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The role of epidermal growth factor and cholinergic receptor agonists and antagonists in MAPK signal transduction in K562 cells

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ABSTRACT

Objective: Muscarinic receptors (M1-M5) are members of the G protein-coupled receptor superfamily and are effective in physiological functions through G proteins. Recent studies suggested that cholinergic receptors mediate cellular activities in hematopoietic cells. The aim of this study was to investigate the potential role of mitogen-activated protein kinases (MAPK) signaling extracellular signal-regulated kinases 1 and 2 (ERK1/2)/phosphorylated ERK1/ (pERK1/2) pathways in chronic myeloid leukemia (K562) cells.

Materials and Methods: Chronic myeloid leukemia cells were cultured. Cells were incubated in the presence of muscarinic receptor agonist, antagonist and epidermal growth factor (EGF). To detect MAPK activation, ERK/pERK protein expression levels were determined by western blot method techniques.

Results: Our study results showed that cholinergic agents and EGFs affect the MAPK pathway in the human K562 cell line.

Conclusion: Cholinergic and EGF receptors may affect the MAPK pathway in K562 cells.

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Keywords: G protein coupled receptor, Carbachol, Epidermal growth factor, Mitogen-activated protein kinase, Chronic myeloid leukemia

1. INTRODUCTION

Acetylcholine (ACh) is a classical neurotransmitter. It has been demonstrated in a variety of neuronal and non-neuronal cells. ACh also plays a role as a mediator of cell communication in non-neuronal cells [1]. ACh has widespread physiological effects such as cytoskeletal regeneration, cell proliferation, differentiation, and programmed cell death (apoptosis) [2].

Acetylcholine is involved in the regulation of many physiological processes, involving muscarinic acetylcholine receptors (mAChR) (M_1-M_5) from the G protein-coupled receptor (GPCR) superfamily [3]. Muscarinic acetylcholine receptors expressed in different tissues and cells mediate intracellular responses by interacting with G protein subtypes [4]. Recent studies have shown that mAChRs functionally mediate cellular activities in non-neuronal and hematopoietic cells. It has also been reported that they are expressed in various cancer cell types, including the brain, mammary, colon, skin, lung, and prostate cancer cells. It is also stated that there are interactions between mAChRs and epidermal growth factor receptors (EGFRs) in different cancer cell types [5, 6]. Different mAChR subtypes are expressed in B and T lymphocytes [2]. Activation of M_1, M_2, and/or M_3 receptors in these cancer cell types causes an increase in cell proliferation. However, it has also been reported that mAChR receptor activation can cause cell cycle arrest and reduce cell proliferation [6]. Muscarinic receptors activate different signal transduction pathways via G proteins. M_1, M_3, and M_5 receptor subtypes interact with αq/11 subunits of G proteins, while M_2 and M_4 receptor subtypes interact with αi/o subunits of G proteins [7,8]. Different researchers showed that many G protein-coupled receptors also activate MAPK [9]. It has been determined that MAPKs are activated by cholinergic receptors. Activation of MAPK via the acetylcholine signal transduction pathway in different cells causes phosphorylation of ERK1/2 , DNA synthesis, and cell proliferation [4]. It has previously

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been shown that mAChRs increase SNU-407 colon cancer cell proliferation via the ERK1/2 pathway. The researchers showed that EGFR, protein kinase C, ERK1/2, and ribosomal S6 kinases (RSK) and mAChR-mediated activation affect proliferation of SUNU 407 colon cancer cells. EGFR activates MAPK, ERK1 and ERK2 (p44 and p42 MAPK), which are activated by mitogens, and their central role in cell proliferation has been established. These receptors activate the transcription of genes necessary for cell survival and cell proliferation [10]. The M_3 mAChR is determined to be expressed and functional in colon cancer cells [11]. The role of these receptors in cancer is largely unknown, they are known to mediate cell growth through different signal transduction pathways [11]. The Ras, small GTP-binding protein, /Raf, a serine/threonine protein kinase, /MEK tyrosine and serine/threonine dual specificity protein kinases,/ERK cascade is an important signaling pathway in MAPKs. Over activation of the Ras /Raf/MEK, /ERK pathway has been reported in 30% of human tumors [12]. In cancer, hormones, growth factors, differentiation factors, and tumor-promoting agents use the Ras/Raf/MEK/ERK signal transduction pathway. This signal transduction pathway starts with Ras activation, and followed by activation of Raf, MEK, and ERK [12]. mAChRs have been shown in animal and human cell lines to stimulate mitogen-activated protein (MAP) kinases, which act by activating extracellular signal-regulated kinases 1 and 2 (ERK1/2) [13]. Our previous studies demonstrated the semi-quantitative RT-PCR method by which K562 cells express M_2, M_3, and M_4 muscarinic receptor subtypes [14]. We also demonstrated the expression of muscarinic receptors at protein level in K562 cells using specific antibodies specific for M_2, M_3, and M_4 subtypes by western blot method [15]. In our previous studies, cholinergic system was found to be functional in K562 cells by muscarinic receptor-mediated protein kinase C , nitric oxide , cylic adenosine mono phosphate, c-fos, an intermediate early gene, and Ca^{+2} measurement assays [14,15]. We also showed that alpha 7 nicotinic receptor expression was determined in K562 cells. The effects of cholinergic agonist ACh and/or atropine (ATR), nicotinic antagonist, methyllycaconitine on cell proliferation and intracellular Ca^{2+} levels in K562 cells were determined. [16]. The epidermal growth factor receptor is one of the key players in the MAP kinase signaling cascade that is activated through the cholinergic system. Different investigators have shown that MAPK/ERK activation by GPCR occurs via Ca^{2+} dependent or independent mechanisms, depending on receptor type and cellular background [17,18]. EGFR is a member of the tyrosine kinase family. Various signaling pathways activate these receptors. Several studies have found that stimulation of various GPCRs activates the epidermal growth factor receptor [19, 20]. The signaling pathways involving mAChRs are not fully clear. It appears to depend on the context of cellular growth [21].

This study aimed to determine the changes in ERK, pERK protein expression levels in the presence of carbachol (CCh) and/or EGF to determine the role of EGFRs in muscarinic receptor-mediated mitogen-activated protein kinase activation in human erythroleukemia cells.

2. MATERIALS and METHODS

Carbachol, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP), atropine (ATR) , Roswell Park Memorial Institute 1640 (RPMI-1640) medium, and epidermal growth factor (EGF) were purchased from Sigma Chemical Co, St. Louis, MO, USA. Fetal bovine serum (FBS) was obtained from Biol. Ind. (Beit Haemek, Israel). The Lowry kit (SMART TM BCA Protein Assay Kit iNtRON Biotechnology (Korean), The Nitra Blue Tetrazolium/5-Bromo-4-Chloro-3-indolyl phosphate (NBT/BCIP) was provided by Promega (Madison, WI, USA). ERK, phosphorylated ERK (pERK) (137F5 – p44/42 MAPK (Erk1/2) Rabbit mAb #4695), Phospho-p44/42 MAPK (ERK 1/2) (Thr202/Tyr204) (197G2) Rabbit mAb #4377 and β-actin antibodies (Sc190657) were supplied by Cell Signaling Technology (CST-USA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Sigma (St Louis, MO, USA) provided the secondary antibodies.

Cell culture

The American Type Culture Collection (ATCC), Manassas, VA, USA, provided the K562 cells. K562 cells were incubated in RPMI-1640 culture medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 200 mM L-Glutamine, at 37°C, in a humidified atmosphere of 5% CO_2 and 95% air, with one half of the medium being replaced every 3-4 days.

Cell proliferation and viability

Cells were stained with 0.4 % trypan blue and counted using a hemocytometer. The trypan blue exclusion test calculated cell viability and proliferation.

K562 cells were seeded into culture flasks containing RPMI-1640 medium under serum-free conditions. After 24 h, these "starved cells" were placed into a medium containing 1% FBS. K562 cells were exposed to CCh in the presence or absence of the M_3 muscarinic selective antagonists 4-DAMP. 4-DAMP was administered for 30 min before CCh/EGF was added to the culture medium. Cells were harvested and washed in phosphate buffered saline for 15 minutes at 400 g before being frozen at –80 °C.

Preparation of homogenates and western blot analysis

Semi-quantitative western blotting assessed the levels of ERK and pERK protein. Cells treated with drugs and cell lysates were prepared in lysis buffer. Lysates were lysed with Dounce homogenizer in a lysis buffer that contained 20 mM HepesKOH, pH 8.0, 0.1 mM, ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonylfluoride, 10 µg/mL leupeptin and 2 µg/mL aprotinin. The Lowry kit (SMART TM BCA Protein Assay Kit, iNtRON Biotechnology Korean), was used to detected protein levels in cell homogenates. Western blot analyses were as described in our previous studies with minor modification [14,15]. 150 µg of protein was loaded onto sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell,

0.45 μM, Germany). The membranes were blocked at room temperature for 60 min. Later, the membranes were incubated overnight at 4°C with antibodies against ERK and pERK (1/100) (Cell Signaling Technology,USA). The blots were washed with Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBS-T) and were later incubated with alkaline phosphatase-conjugated secondary antibodies for 1 h at room temperature (20°C). β-actin antibody was used for loading control in each blot (Santa Cruz, CA, USA). Secondary antibody and dilutions were used: goat-antirabbit 1:10,000 (Sigma, St Louis, MO, USA). Nitra blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) was used to detect the antibody-antigen complex. The free edition of ImageJ software quantified band intensities by optical density. The apparent molecular weights of ERK, pERK, and β-actin are 42 kDa, 44 kDa, and 47 kDa, respectively.

3. RESULTS

K562 cells were incubated in RPMI-1640 culture medium containing 10% fetal calf serum, L-Glutamine, at 37°C, in a humidified atmosphere of 5% CO_2 and 95% air. K562 cells were observed by Phase contrast light microscopy (Figure 1).

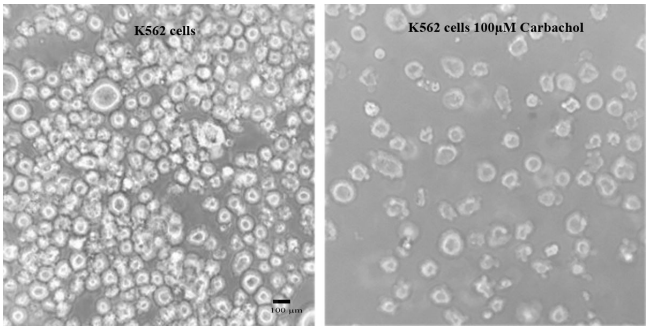

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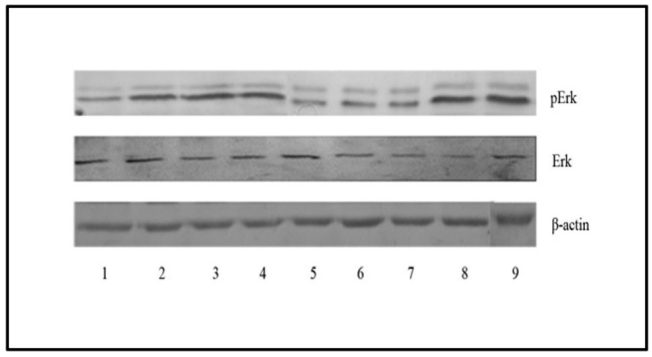
***Figure 1**. Phase contrast light microscopy of K562 cells. K562 cells were seeded at a density of 1X10^5 cells/ml in RPMI 1640 medium containing L-glutamine and 1% fetal calf serum. a) K562 cells b) Carbachol treated K562 cells*

Effect of CCh and EGF on pERK/ERK expression in K562 cells

K562 cells were seeded into culture flasks containing RPMI-1640 medium under serum-free conditions. After 24 h, these "starved cells" were placed into a medium containing 1% FBS.

K562 cells were exposed to 100μM CCh or 16nM EGF in the presence or absence of the M_3 muscarinic selective antagonists 4-DAMP at 0, 30, and 120 min. Expression of pERK/ERK was detected by western blot analysis of whole lysates of K562 cells. CCh increased the time-dependent expression of pERK/ERK, while EGF caused a decrease at 30 min. The epidermal growth factor was determined to decrease pERK/ERK expression in 30 minutes (Figure 2).



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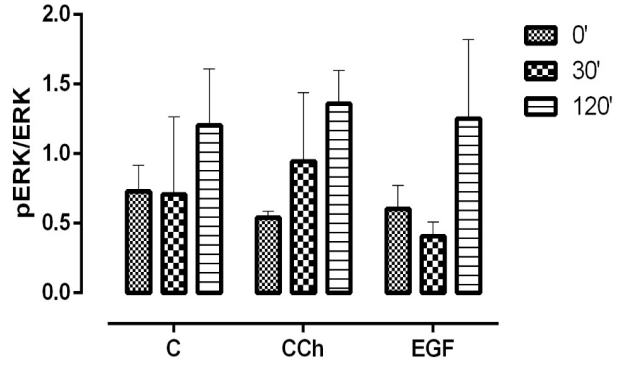

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***Figure 2.** Effects of CCh /EGF on ERK expression. K562 cells were treated with 100 μM CCh /or 16nM EGF for 0, 30 min, and 120 min. 1) Control 0 min 2)CCh 0 min 3)EGF 0 min 4) Control 30 min 5) CCh 30 min 6) EGF 30 min 7) Control 120 min 8) CCh 120 min 9) EGF 120 min. Western blotting for β-actin was used as the control for the assay. a) Typical western blot figure is representative of three separate experiments. b) The results were shown as ± SEM by taking the average of 3 independent experiments. Statistical analysis was performed with Two-way ANOVA; P>0.05 (ns).*

In order to determine the role of EGF in muscarinic receptor-mediated mitogen-activated protein kinase activation, we investigated the effect of cholinergic agonist/antagonists and EGF on pERK/ERK. Results are shown in Figure 3. pERK/ERK expression decreased compared to the control group (P<0.05) (Figure 3). Exposure of K562 cells to the M_3 muscarinic receptors the antagonist 4-DAMP significantly inhibited pERK and ERK responses to CCh (P<0.05) (Figure 3). Our results show that pretreatment of K562 cells with the M_3 selective muscarinic receptor antagonist 4-DAMP (10^{-6} M, 30 min) reduced pERK/ERK expression compared to control (P<0.05).

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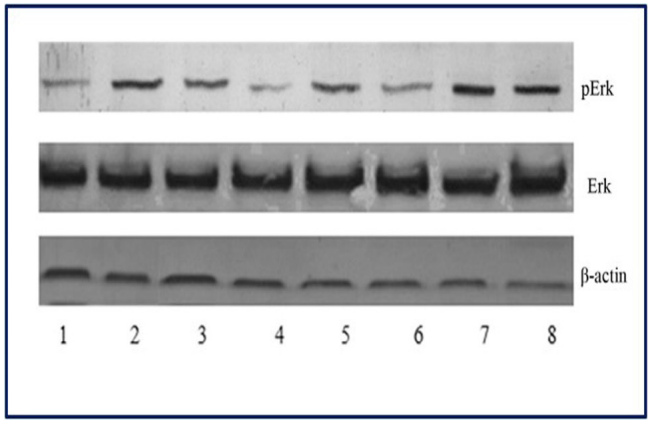

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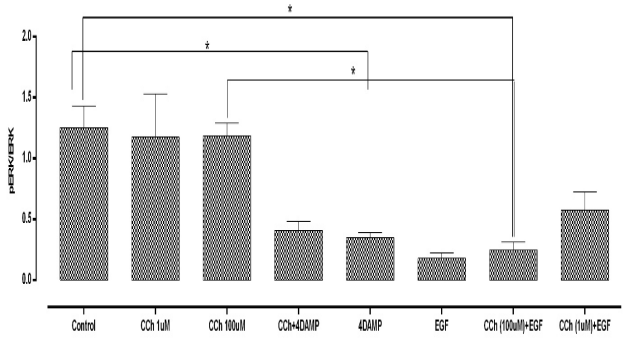

Figure 3. *Effects of CCh, 4-DAMP, and EGF on pERK/ERK expression, K562 cells were treated with 100 µM CCh for 30 min. The 10 µM 4-DAMP antagonist was added 30 min prior to CCh. 1) Control, 2) 1µM CCh 3) 100 µM CCh 4) CCh+4-DAMP 5)4-DAMP 6) 16nM EGF 7) 100 µM CCh +EGF 8) 1 µM CCh +EGF. a) Representative western pERK1/2 and ERK1/2 phosphorylation blots are shown, western blotting for β-actin was used as the control for the assay. b) The results were shown as the mean of the 3 independent experiments as ± standard error (SEM) (P<0.05). Statistical analysis was performed with Two-way ANOVA (P<0.05).*

Strong inhibition of pERK/ERK by both 4-DAMP and EGF was observed. pERK/ERK ratio was significantly inhibited in the presence of CCh+EGF compared to CCh.

4. DISCUSSION

The present study showed that mAChR agonists, antagonists, and/or EGF caused a change in MAPK in K562 cells. Expression of pERK/ERK in K562 cells was decreased in the EGF, 4-DAMP, and EGF+CCh groups compared to the control. We have previously shown that cell proliferation is inhibited when K562 cells are exposed to CCh in a cell growth medium supplemented with 1% or 10% serum [15]. However, it has been shown that CCh stimulates K562 cell proliferation (increased DNA synthesis) in a serum-free medium. We also showed that growth inhibition elicited by CCh in K562 cells is reversed by atropine and 4-DAMP. CCh also caused a decrease in the expression of M_2 and M_3 proteins in K562 cells. [15]. This study showed that blocking M_3 mAChR signaling using the selective antagonist 4-DAMP inhibited MAPK pathway protein expression. 4-DAMP appeared to suppress M_3R mAChR-mediated phosphorylation of ERK. Because EGF also inhibited ERK expression levels after incubation with CCh in K562 cells, the change in ERK expression was independent of EGFR activation. Kuol et al., showed the effect of atropine and 4-DAMP on the phosphorylation of protein kinase B (AKT) and ERK. They also demonstrated EGFR activation in CT-26 cells. They also showed that blocking all muscarinic receptors with atropine and M_3R with 4-DAMP significantly suppressed CT-26 cell proliferation in a dose-dependent manner and induced apoptosis through inhibition of the EGFR/AKT/ERK signaling pathways. They suggested that atropine exerts its effect by inhibiting the EGFR/AKT/ERK pathway, and 4-DAMP by suppressing the AKT/ERK signaling pathway [22]. Different researchers have noted that mAChR activation can stimulate or inhibit cellular growth depending on previous levels of cellular activity [21, 23]. According to Metzger et al., CCh inhibited EGF-induced HaCaT cell migration [24]. Prenzel et al., stated that M_3R activation promotes CRC progression by both EGFR-dependent and independent mechanisms [25]. Yu et al., found a close link between M_3R expression and AKT and ERK phosphorylation and EGFR activation in gastric cancer cells [26]. Different researchers have reported that M_3 mAChR mediated EGFR activation, signaling pathway is activated in different types of cancer, thereby phosphorylating ERK1/2 and AKT [27, 28]. In different studies, the ability of muscarinic agonists to stimulate growth and M_3 receptor antagonists to inhibit tumor growth has been demonstrated for breast, melanoma, lung, stomach, colon, pancreatic, ovarian, prostate, and brain cancer. While the cholinergic system is found in a wide variety of cancers, mAChRs have been shown to be organ-specific [29]. Kanlı et al., showed that pilocarpine (M_3 AChR agonist) exhibits antiproliferative effects in the presence of 1% and 0% FBS [30]. Antiproliferative effects of pilocarpine were not reversed by 4-DAMP (M_3 AChR antagonist) in K562 cells. Pilocarpine did not change M_3 mAChR expression. But 4-DAMP+pilocarpine group caused significant increase in M_3 mAChR expression compared to the control group [30, 31]. Treatment with CCh for 48h decreased the K562 cell number, indicating that CCh had a very fast and irreversible effect to promote cells to necrotic cell death [32]. The results of present study show that besides the inhibitory effect of cholinergic receptor antagonists, M_3R blocking may exhibit antitumor effects through a variety of mechanisms, including antitumor response through suppression of the pErk/ERK signaling pathway. Because these receptors can activate multiple signaling pathways, the growth-promoting and inhibiting effects of muscarinic agonists/antagonists in cancer cells are still unclear. This study showed that cholinergic and EGFRs may affect the MAPK pathway in human chronic myeloid erythroleukemia cells.

Compliance with the Ethical Standards

Ethics Committee approval: This research using cell lines does not require ethical approval.

Financial support: The authors have no relevant financial information to disclose.

Conflict of interest: The authors have no potential conflicts of interest to disclose.

Authors contributions: SGA and BA: Carrying out the experiments, H C, B A, and S GA: Analyzing the results and conducting the project, H C: Writing – Original draft preparation, writing – reviewing and editing. All authors read and approved the final version of the article.

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