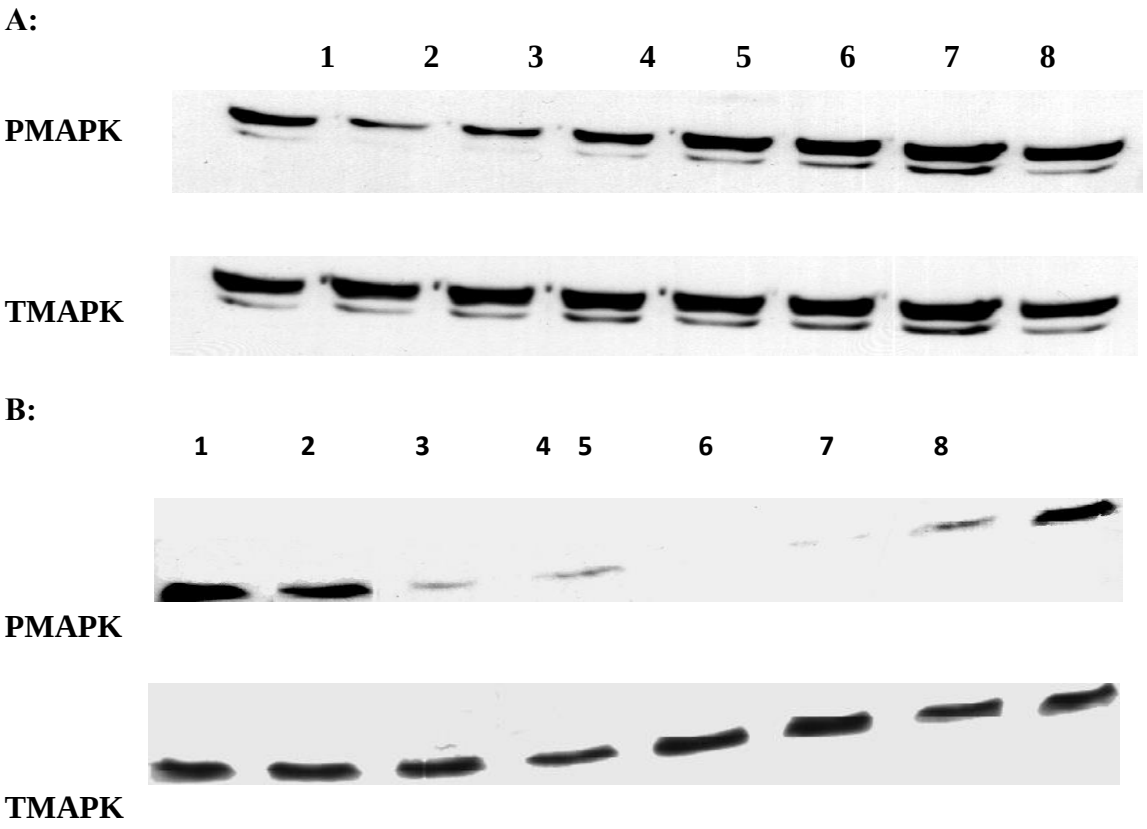

***Fig. 5**. Western blot analysis of the effect of E_2 and ICI 182, 780 on MAPK activation in MCF-7/EST breast cancer cells for 24 h (A) and 1 h (B). Cells were exposed to increasing concentrations of E_2 and ICI 182,780 for 24 h and 1 h, and proteins were extracted. Proteins were resolved by 10 % SDS-PAGE as described in the "Materials and Methods" section. Lane 1 represents the cells treated with vehicle alone. Lanes 2,3,4,5,6,7 and 8 represent the cells treated with 10 pM E_2, 100 nM ICI 182,780, 500 nM ICI 182,780, 1 μM ICI 182,780, 10 pM E_2 + 100 nM ICI 182,780, 10 pM E_2 + 500 nM ICI 182,780 and 10 pM E_2 + 1 μM ICI 182,780, respectively. Upper bands show the activation of MAPK visualized using anti-active MAPK rabbit polyclonal antibody and chemiluminescence. Lower bands show the total MAPK expression performed by stripping and reprobing the membrane. Significant difference exists among control and E_2 treated groups.*

DISCUSSION

Estrogen acts as a stimulatory mitogen in cells to transactivate the genes inducing cell growth and proliferation. The breast cancer growth and development are attributable to the stimulatory effect of estrogen on the mammary cells. The growth and proliferation of the most of the human breast cancer cells depend on the estrogenic stimulation [1,2,13, 16, 30]. Estrogen has mitogenic effect on the ER positive human breast cancer cell line, MCF-7, without any other growth factors or hormones. Mitogenicity associated with ER-mediated cellular events is partly believed to be the mechanism by which estrogens contribute to cancers by causing DNA adducts during the detoxification reactions [13,31].

Most of the biological actions of estrogen are mediated through two different nuclear receptors, ERα and ERβ, each transcribed by distinct genes [30,31]. The E2 binds to nER to induce the genes having ERE on their promoters. In addition to this well-known classical pathway, several other molecular mechanisms of the estrogen signaling were proposed in that nER may be activated by extracellular signals, different response elements might be activated by E2 and a putative membrane receptor may be activated by E2 to induce the genes [32,33]. These distinct effects of estrogen may very well account for the development of breast cancers because estrogen appears to be major endogenous hormone to regulate both normal and cancerous breast tissue [13,31].

In our present study, we investigated the role of EST in the most prevalently used breast cancer cell line, MCF-7, because E2 has been shown to induce cell growth and proliferation in this cell line [12,14,24]. The binding of E2 to nER is inactivated by the action of EST, leading to less cell growth arrest and inhibition. The fact that normal human mammary epithelial cell express EST whereas MCF-7 cells do not have EST expression [5] indicates that the presence of EST function in MCF-cells may alter the growth stimulatory effects of E2 as well as signaling pathways activated by E2 in MCF-7 cells. Preliminary studies showed that the effects of estrogen is inactivated by EST in MCF-7 / EST cells, resulting with a decreased cellular growth and proliferation [7,12,16], because the sulfated estrogen (ES) produced by EST does not bind to its nER and it is removed from the cells to be excreted.

The dependence of breast cancer cells to distinct cellular signaling pathways is a well-known concept. The MAPK pathway is activated in almost all breast cancer cell lines, including MCF-7 cells [15,17,18,20]. As it has been mentioned before, E2 in a non-genomic fashion activates MAPK cascades to induce cell growth and protein synthesis via a membrane receptor. Because EST metabolically inactivate the action of E2 in MCF cells, we hypothesized that human ER responsive breast cancer cell line MCF-7 transfected with EST enzyme (MCF-7 / EST) expresses a unique membrane-associated estrogen receptor (mER) different from the nuclear ER (nER) present in the normal mock transfected MCF-7 cells (MCF-7/ PCDNA). This novel mER is rapidly activated by E2 in order to induce cellular growth and proliferation as well as MAPK signaling pathway that is not associated with the nER.

We previously demonstrated that the growth and proliferation of MCF-7/EST cells is reduced compared to that of control cells [16]. This study shows that the presence of EST activity stimulates the cellular MAPK pathway. The concentrations of E2 needed to induce MAPK activation were much higher in MCF-7/PCDNA cells than MCF-7/EST cells, indicating that low amounts of E2 are responsible for the activation of MAPK. The rapid actions of low (pico to nanomolar) E2 concentrations on the MAPK activation in the MCF-7/EST cells may be attributed to a putative membrane ER (mER), because membrane-associated effects of E2 through a putative membrane receptor may need lower E2 amounts to activate cellular signaling pathways. In addition, the absence of the expression of the ER in MCF-7/EST cells might be explained by the fact that EST metabolically inactivates the E2 by sulfating it, and E2S cannot bind to its nER to induce cell growth [3-5]. The sulfated estrogen is removed from the cells and does not enter to the cell again**.** The possibility of the effect of sulfatase and aromatase enzymes, which removes sulfate from estrogen thereby reactivating it and produces estrogen, respectively, is very low in these cells, because the EST enzyme has higher substrate specificity than sulfates and aromatase enzymes, meaning that the Km value of the EST enzyme is at nM ranges whereas the Km values of sulfatase and aromatase enzymes are at μM ranges

[10,16,29]. This fact indicates that EST sulfates E_2 in the physiological concentrations at which E_2 is functionally active in the cell. In MCF-7/PCDNA cells, estrogen by binding to its nER activates MAPK at the transcription level. The ER+E_2 complex induces the transcription of the immediate-early genes, which in turn activate MAPK. Because E_2 will not be inactivated by EST enzyme in these cells, E_2 stimulates the production and the secretion of the growth factors inducing cellular signaling cascades.

Many human cancers have been shown to have up-regulated MAPK pathway [17-19]. MAPK signaling cascade is activated by phosphorylation in response to mitogenic signals and hormones. The classical Ras-Raf-MAPK-Erk pathway is activated by E_2 in MCF-7 cells [20,21]. We demonstrate that E_2 did not activate Raf-1 kinase activity in both MCF-7/EST and MCF-7/PCDNA cells. This may suggest that in both cell lines other signaling pathways and mechanisms than classical Ras-Raf-MAPK-Erk pathway may activate the MAPK. It could be GPCR signaling, PKA, PI3K or Ca++ -mediated signaling [32,33]. It has been demonstrated that Raf-1 might not be required for proliferation but rather plays an essential role in the protecting cells from apoptosis, independent of MEK-MAP kinase cascade. Another subtype of Raf, B-raf, may be involved in the MAPK activation of these two cells because B-raf is activated by another Ras related protein, Rap-1 in the GPCR signaling.

The specific nER antagonist ICI 182,780 prevents the binding of E2 to its nER and also inhibits the E2-induced MAPK activation in MCF-cells, leading to the inhibition in the cell proliferation and growth [32]. We show that ICI 182,780 inhibited the MAPK activation in both cell lines. However, the inhibition in the MAPK activation was observed at 1 hour in MCF-7/EST cells whereas ICI 182,780 inhibited MAPK activity at 24 h in MCF-7/PCDNA cells. The ability of ICI 182,780 to inhibit the activation of MAPK at only 1 hour in MCF-7/EST cells may be attributable to a novel membrane estrogen receptor that is related to nER and is inhibited by ICI 182,780, because it has been suggested that mER and nER proteins might be derived from single transcript [9]. Estradiol is not inactivated by EST in these cells and binds to this mER to activate MAPK pathway. In addition, the genomic action of E2 on the MAPK activation is excluded in these cells due to the fact that nER antagonist ICI 182, 780 did not prevent the activation of MAPK caused by E2 at 24h, which clearly contradicts the transcriptional action of nER in these cells. In contrast, in MCF-7/PCDNA cells, the inhibitory actions of ICI 182,780 on the MAPK activation at only 24 hour suggests that ICI 182,780 - sensitive nER is necessary to mediate the effects of E2 on the MAPK activity.

CONCLUSION

The results from our study demonstrate that activation of MAPK by estradiol in MCF-7 breast cancer cells is mediated via a pathway that does not involve RAF-1 protein kinase activation. Induction of MAPK signal transduction cascade in the presence of estrogen confers the stimulatory effects of estrogen on the cellular growth and proliferation. The activation of MAPK by low amounts of estradiol in MCF-7/EST cells as well as the absence of nER expression by estradiol indicate another mechanism of action of estradiol in a complex chain of events that induce a novel mER that may have significant roles on the cellular signal transduction pathways. This information may open new avenues to elucidate the function of mER in regulating cellular events as well as may provide new insights in the treatment of the breast cancers because it is very important to determine how breast tumors respond to estrogen in patients who are candidates for hormone

therapies. Understanding the E2-initiated signaling cascades in breast tumor cells will help predict responses to such therapies so that an established guideline will be determined. The patho-physiological differences in mER and nER profiles in tumor cells could also be a novel target for anti-tumor therapies for this detrimental disease.

Acknowledgements. I sincerely acknowledge a partial support I received from TUBITAK, Turkey for this research project.

REFERENCES

[1] Russo, J., Tahin Q., Lareef, M.H., Hu, Y.F., Russo, I.H. (2002): Neoplastic transformation of human breast epithelial cells by estrogens and chemical carcinogens. Environ Mol Mutagen. 39(2-3):254-63.

[2] Skibinski, A., Kuperwasser, C. (2015): The origin of breast tumor heterogeneity. Oncogene 34(42): 5309-16.

[3] Barbosa, A.C., Feng, Y., Yu, C., Huang, M., Xie, W. (2019): Estrogen sulfotransferase in the metabolism of estrogenic drugs and in the pathogenesis of diseases. Expert Opin Drug Metab Toxicol 15(4):329-339.

[4] Falany, C.N., Wheeler, J., Oh, T.S., Falany, J.L. (1994): Steroid sulfation by expressed human cytosolic sulfotransferases. J. Steroid Biochem Mol Bio 48: 369-375.

[5] Falany, C.N., Falany, J.L. (1996): Expression of cytosolic sulfotransferases in normal mammary epithelial cells and breast cancer cell lines. Cancer Res 56: 1551-1555.

[6] Song, W.C. (2001): Biochemistry and reproductive endocrinology of estrogen sulfotransferase. Ann N Y Acad Sci 948: 43-50.

[7] Qian, Y., Deng, C., Song, W.C. (1998): Expression of estrogen sulfotransferase in MCF-7 cells by cDNA transfection suppresses the estrogen response: potential role of the enzyme in regulating estrogen-dependent growth of breast epithelial cells. J Pharmacol Exp Ther 286(1): 555-60.

[8] Roy, A.K. (1992). Regulation of steroid hormone action in target cells by specific hormone-inactivating enzymes. Prc. Soc. Exp. Biol. Med. 199: 265-272.

[9] Razandi, M., Pedram, A., Greene, G.L., Levin, E.R., (2017): Cell membrane and nuclear receptors (ERs) originate from a single transcript: studies of ER alpha and ER beta expressed in Chinese hamster ovary cells. Mol Endocrinol 13: 307-319.

[10] Falany, C.N., Krasnykh, V., Falany, J.L. (1995): Bacterial expression and characterization of a cDNA for human liver estrogen sulfotransferase. J Steroid Biochem Mol Biol 52(6):529-39.

[11] Aksoy, I.A., Wood, T.C., Weinshilboum, R. (2004): Human liver estrogen sulfotransferase: identification by cDNA cloning and expression. Biochem Biophys Res Commun 200(3):1621-9.

[12] Falany, J.L., Falany, C.N. (1997): Regulation of estrogen activity by sulfation in human MCF-7 breast cancer cells. Oncol Res 9: 589-596.

[13] Henderson, B.E., Feigelson, H.S. (2000): Hormonal carcinogenesis, Carcinogenesis, 21: 427-433.

[14] Clocca, D.R., Vargas Rolg, L.M. (1995): Estrogen receptors in human target tissues: biological and chemical implications. Endoc Rev 16: 35-62

[15] Santen, R.J., Song, R.X. (2002): The role of mitogen-activated protein (MAP) kinase in breast cancer. J Steroid Biochem Mol Bio 80: 239-256.

[16] Macrina, N., Hex, C. (2002): Regulation of MCF-7 breast cancer cell growth by beta-estradiol sulfation. Breast Cancer Res Treat 74(2):167-76.

[17] Pearson, G., Robinson, F,. Beers Gibson, T., Xu, B.E., Karandikar, M., Cobb, M.H. (2001): Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22(2):153-83.
[18] Kolch, W. (2000): Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J 15;351 Pt 2:289-305
[19] Robinson MJ, Cobb MH. 1997. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 9(2):180-6.
[20] Adeyinka, A., Nui, Y., Cherlet, T., Snell, L., Watson, P.H., Murphy, L.C. (2002): Activated Mitogen-activated Protein Kinase Expression during Human Breast Tumorigenesis and Breast Cancer Progression. Clin Cancer Res 8(6):1747-53.
[21] Lewis, T.S., Shapiro, P.S., Ahn, N.G. (1998): Signal transduction through MAP kinase cascades. Adv Cancer Res 74:49-139.
[22] Zhang, Z., Maier, B., Santen, R.J., Song, R.X. (2012): Membrane association of estrogen receptor alpha mediates estrogen effect on MAPK activation. Biochem Biophys Res Commun 294(5):926-33.
[23] Atanaskova, N., Keshamouni, V.G., Krueger, J.S., Schwartz, J.A., Miller, F., Reddy, K.B. (2012): MAP kinase/estrogen receptor cross-talk enhances estrogen-mediated signaling and tumor growth but does not confer tamoxifen resistance. Oncogene 21(25):4000-8.
[24] Keshamouni, V.G., Mattingly, R.R., Reddy, K.B. (2002): Mechanism of 17-beta-estradiol-induced Erk1/2 activation in breast cancer cells. A role for HER2 AND PKC-delta. J Biol Chem 277(25):22558-65.
[25] Filardo, E.J., Quinn, J.A., Frackelton, A.R., Bland, K.I. (2002): Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol Endocrinol 16(1):70-84.
[26] Marquez, D.C., Pietras, R.J. (2001): Membrane-associated binding sites for estrogen contribute to growth regulation of human breast cancer cells. Oncogene 20(39): 5420-30.
[27] Revelli, A., Massobrio, M., Tesarik J. (1998): Nongenomic actions of steroid hormones in reproductive tissues. Endocr Rev 19(1):3-17.
[28] Migliaccio, A., Castoria, G., Di Domenico, M., De Falco, A., Bilancio, A., Auricchio, F. (2015): Src is an initial target of sex steroid hormone action. Ann N Y Acad Sci 963:185-90.
[29] Falany, C.N., Wang, J., Falany, J.L., Frost, A. (2000): Sulfotransferase expression in normal and cancerous human breast tissues. Breast Cancer Res Treat 64:28.
[30] Katzenellenbogen, B.S., Choi, I. (2000): Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. J Steroid Biochem Mol Biol 74:279-85.
[31] Clemons, M., Goss, P. (2001): Estrogen and the risk of breast cancer. New Eng J Med, 344: 276-85.
[32] Kelly, M.J., Levin, E.R. (2001): Rapid actions of plasma membrane estrogen receptors. Trends in Endocrinol Metabol 12(4): 152-56.
[33] Hall, J.M., Couse, J.F., Korach, K.S. (2017): The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 296: 36869-36872.