Research in Pharmaceutical Sciences, February 2018; 13(1): 57-64
Received: May 2017
Accepted: July 2017

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Estrogen stimulates adenosine receptor expression subtypes in human breast cancer MCF-7 cell line

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Abstract

Estrogen is a steroid hormone that plays a key role in the development and regulation of reproductive system. It has been shown that estrogen is related to breast cancer development through binding to its receptors. In order to uncover the estrogen effects on adenosine receptor expression, estrogen-positive MCF-7 cells were used to treat with agonist and antagonist of estrogen and then the mRNA expression of adenosine receptor subtypes were evaluated. Estrogen-positive MCF-7 cells were treated with various concentrations of 17β estradiol (E2) as an estrogen agonist, and ICI 182,780 as an estrogen antagonist. The gene expression of adenosine receptor subtypes were detected by real time RT-PCR. The results of MTT assay showed that E2 increased cell viability in a dose dependent manner. The expression pattern of all adenosine receptor subtypes are as follow; A2b > A1 > A2a > A3 in untreated MCF-7 cells. Obtained results showed that E2 incubation at 0.001-0.01 μM led to up-regulation of A1ARs, A2aARs and A3ARs dose dependently. E2 at 0.001 μM also had no significant effect on A2bARs expression but, at higher doses induced a considerable decrease in mRNA A2bARs expression. Treatment with antagonist confirmed that up-regulation of these receptors is mediated by estrogen receptor. Taken together, our results indicate that treatment of MCF-7 cells with E2 led to up-regulation of adenosine receptors. However, these effects were partially restored by treatment with antagonist suggesting that such effects are mediated by estrogen receptors.

Keywords: Breast cancer; MCF-7 cells; 17β Estradiol; Estrogen; Adenosine receptors

INTRODUCTION

Estrogen is a steroid sex hormone that is responsible for the development and regulation of the female reproductive system and secondary sex characteristics. Generally, it has been accepted that estrogen is commonly used to manage menopausal symptoms (1). Recently it has been shown that despite beneficial effect of estrogen, it increases some human cancer risk (2-5).

For instance, menopausal hormone replacement therapy of combined estrogen-progestin regimen appears to increase risk of breast malignancy (6). In addition, catechol metabolites of estrogen have been indicated that plays an important role as carcinogens by causing genotoxic stress and DNA damages (7,8). Therefore, some cohort studies have suggested that such therapy should be omitted or modified (9). Breast cancer as one of the most lethal human cancers is generally accepted as a consequence of estrogen therapy in line with other cause of breast cancer. Approximately 246,660 of new cases were diagnosed in 2016 and also it was the second leading cause of cancer-related deaths among women in USA (10).

Normal breast tissues have several common developmental stages such as embryonic, pubertal, pregnancy, lactation, and post-lactation stages (11). It has been shown that repetitive cycle of maturation and involution occurs and mostly is regulated by estrogen in each pregnancy (12).

Estrogens also regulate the proliferation of breast cancer cells and alter their normal properties (13). However, its precise mechanism of action is partially understood.

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Access this article online

Website: http://rps.mui.ac.ir

DOI: 10.4103/1735-5362.220968

In order to clarify estrogen mechanism of action, Frasor, *et al.* have shown that large number of positive proliferation regulators were up-regulated while, also some growth inhibitory factors were down regulated in response to estrogen treatment of estrogen-positive MCF-7 human breast cancer cells (14).

Adenosine is a purine nucleoside which plays as a signaling molecule affecting several key physiological processes, including cell proliferation, differentiation and apoptosis (15,16). Recently it has been shown that adenosine exists in a higher concentration in cancerous tissue as compared with normal tissues (17). As a result, it has been indicated that adenosine is able to interfere with the recognition of tumor cells by immune system (18-20). Nowadays it has been generally accepted that adenosine functions are mostly mediated by its interaction with a group of G-protein-coupled receptor subtypes, namely A1, A2a, A2b and A3 (21). Adenosine receptors have recently emerged as one of the main players in tumor growth, invasion and metastasis (22,23). Moreover it has been indicated that adenosine signaling is strongly affected by estrogen treatment (24).

Despite the wide variety of studies investigating the role of A1 adenosine receptors (A1ARs) in human cancers, its role is not fully understood. However, it has been shown that A1ARs is associated with carcinogenesis and also it is up regulated in peritoneal colon tissues, leukemia Jurkat, human primary breast tumor and human melanoma A375 cell lines. In addition, cell cycle analysis has shown that knock down of A1ARs affect G1 checkpoint, leading to increasing in accumulation of cells in G2/M phase, which confirmed its stimulatory effects on cell proliferation (22).

A2a adenosine receptors (A2aARs) as the second member of adenosine receptors, appears to be expressed in various human tumor cells including neuroblastoma, glioma, lymphoma, leukemia, melanoma, colon carcinoma, human glioblastoma cells, and human breast cancer cells (25-29). Large body of evidence has shown that agonist activation of adenosine A2A receptors led to reduction in neuronal apoptosis as well as stimulation of the proliferation of MCF-7 cells (30,31). However, activation of this receptor has been shown to promote cell death of human A375 melanoma cells as well as activation of apoptosis in Caco-2 human colonic cancer cell line (26). ZLin, *et al*. also have shown that estradiol treatment of estrogen α-positive breast cancer cells leads to up-regulation of A2aARs messenger RNA (mRNA) and protein levels and also its effect was reversed by the estradiol antagonist ICI 182,780 (32). The exact mechanism underlying ICI 182,780 effects is partially unclear; however it has been shown that it binds to estrogen by an affinity of more than 17β estradiol (E2) thereby reducing E2 effects (33). Gene transcript reveals that the A2bARs are expressed in a wide variety of human tissues and cell types (34). However, this receptor seems to be activated just at high concentrations of adenosine, which is shown under pathophysiological conditions (35).

A3 adenosine receptors (A3ARs) have been shown to present in various types of tumor cells, including human leukemia, Jurkat lymphoma, rat lymphoma, human melanoma, mouse pineal gland tumor cells, human glioblastoma, and human prostatic cells (36). A large body of data has shown that A3ARs expression is highly sustained in tumor cells (36). Despite several lines of studies, the expression level of all subtypes of adenosine receptor in response to estrogen is not clear. Therefore, the present study intends to shed some light on the expression level of adenosine receptor subtypes in MCF-7 cells treated with E2 and ICI 182,780 as the estrogen agonist and antagonist, respectively.

MATERIALS AND METHODS

Materials

Culture media, penicillin/streptomycin, growth supplements, and 0.25 % trypsin-EDTA solution were obtained from Gibco (BRL, Eggenstein, Germany). MTT, Activated charcoal, ICI 182,780, and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (Sigma -Aldrich, St. Louis, Missouri, USA).

Cell culture

The human breast cancer cell line MCF-7 was obtained from Pasture Institute of Iran

(Tehran, Iran). This cell line was cultured as a monolayer in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (BRL, Eggenstein, Germany) in 5% CO2 at 37 ºC. The cultures were then incubated with 0, 0.001, 0.01, and 0.1 mmol/L of E2 for 12 h (Sigma, St. Louis, Missouri, USA). To further study the effects of estrogen antagonist (ICI 182,780), the cells were pre-treated with various concentration of charcoal to deactivate any probable estrogen, and then 1 h later cells were treated with 1 μM ICI 182,780.

MTT cell viability assay

The MTT test was conducted to measurement of viable cells. It is a simple method to measure cytotoxicity, proliferation, and activation used in cell culture. This experiment is based on the ability of mitochondrial dehydrogenase enzymes of living cells to oxidize MTT making a purple formazan precipitate. This test was carried out as follow, briefly, 5×10^3 cells were seeded into a 96-well plate. After 24 h, different concentrations of E2 (0.001, 0.01, 0.1, 1 and 10 μM) were added into each well and then after 12 h of incubation with E2, 20 μL of MTT compound at final concentration of 5 mg/mL was added, and then 4 h later DMSO was added in order to dissolve violet formazan crystals via metabolically viable cells. Absorbance of each well was measured at 570 nm using a microplate reader (Biotek, Winooski, VT) to indicate the number of viable cells.

Analysis of gene expression by real-time quantitative PCR

Total RNA was extracted from treated and untreated cells using RNX-plus kit according to the manufacturer's instruction. Total RNA (1 μg) was reverse transcribed to cDNA with random hexamer primers using Quantitect Rev, transcription kit (Takara, Japan) and then in order to quantitatively examine adenosine receptor subtypes gene expression quantitative real-time RT-PCR was performed on a light cycler instrument (Corbette Research, Australia).

5-fold serial dilution of cDNA from collected cells was made to construct standard curves. Reaction mixture (25 μL) containing 2 μL of cDNA template, 1.5 μL each of forward and reveres primers and Quantitect SYBR Green master mix (Qiagen, Germany) amplified based on SYBR Green method. Direct detection of PCR products was monitored by measuring the fluorescence produced due to SYBR Green dye binding to dsDNA after every cycle. Each cycle of amplification was as follow: denaturation at 95 °C for 10 min and 40 cycles at 95 °C for 10 s, 60 °C for 20 s. Primers used in the present study were designed by Beacon Designer® version 8.0 and their sequences were indicated in Table 1. Finally the quantification of adenosine receptor subtypes gene expression was normalized to the endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data are expressed as mean ± standard deviation. All experiments were done in triplicate. Statistical analysis was conducted by the statistical program SPSS 18.0. For statistical analysis, the Student's t-test and one-way analysis of variance (ANOVA) were applied to compare the treated cells with the untreated cells. Statically significant differences considered when $P < 0.05$ was achieved.

Table 1. The sequence of primers**.**

Gene	Primer sequence	Product size
A1	**F** CCA CAG ACC TAC TTC CAC ACC **R** CCA TCT TGT ACC GGA GAG GG	136
A2a	**F** CTC CGG TAC AAT GGC TTG GT **R** TGG TTC TTG CCC TCC TTT GG	137
A2b	**F** CTG TGT CCC GCT CAG GTA TAA **R** CAG CAG CTT TCA TTC GTG GTT	180
A3	**F** TTG CCT ACT GCT TAT CTT **R** TCT TGT ATC TGA CGG TAA	98
GAPDH	**F** CTC CCG CTT CGC TCT CTG **R** TCC GTT GAC TCC GAC CTT C	116

GAPDH; glyceraldehyde 3-phosphate dehydrogenase.

RESULTS

The effect of 17β estradiol on proliferation of breast cancer MCF-7 cells

To uncover proliferative effects of E2 on MCF-7 cell lines, MTT assay was performed. It has been accepted that MTT as a calorimetric experiment, measures the activity of succinate dehydrogenase which converts tetrazolium dye to formazan. Therefore, this assay generally used as an index of viable cell and proliferation. As shown in Fig. 1, E2 has a concentration-dependent stimulation effect on the proliferation of MCF-7 cells. A statistically significant increase was found in the cell viability at 0.001–1 μM for 12 h. These data exhibited that cell viability was 100, 133, 130, 129, 121 and 112 percent of controls for concentration of 0, 0.001, 0.01, 0.1, 1, and 10 μM of E2, respectively ($P < 0.05$). As shown in Fig. 1, the cytotoxic effect at concentration of 10 μM was observed. Therefore to continue the current study treatment of MCF-7 cells with concentration of 0, 0.001, 0.01, 0.1 μM for 12 h was performed.

Relative gene expression of adenosine receptors in MCF-7 cells

The basal gene expression levels of adenosine receptors, A1, A2a, A2b, and A3, were assessed by RT-PCR. The results showed a profile of gene expression levels of all four adenosine receptor subtypes (A2b > A1 > A2a > A3) in this cells (Fig. 2). The mRNA expression of the A2bARs was higher than other adenosine receptors. While the A3R was the lowest mRNA expression as compared to all adenosine receptor subtypes.

The effect of 17β estradiol on A1 adenosine receptors gene expression in MCF-7 cells

The effect of E2 and its antagonist (ICI 182,780) on A1ARs gene were examined by real time PCR in MCF-7 cells (Fig. 3). E2 treatment at concentration of 0.001 to 0.1 μM induced an up-regulation of A1ARs mRNA in MCF-7 cells ($P < 0.05$). However treatment of ICI 182,780 reversed E2 effects, confirming that this effect was due to an interaction of E2 with its receptor which was inhibited by antagonist ($P < 0.05$).

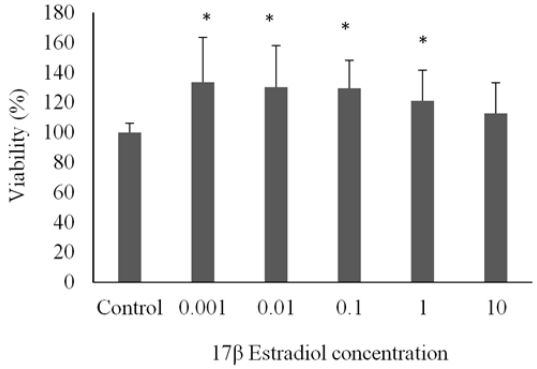

Fig. 1. Proliferative effect of 17β estradiol on human breast cancer cell line MCF-7. 17β estradiol increased cell viability at concentrations less than 10 μM. Results (mean ± SD) are presented as the percent of control values of three independent experiments ($^{*}P < 0.05$ compared with control).

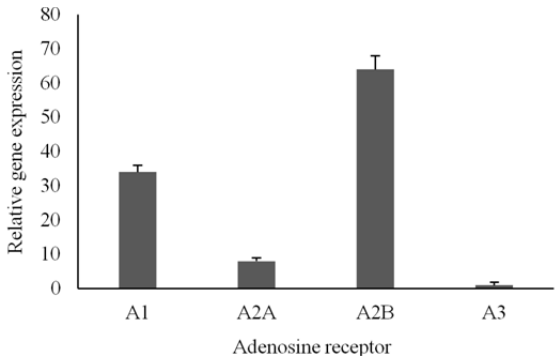

Fig. 2. The relative gene expression of adenosine receptors were performed using real time RT-PCR.

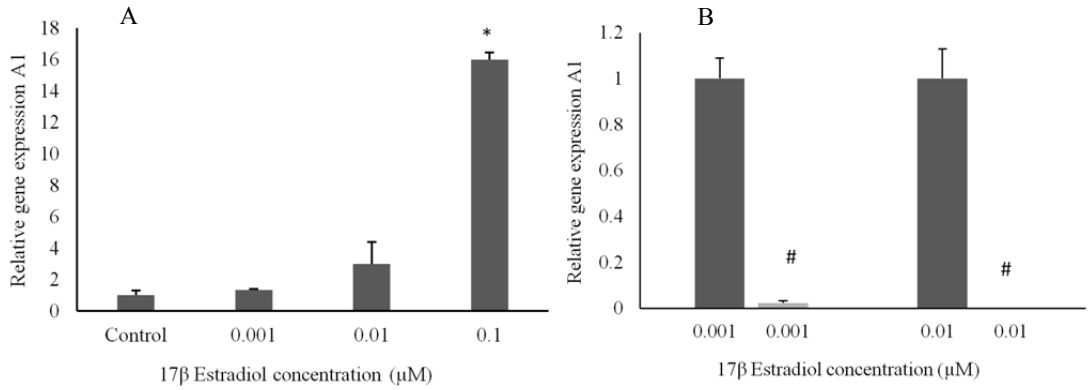

Fig. 3. Relative gene expression of A1 adenosine receptors in MCF-7 cells treated with different concentrations of 17β estradiol. (A) mRNA levels of A1 adenosine receptors in MCF-7 cells treated with different concentration of 17β estradiol ($^{*}P < 0.05$ compared to control and concentrations lower than 0.1 μM). (B) Relative mRNA content of A1 adenosine receptors in MCF-7 cells pretreated with two concentrations of 17β estradiol for 1 h followed by 1 μM ICI 182,780 ($^{\#}P < 0.001$ compared with corresponding control).

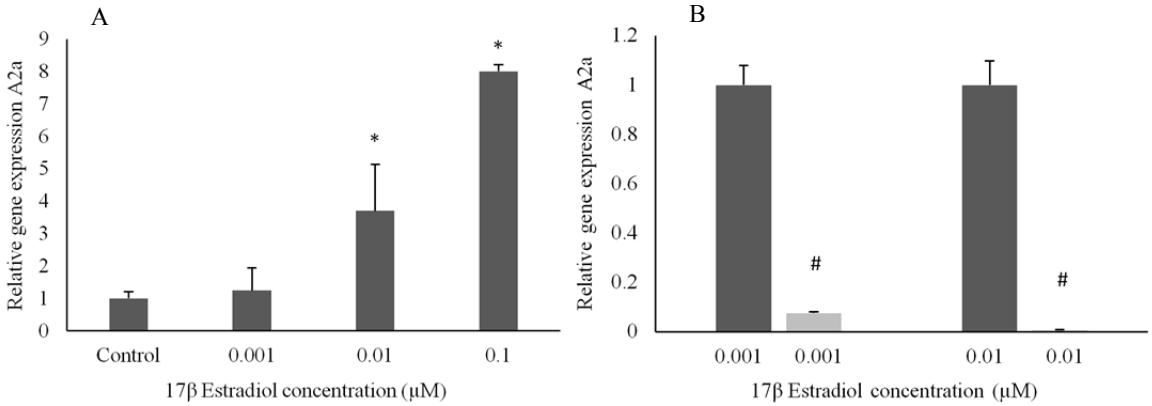

Fig. 4. Relative gene expression of A2a adenosine receptors in MCF-7 cells treated with different concentrations of 17β estradiol. (A) mRNA levels of A2a adenosine receptors in MCF-7 cells treated with different concentration of 17β estradiol ($^{*}P < 0.05$ compared to control and concentrations lower than 0.1 μM). (B) Relative mRNA content of A2a adenosine receptors in MCF-7 cells pretreated with two concentrations of 17β estradiol for 1 h followed by 1 μM ICI 182,780 ($^{\#}P < 0.001$ compared with corresponding control).

The effect of 17β estradiol on A2 adenosine receptors expression in MCF-7 cells

Similar to A1ARs, the effect of E2 and ICI 182,780 on A2aARs gene was performed (Fig. 4). E2 incubation at different concentration of 0.001, 0.01 and 0.1 μM stimulates A1ARs mRNA up-regulation in MCF-7 cells in a dose dependent manner ($P < 0.05$). However 1 h treatment of ICI 182,780 in a dose of 1 μM prevented E2 effects suggesting that expression of A2aARs attenuated by E2 receptor which was inhibited by antagonist ($P < 0.05$).

The effect of 17β estradiol on A2b adenosine receptors expression in MCF-7 cells

As shown in Fig. 2, A2bARs mRNA expression was higher compared to other subtypes. Therefore, E2 treatment at lower doses (0.001 μM) led to a non-significant increase in A2bARs expression (Fig. 5A). Treatment of cells with higher dose of E2, however, induced a sharp decrease in mRNA A2bARs expression in a dose dependent manner. Treatment of cells with 1 μM of ICI 182,780, blocked E2 effects (Fig. 5B) ($P < 0.05$).

The effect of 17β estradiol on A3 adenosine receptors gene expression in MCF-7 cells

As shown in Fig. 2, A3R mRNA expression was the lowest in comparison with other adenosine receptors. The results showed that E2 incubation (0.1 μM) led to a significant increase in A3R expression (Fig. 6A). Post-treatment of cells with 1 μM of ICI 182,780, significantly reduced effect of E2 (Fig. 6B) ($P < 0.05$).

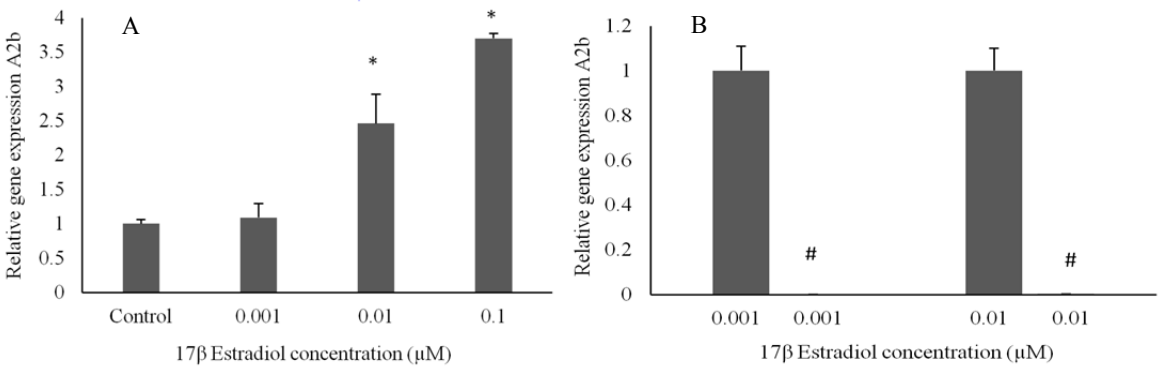

Fig. 5. Relative gene expression of A2b adenosine receptors in MCF-7 cells treated with different concentrations of 17β estradiol. (A) mRNA levels of A2b adenosine receptors in MCF-7 cells treated with different concentration of 17β estradiol ($^{*}P < 0.05$ compared to control and concentrations lower than 0.1 μM). (B) Relative mRNA content of A2b adenosine receptors in MCF-7 cells pretreated with two concentrations of 17β estradiol for 1 h followed by 1 μM ICI 182,780 ($^{\#}P < 0.001$ compared with corresponding control).

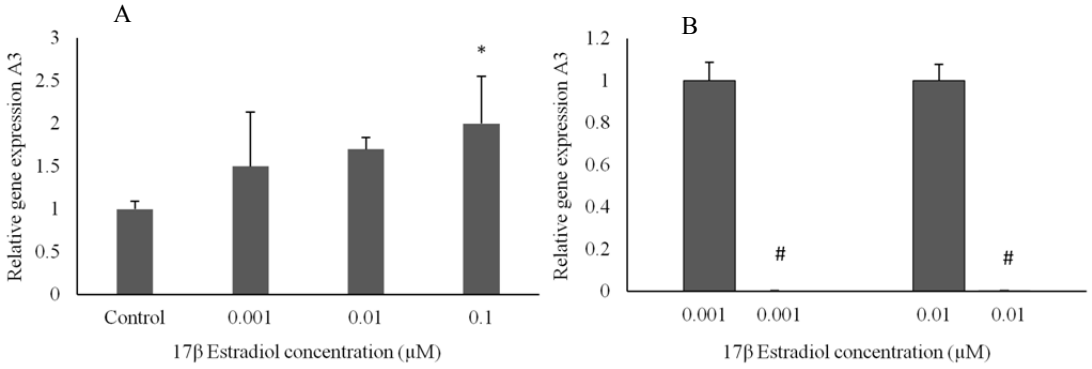

Fig. 6. Relative gene expression of A3 adenosine receptors in MCF-7 cells treated with different concentrations of 17β estradiol. (A) mRNA levels of A3 adenosine receptors in MCF-7 cells treated with different concentration of 17β estradiol ($^{*}P < 0.05$ compared to control and concentrations lower than 0.1 μM). (B) Relative mRNA content of A3 adenosine receptors in MCF-7 cells pretreated with two concentrations of 17β estradiol for 1 h followed by 1 μM ICI 182,780 ($^{\#}P < 0.001$ compared with corresponding control).

DISCUSSION

Estrogen is a steroid hormone playing a pivotal role in the development and regulation of the female reproductive system. Nowadays it is commonly used for the management of some female related disorders (1). However, it has been reported that estrogen is related to some human cancer (2-5). Breast cancer as the second leading cause of cancer is a consequence of estrogen therapy (10). It has been shown that estrogen contributes in the proliferation of breast cancer with an almost unknown mechanism (13). In order to elucidate the mechanism underling proliferative effect of estrogen, the current study was designed to use estrogen receptor estrogen-positive MCF-7 human breast cancer cells to indicate expression profile of adenosine receptors in response to estrogen treatment. The selection of this super family of receptor was because of their involvement in a wide variety of human cancers (22). After establishing of proper concentration of E2 using MTT assay, the effect of E2 on the expression of A1ARs was studied. The results showed that E2 sharply up-regulates A1ARs expression in a dose dependent manner. Due to the presence of estrogen in such cell line, it is more likely that up-regulation of A1ARs expression is a consequence of cascade activation produced by estrogen treatment. In addition, antagonist treatment of cells blocked E2 response to suggesting that A1ARs is located in the downstream of estrogen signaling. On the other hand, a growing body of evidence supports that

A1ARs participates in carcinogenesis of some human cancers (22). Furthermore, Synowitz, *et al*. observed that adenosine play a key role in glioblastoma tumor growth through A1ARs (37). Therefore, this study along with other related studies suggest that considerable attention should be paid to A1ARs during estrogen treatment for curing some cancers.The second member of the adenosine receptor family is A2aARs. The results of the present study exhibited that similar to A1ARs, A2aARs is up-regulated in response to E2 incubation in a concentration dependent fashion. Similar to this study, Lin, *et al*. have shown that incubation of MCF-7 cells with estradiol resulted in up-regulation of A1ARs mRNA and protein levels and also its effect was prevented by the estradiol antagonist (32). Moreover, it has been illustrated that A2aARs are present in various human tumor cells. In addition, A2aARs activation stimulates MCF-7 cells proliferation (30, 31).

The third sub-type of adenosine receptors is A2bARs which has similar expression to A2aARs. The obtained data showed that E2 dose dependently up-regulated A2bARs expression. Moreover, it has been exhibited that this member of adenosine receptors is strongly involved in cancer, renal disease, and diabetes (38). In addition, Haji ahmadi, *et al.* have observed that A2bARs agonist significantly reduced cell viability in a dose-dependent manner in ovarian cancer cell lines. They demonstrated that A2bARs agonist induces apoptosis via the mitochondrial signaling pathway (39). A3ARs is another member of adenosine receptors whose pattern is somehow different from A1ARs and A2aARs. A3ARs has the highest expression in untreated MCF-7 cells up-regulated at 0.1 μM of E2. Previous studies have reported that A3ARs is expressed in various types of human cancer cells and also it is the only adenosine subtype to be over-expressed in inflammation (36, 40). As an example, A3ARs is expressed in cancer tumor in comparison with adjacent normal cells in colorectal cancer (36). The effect of estrogen on MCF-7 cells is not restricted to adenosine receptor subtypes. As an example Frasor, *et al*. have reported that estrogen treatment altered wide variety of genes expression. They showed that more than 8000 genes were down-regulated whereas some of genes being positive proliferation regulators such as survivin, growth factors and cell cycle elements were up-regulated (14).

CONCLUSION

In order to clarify the effect of estrogen on the mRNA expression of adenosine receptor subtypes, the present study used estrogen-positive MCF-7 breast cancer cells. The results showed that E2 as an agonist of estrogen significantly up-regulated all subtypes of adenosine receptors. However, using estrogen antagonist, ICI 182,780, prevented the effect of E2 confirming that up-regulation of these receptors might be mediated by estrogen receptor.

ACKNOWLEDGEMENTS

The content of this paper is extracted from the MSc thesis (No. 394781) submitted by Azam Mohamadi which was financially supported by the Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

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