

Crosstalk between adenosine receptor (A_{2A} isoform) and $ER\alpha$ mediates ethanol action in MCF-7 breast cancer cells

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Abstract. Alcohol consumption increases the risk of breast cancer but the underlying mechanisms are not well understood. We have shown previously that ethanol activates ER signalling pathway in a cAMP/PKA-mediated ligand-independent manner. Since the activation of A_{2A} adenosine receptor ($A_{2A}AR$) by ethanol has been reported in other cell types, here we tested if cross-talk between this Gs-coupled receptor and $ER\alpha$ could be involved in ethanol effects in breast cancer cells. Our study shows that $A_{2A}AR$ is expressed and functional in the hormone-dependent breast cancer cell line MCF-7. Interestingly, activation of this receptor by the selective agonist CGS21680 stimulates the transcription of progesterone receptor, a well known estrogen target gene. CGS21680 also stimulates the pER ϵ Luc reporter activity in transfected MCF-7 cells, an effect antagonized by the anti-estrogen ICI182,780. Moreover, CGS21680 stimulates the proliferation of MCF-7 cells similarly to E_2 . Finally, the $A_{2A}AR$ antagonist MSX-3 inhibits the ethanol-induced activation of $ER\alpha$ signalling pathway. These results demonstrate cross-talk between $A_{2A}AR$ and $ER\alpha$ that is involved in ethanol action. This could open new perspectives for the therapy of estrogen-dependent breast cancer.

Introduction

Adenosine is a natural metabolite that plays a role in vasodilatation, cardioprotection after ischemia, inhibition of platelet aggregation, mast cell activation, and cell growth (1). Four adenosine receptors (ARs) (A_1AR , $A_{2A}AR$, $A_{2B}AR$ and A_3AR) have been cloned (2). Each of them regulate the activity of adenylyl cyclase: the A_1 and A_3 receptors mediate

a decrease in cAMP via Gi/o whereas the two A_2 receptors mediate an increase in cAMP via Gs (3). Extracellular adenosine activates A_1 and A_{2A} receptors whereas at high concentrations, it acts mainly on A_3AR .

Adenosine and AR seem to play different roles in various types of cancer (4). In breast cancer, selective A_1AR and selective A_3AR agonists inhibit cell proliferation of both hormone-dependent and hormone-independent breast cancer cell lines (5-8). An inhibitor of the ecto-5'-nucleotidase CD73, an enzyme that generates adenosine, decreases proliferation of breast cancer cells MDA-MB231 *in vitro* as well as *in vivo* in xenografts (9), while ecto-5'-nucleotidase overexpression promotes invasion, migration and adhesion of breast cancer cells (10). Breast carcinoma tissues show higher A_1 and A_3AR expression in the tumor vs. adjacent non-neoplastic tissue or normal tissue (11,12). Moreover, a depletion of A_1AR in MDA-MB-468 human breast tumor cells significantly induces apoptosis (12).

Breast cancer is the most common cancer in women. About 60% of all patients have hormone-dependent breast cancer. These tumors express estrogen receptors (ER) and require estrogens for their growth (13). $ER\alpha$, a member of the superfamily of steroid nuclear receptors, mediates the effects of these hormones on tumor development. In the best understood mode of action, the liganded $ER\alpha$ receptor binds to specific estrogen response elements (EREs) within target genes and recruits a p160/p300 coactivator complex to the promoter (14,15). This coactivator complex promotes gene transcription by remodeling chromatin and by contacting the basal transcription machinery. A hormone-independent activation of $ER\alpha$ also occurs in human breast cancer cells. Indeed, $ER\alpha$ activity can be stimulated by several kinase pathways (16). For instance, the mitogen-activated protein kinase (MAPK) pathway activated by epidermal growth factor directly phosphorylates $ER\alpha$ in the absence of ligand and induces ER transactivation (17,18). In MCF-7 breast cancer cells, ethanol induces a ligand-independent activation of $ER\alpha$ mediated by the cAMP/PKA pathway (19). However, the mechanisms involved in the ethanol-induced activation of cAMP/PKA are unknown. Interestingly, in NG108-15 cells (neuroblastoma x glioma hybrid cells), acute ethanol exposure induces an increase in extracellular adenosine that activates A_2 receptors to stimulate cAMP production (20). Such a mechanism could exist in MCF-7 cells.

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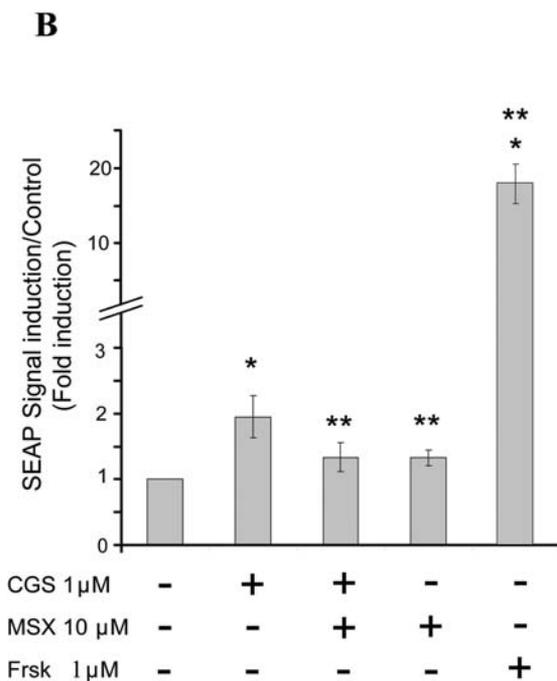
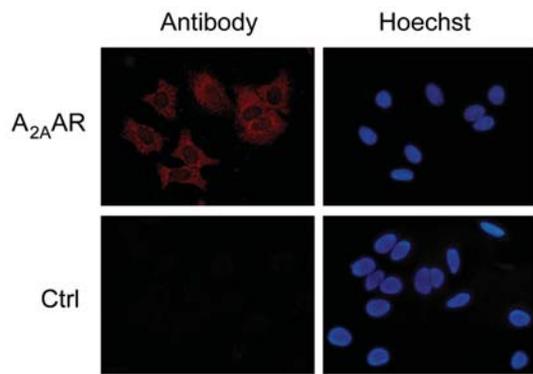


Figure 2. A_{2A}AR protein expression in MCF-7 cells. (A) Immunodetection of A_{2A}AR in MCF-7 cells leading to membrane staining. In control cells the primary antibody was omitted. (B) Expression of the SEAP reporter gene following treatment of pCRE-SEAP and pCMV-βGal co-transfected MCF-7 cells with the A_{2A}AR agonist CGS21680 for 24 h in presence or absence of the A_{2A}AR antagonist MSX-3. Forskolin (Frsk) treatment was used as a positive control. SEAP reporter activity was normalized with the β-Gal activity. *Significantly different from untreated control cells (P<0.05). **Significantly different from CGS-treated cells (P<0.05).

agonist CGS21680 induced 1.95-fold increase in SEAP expression (Fig. 2B). This effect was inhibited by the antagonist MSX-3 (Fig. 2B).

A_{2A}AR activation stimulates ERα signalling pathway. To test the hypothesis of a cross-talk between A_{2A}AR and ERα, we studied if the expression of the estrogen target gene PR (19,21) could be stimulated by an A_{2A}AR agonist. We observed an increased level of PR mRNA following a 24 h treatment with 1 μM CGS21680. This effect was abolished in

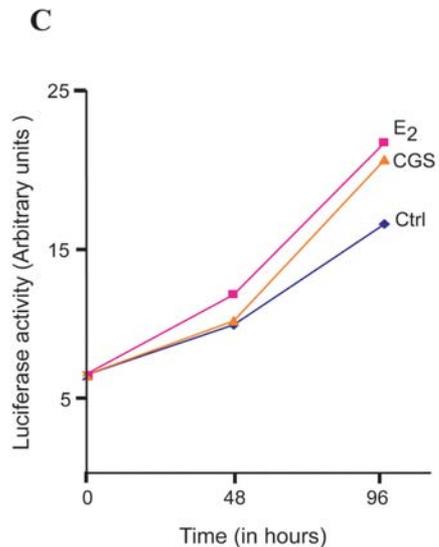
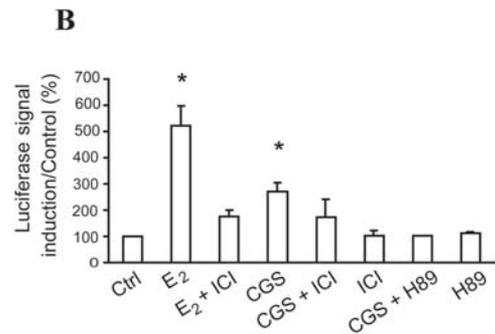
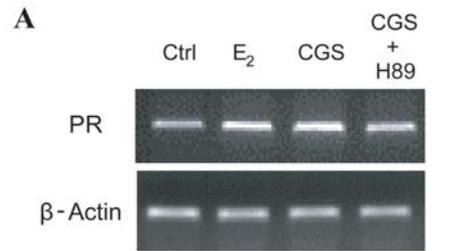


Figure 3. A_{2A}AR activation stimulates ERα target gene expression. (A) Semi-quantitative RT-PCR analysis of PR mRNA. MCF-7 cells were treated for 24 h with either E₂ (1 nM) or the selective A_{2A}AR agonist CGS21680 (1 μM) in presence or absence of the PKA inhibitor H89 (10 μM). β-actin mRNA levels were determined as an internal control. (B) Expression of the luciferase reporter gene following 24 h treatment of pERE-tkLuc and pCMV-βGal co-transfected MCF-7 cells with the A_{2A}AR agonist CGS21680 or E₂, in presence or absence of ICI182,780 or H89. Luciferase reporter activity was normalized with the β-Gal activity and expressed as percentage of the control assumed as 100%. *Significantly different from untreated control cells (P<0.05). (C) Cell proliferation analysis. MCF-7 cells were treated with CGS21680 (A_{2A}AR agonist, 1 μM), E₂ (1 nM), or ethanol (control, 0.1%). Results are expressed in arbitrary units. The mean of 3 different experiments is shown without standard error of the mean for a better observation of the different points.

the presence of the PKA inhibitor H89 (Fig. 3A). In order to definitely demonstrate the activation of ERα target gene expression by the A_{2A}AR agonist, MCF-7 cells were transfected with the reporter construct pEREtLuc and treated by different compounds (Fig. 3B). Similarly to E₂ (1 nM) used as a control, the A_{2A}AR agonist CGS21680 (1 μM) significantly

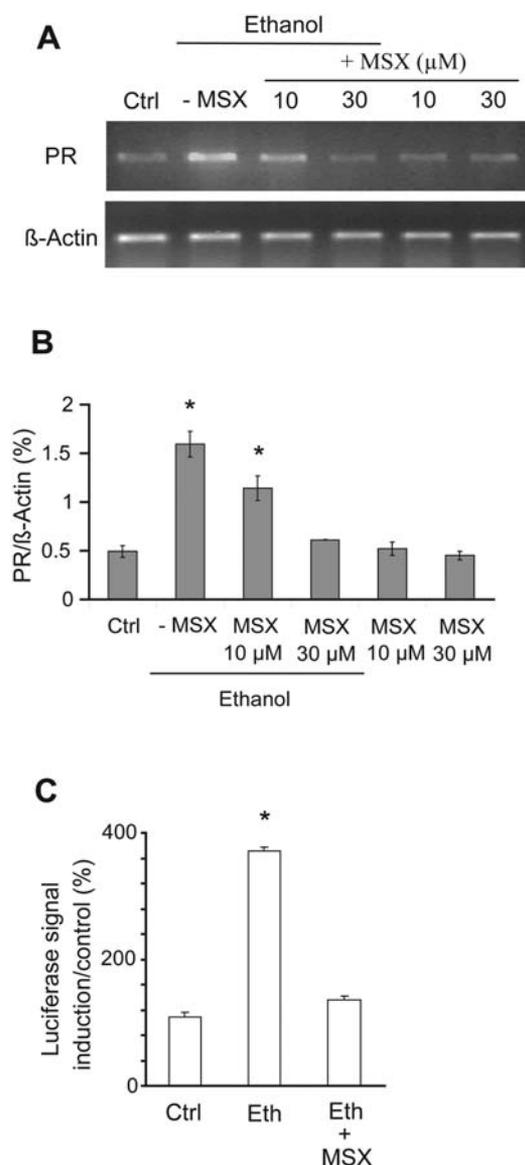


Figure 4. A_{2A}AR mediates ethanol-induced activation of ER signalling pathway. (A) Semi-quantitative RT-PCR analysis of PR mRNA. MCF-7 cells were treated for 24 h with ethanol (0.3%) and a selective A_{2A}AR antagonist, MSX-3 (MSX) (10 and 30 μ M). β -actin mRNA levels were determined as an internal control. (B) The histogram represents the mean \pm SE of four separate RT-PCR experiments in which the band intensities of PR were evaluated in terms of optical density arbitrary units and normalized with the β -actin signal intensity. *Significantly different from untreated control ($P < 0.05$). (C) MCF-7 cells were cotransfected with pEREtkLuc and pCMV- β Gal. The cells were treated for 24 h with ethanol (0.3%) in the presence or absence of MSX-3. Luciferase reporter activity was normalized with the β -Gal activity and expressed as percentage of the control assumed as 100%. In each experiment, the activities of transfected plasmids were assayed in triplicate transfections. *Significantly different from untreated control ($P < 0.05$).

increased luciferase activity (3-fold). This effect was mediated by the activation of ER α since it was antagonized by the pure antiestrogen ICI182,780 (100 nM) which also inhibited the response to E₂. Besides, the stimulation of ERE-directed expression by CGS21680 was inhibited in presence of the PKA inhibitor H89 (Fig. 3B).

We also determined if the cross-talk between A_{2A}AR and ER α could stimulate cell proliferation. After 96 h, CGS21680

(1 μ M) stimulated MCF-7 cell proliferation (Fig. 3C). A similar effect was observed with E₂ (1 nM) (Fig. 3C). Taken together, these results demonstrate cross-talk between A_{2A}AR and ER α .

A_{2A}AR mediates ethanol-induced activation of ER α signalling pathway. We have recently shown in MCF-7 cells that ethanol activates the cAMP/PKA pathway and induces a ligand-independent activation of ER α (19). Interestingly, ethanol has been reported to stimulate cAMP production in both NG 108-15 and lymphoma cells via the A_{2A}AR (20). In order to test the involvement of A_{2A}AR in ethanol-induced activation of ER α signalling pathway in MCF-7 cells, PR gene expression was studied after treatment with ethanol for 24 h, in presence or absence of MSX-3 (10 and 30 μ M). Ethanol was used at 0.3% since it is the most efficient dose *in vitro* (19). The antagonist induced a dose-dependent inhibition of ethanol-induced increase in PR expression (Fig. 4A and B).

We also studied pEREtkLuc transfected MCF-7 cells (Fig. 4C). This transfection assay showed an inhibition of ethanol action in the presence of the A_{2A}AR antagonist MSX-3 (30 μ M).

Discussion

Although the role played by adenosine and its receptors in breast cancer is not well understood, several studies indicate that adenosine is able to promote tumor cell proliferation and migration (10,23). Nevertheless, the underlying mechanisms remain obscure. In this study, we tested the hypothesis of cross-talk between A_{2A}AR and ER α in the hormone-dependent breast cancer cell line MCF-7. Such cross-talk could explain some tumorigenic processes involving adenosine or compounds as ethanol which could increase adenosine levels (20).

Our results based on both mRNA and protein detection show that A_{2A}AR is expressed in MCF-7 cells. Besides, in the transfection assays using a pCRE-SEAP reporter construct, the data obtained following treatment of the cells with a selective A_{2A}AR agonist are in agreement with the increase in cAMP usually observed following A_{2A}AR activation (3). Hence, the A_{2A}AR pathway is functional in MCF-7 cells. These findings contrast with a previous study concluding that MCF-7 cells did not contain adenosine receptors as judged by the lack of effect of non-selective agonists on adenylyl cyclase activity or intracellular Ca²⁺ levels (24). However, the same author reported recently the presence of the transcript of all adenosine receptors in the same cell line, with A₃ and A_{2B} isoforms being functional as deduced from cAMP production (25).

Then, different approaches led to the demonstration of cross-talk between A_{2A}AR and ER α . First, the treatment of MCF-7 cells with an A_{2A}AR agonist induced an increase in PR mRNA level that is often studied as an ER target gene (19,21). This result was confirmed by a more precise test using transfection assays with a pEREtkLuc reporter construct. In this study it was surprising to observe that the stimulation of luciferase activity was in the same range in case of exposure to either an A_{2A}AR agonist or E₂ itself. This similar intensity was correlated with the results of proliferation analyses.



we observed that the A_{2A}AR antagonist MSX3 both the increase in PR expression and the ERE-mediated increase in luciferase activity in transfection assays that are usually observed following exposure of MCF-7 cells to ethanol. This demonstrated the important role of A_{2A}AR in the ethanol-induced activation of ER α signalling pathway (19). So, ethanol acts in a similar A_{2A}AR-dependent manner in breast cancer cells and in NG 108-15 and lymphoma cells (20).

To conclude, our results demonstrate cross-talk between A_{2A}AR and ER α that is involved in ethanol action on MCF-7 cells. Nevertheless, the link between ethanol and A_{2A}AR remains to be determined. In S49 lymphoma cells, ethanol exposure increases extracellular adenosine by inhibiting the adenosine uptake via the nucleoside transporter (20). A similar process could be involved in ethanol-treated MCF-7 cells to explain A_{2A}AR activation. Besides, A_{2A}AR is linked to estrogen signalling pathway, it can be activated by extracellular adenosine whose concentration is elevated in breast tumor environment (23), therefore A_{2A}AR could constitute an interesting target for the design of new therapeutic agents.

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