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Effects of carbachol on apoptosis in human chronic myelogenous leukemic K562 cell line

İnsan kronik miyeloid lösemi K562 hücrelerinde karbakolün apoptoza etkisi

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ABSTRACT

Objectives: Muscarinic receptors mediate diverse actions of acetylcholine in the central nervous system and in non-nervous tissues innervated by the parasympathetic nervous system. Our study aims to evaluate the potential association of the M_3 muscarinic receptor with K562 cell proliferation and death.

Materials and Methods: Cell proliferation was evaluated by bromodeoxyuridine (BrDU) incorporation. To show early, late apoptosis and cell death, cells were labelled with Annexin V, propidium iodide (PI) and analyzed by flow cytometry. Nuclear extracellular signal-regulated kinase (ERK/pERK) expression was measured by western blot analysis.

Results: Treatment with carbachol (CCh) for 48h decreased cell number. Exposing K562 cells to CCh for 24h decreased the number of early apoptotic cells but did not change the number of late apoptotic and necrotic cells. CCh treatment for 48h increased the number of necrotic cells, but decreased the number of early and late apoptotic cells. In response to CCh, nuclear ERK expression was increased and this effect was reversed by 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4DAMP). Nuclear pERK expression was decreased in CCh treated cells, 4DAMP did not reverse the effect.

Conclusion: Our data suggest that cholinergic agonist CCh affects cell proliferation in K562 cells not only through muscarinic receptors but also through other cholinergic receptors**.**

Keywords: Muscarinic receptors, K562 cells, Carbachol, Cholinergic system

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ÖZ

Amaç: Muskarinik reseptörler merkezi sinir sisteminde asetilkolinin çeşitli etkilerine aracılık ettiği gibi, parasempatik sinir sistemi ile etkileşen sinirsel olmayan dokulara da aracılık ederler. Çalışmamızda, M_3 muskarinik reseptör alttipinin K562 kanser hücre çoğalması ve ölümündeki rolünü belirlemeye çalıştık.

Gereçler ve Yöntemler: Hücre çoğalması bromodeoksiüridin (BrDU) yöntemi ile belirlenmiştir. Hücre ölümü, erken ve geç apoptoz Anneksin V ve propidyum iodid (PI) varlığında akış sitometrisi yöntemi ile gösterilmiştir. Nuklear dış sinyal düzenleyici kinaz (ERK/fosfo-ERK) ekspresyonu western emdirimi yöntemi ile belirlenmiştir.

Bulgular: Çalışmamızda 48 saat karbakol(CCh) ile muamele edilen K562 hücre sayısında azalma belirlenmiştir. Hücreler 24 saat CCh ile muamele edildiklerinde erken apoptotik hücre sayısında azalma gözlemlenirken geç apoptoz ve nekrotik hücre sayısında değişim olmamıştır. Bununla birlikte, 48 saat boyunca CCh ile muamele olan hücrelerde, erken ve geç apoptotik hücre sayısı azalırken, nekrotik hücrelerin sayısı artmıştır. CCh ile muamele edilen hücrelerde nüklear ERK ekspresyonu artarken bu etki 1,1-dimetil-4-difenilasetoksipiperidinium iodid (4DAMP) ile geri çevrilmiştir. Aynı koşullarda CCh ile muamele edilen hücrelerde nuklear pERK ekspresyonu azalmış, bu etki 4DAMP ile geri çevrilmemiştir.

Sonuç: Bulgularımız, K562 hücre proliferasyonundaki kolinerjik etkinin yalnızca muskarinik mekanizma ile değil diğer kolinerjik reseptörlerin de katkısıyla gerçekleştiğini düşündürmektedir.

Anahtar kelimeler: Muskarinik reseptör, K562 hücreleri, Karbakol, Kolinerjik sistem

Introduction

G-protein-coupled receptors (GPCRs), the largest family of cell-surface molecules involved in signal transmission, have recently emerged as crucial players in tumour growth and metastasis. These receptors control key physiological functions, including neurotransmission, hormone and enzyme release from endocrine and exocrine

glands, immune responses, cardiac – and smooth-muscle contraction and blood pressure regulation, just to name a few. Their dysfunction contributes to some human diseases; therefore, GPCRs represent the target of 50–60% of all current therapeutic agents, either directly or indirectly [1].

Five subtypes (M_1-M_5) of receptors with seven transmembrane segments are integral membrane proteins, bind with acetylcholine (ACh) in the extracellular segment, and thereafter interact with and activate GTP-binding regulatory proteins (G proteins) in the intracellular segment [2]. Three muscarinic receptor subtypes (M_1R, M_3R, and M_5R) stimulating cellular signaling are conditional oncogenes when expressed in cells capable of proliferation [3]. Many studies have shown that cholinergic agonist carbachol (CCh) induced cancer cell proliferation [4,5].

Leukemia is a clonal disorder characterized by blocked normal differentiation and cell death of hematopoietic progenitor cells. Chronic myelogenous leukemia (CML), is a hematopoietic stem cell disorder with increased production of granulocytes at all stages of differentiation, leading to a myeloproliferative syndrome [6,7]. The K562 cell line derived from a CML patient during blast crisis was examined for properties of B and T lymphocytes and cell lines. Although K562 cells have some T cell properties, these are not exclusive [8]. K562 cell lines are good models for studying cell proliferation and apoptosis in CML. We have previously demonstrated that CCh decreased proliferation of K562 cells supplemented after starvation with 1% serum in 24h, an effect prevented by atropine [9].

Apoptosis is one of the ways for cell death that is referred to as programmed cell death (PCD). It plays important roles in embryonic development, immune system maturation and cytotoxic effector function, and carcinogenesis [10]. Apoptosis is executed by two pathways. Death receptors, such as Fas and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) DR4 and DR5 trigger the extrinsic/death receptor pathway [11]. The response to radiation or cytotoxic drug-induced cellular stress activates the intrinsic/mitochondria-dependent pathway [12]. The development of new therapies for the treatment of cancer has been possible by studying apoptotic processes [13].

Programmed cell death uses adenosine triphosphate (ATP), synthesizes new RNA and protein and thus forms active cellular suicide. PCD activates endogenous endonucleases that degrade the cell's DNA. Thereafter, the genetic template required for cellular homeostasis is destroyed [14]. However, there are various ways for cell death, for example hypoxia and exposure to certain toxins cause the form of cell death termed "necrosis". Increased membrane permeability, cell swelling, and rupture are the early events; whereas, loss of plasma membrane integrity is a relatively late event in PCD. PCD seems to play an important role in several physiological situations [15].

In neurons, it has been shown that muscarinic receptors act via activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) which are referred to as mitogen-activated protein (MAP) kinase 1 [16]. M_3R has an important role in the endogenous and exogenous ACh-induced cell proliferation and phosphorylation of ERK and AKT in gastric cancer cells [17].

Materials and Methods

Carbachol, 4DAMP, atropine, Roswell Park Memorial Institute 1640 (RPMI-1640) medium were purchased from Sigma Chemical Co, St. Louis, MO, USA. Fetal bovine serum (FBS) was obtained from Biol. Ind. (Beit Haemek, Israel). Nitro Blue Tetrazolium/5-Bromo-4-Chloro-3-indolyl – phosphate (NBT/BCIP) were purchased from Promega (Madison, WI, USA). phosphorylated ERK (pERK), β-actin antibodies were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibodies were purchased from Sigma (St Louis, MO, USA).

Cell culture

K562 cells were grown in suspension using RPMI medium supplemented with 10% fetal calf serum at 37°C in a 5% CO2 humidified atmosphere. Cells were usually seeded at a density of 10^5 cells/ml and one half of the medium was replaced every 3-4 days. These cells were then starved by seeding into flasks containing RPMI-1640 medium with 0% FBS. Afterwards, these "starved cells" were placed into a medium containing 1% serum, with and without 100 µM CCh, 10 µM 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4DAMP), 10 µM atropine for different times.

Cell counting

The change in K562 cell count in response to CCh stimulation was evaluated by assessing proliferation and cell viability by the trypan blue exclusion test and bromodeoxyuridine (BrDU) labeling, respectively. The contribution of muscarinic receptors was investigated by using the non-specific muscarinic antagonist, atropine. Cell counting was carried out using an ELISA reader (Multiskan Microplate Reader, Thermo Scientific, USA). K562 cells were treated with 0-100 µM CCh and/or antagonist atropine (10 µM) for 48h and aliquots were

removed at indicated times. Antagonist was added 30 min prior to CCh. The average of 3 experiments performed in duplicate was used for data analysis.

Flow cytometric analysis

In order to detect early and late apoptosis and cell death, cells were stained with FITC conjugated Annexin V and propidium iodide (PI) according to the manufacturer's instructions. Samples were then analyzed by flow cytometry (BD FACS Canto, Becton, Dickinson and Company, USA).

Western blot analysis

K562 cells were treated with 100 μM CCh and/or M_3R-selective antagonist 4DAMP (10 μM) for 5 minutes. The duration of CCh treatment was determined by our previous studies (unpublished data). Antagonist, 4DAMP (10 µm) was added 30 min prior to CCh. After addition of CCh and 4DAMP, cells were washed with phosphate-buffered saline (PBS) and were later frozen at −80 °C until further treatment. The frozen cells were homogenized in ice-cold 10 mM Tris–HCl (pH 7.2) buffer containing 1 mM EDTA and protease inhibitors (0.2 mM PMSF, 1 g/ml leupeptin, 1 M pepsitatine, 10 g/ml soybean trypsin inhibitors) with a 9-gauge needle. The samples were centrifuged at 300×g for 5 min at 4 °C. The resulting supernatant was centrifuged at 13000xg for 20 min at 4°C. The pellets were resuspended and washed twice in the same buffer and stored at −80 °C. The protein content of pellets was determined by the Lowry method [18]. 50 µg of protein was loaded onto sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell, 0.45 m, 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 (1/500). The blots were washed with 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). The antibody–antigen complex was detected with nitra blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP). The densitometric analyses were carried out with Bio-Rad Molecular Analyst software (free edition, www.totallab.com).

Results

Effect of CCh on K562 cells proliferation

In this study, we showed that there was a decrease in cell number in the group treated with 100 µM CCh for 48h when compared to the control group. However, this decrease was not reversed by atropine treatment (Figure 1).

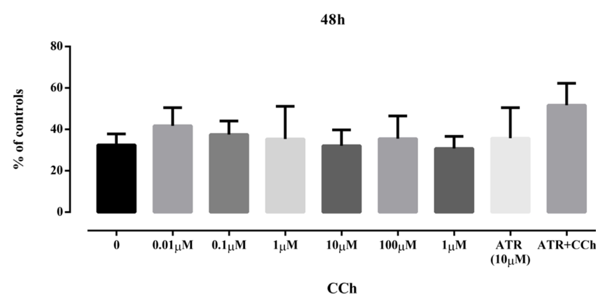

Figure 1. Effect of CCh on K562 cells proliferation. K562 cells were treated with 0-100 µM CCh, 10 µM atropine, 100 µM CCh and 10 µM atropine for 48 hours. Control cells were not treated with CCh. The results were shown as ± SEM by taking the average of 3 experiments. ATR: Atropine.

Effect of CCh on K562 cells apoptosis

We demonstrated that after exposing K562 cells to 100 µM CCh for 24 h the number of early apoptotic cells was decreased by ~10 % but no change was observed in the number of late apoptotic and necrotic cells. After treatment with CCh for 48h, the number of necrotic cells was increased by 1.5-fold whereas apoptotic cells were decreased by ~ 1.55-fold compared to control cells (Figure 2).

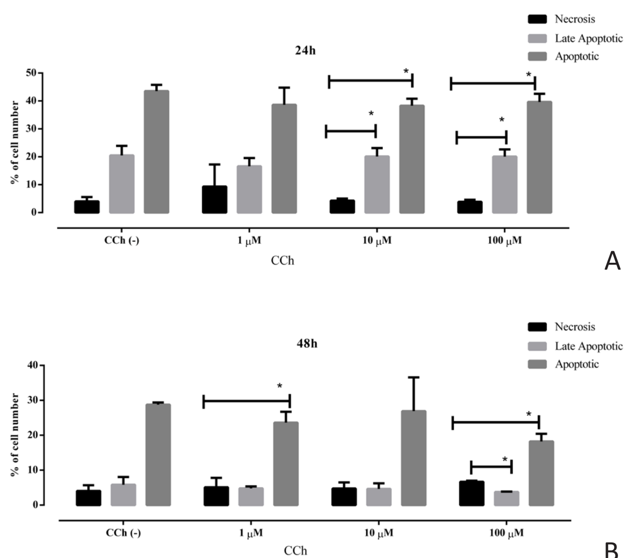

Figure 2. Effect of CCh on K562 cells apoptosis. K562 cells were treated with 0-100 µM CCh for (A) 24h and (B) 48h. Control cells were not treated with CCh. The results were shown as ± SEM by taking the average of 3 experiments. *P < 0.05 for significant change compared with the CCh (-) group.

Effect of CCh on nuclear ERK expression

In cells treated with CCh for 5 min, nuclear ERK expression was increased by 14% compared to the control, but this increase was found to be 3% in cells treated with 4DAMP prior to CCh (Figure 3). On the other hand, in cells treated with CCh for 5 min, expression of nuclear pERK was reduced by 22% compared to the control, however this effect was not antagonized by 4DAMP (Figure 4).

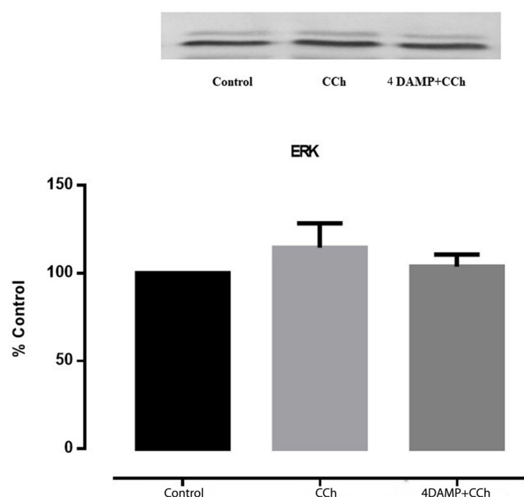

Figure 3. Effect of CCh on nuclear ERK expression. K562 cells were treated with 100 µM CCh for 5 min. Antagonist, 4DAMP (10 µm) was added 30 min prior to CCh. Control cells were not treated with CCh. Percent increase in ERK expression relative to control in cells. The results were shown as ± standard error (SD) by taking the average of 3 experiments.

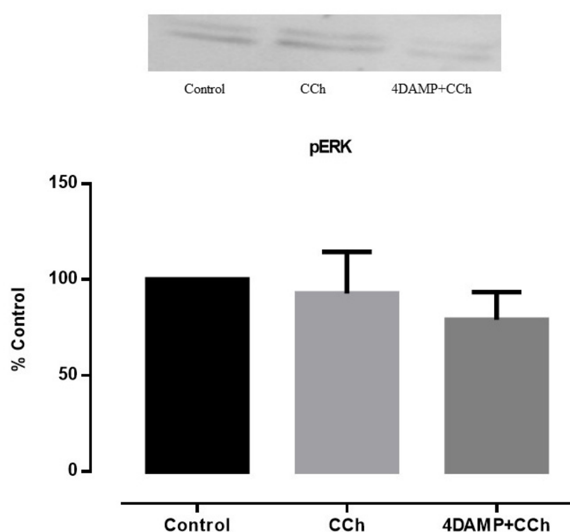

Figure 4. Effect of CCh on nuclear pERK expression. K562 cells were treated with 100 µM CCh for 5 min. Antagonist, 4DAMP (10 µm) was added 30 min prior to CCh. Control cells were not treated with CCh. Percent increase in pERK expression relative to control in cells. The results were shown as ± standard error (SD) by taking the average of 3 experiments.

Statistical analysis

All data were expressed as mean±SEM. Multiple t tests were used for the analysis of data. For all statistical calculations, significance was considered to be a value of $P<0.05$.

Discussion

Acetylcholine serves as a neurotransmitter both in the central and the peripheral nervous systems, where it controls functions including muscle contraction, neurotransmission among others. Recent studies demonstrating that ACh also regulates cell proliferation [19] and apoptosis [20] initiated research on the role of nAChRs and mAChRs in the development and progression of cancer and in stem cell physiology. Based on these studies, we asked whether the M_3R subtype may affect the proliferation of the K562 cell line.

Muscarinic receptor agonists stimulate cell proliferation, survival, migration, and invasion, as shown by *in vitro* studies using human colon cancer cells. These effects are regulated by complex mechanisms involving interacting post-M_3R signaling pathways which activate post-receptor signaling cascades [21]. Colon cancer cell proliferation is regulated by rapid, reversible activation of ERK1/2; whereas, cell survival and resistance to radiation is regulated by PI3K/AKT activation [21,22]. M_3R activation stimulates colon cancer growth in animal models relevant to human colon cancer [23]. Similarly, M_3R deficiency attenuates tumor formation [24,25]. Thus, M_3R expression and activation seem to play important roles in in the progression of colon neoplasia. [26].

Expression of mRNAs of muscarinic receptors (M_2, M_3 and M_4) has been shown in K562 cells by using reverse transcription polymerase chain reaction and immunoblotting, but the roles of muscarinic receptors have not been clarified yet [27,28]. There is previous evidence suggesting that muscarinic receptors can regulate cell proliferation depending on the growth context of the cell. Whether cells are in a quiescent state or growing appear to determine the type of effect. Our previous data showed that *in vitro* proliferation of K562 cells was thoroughly dependent on the presence of fetal bovine serum that contained various growth factors. Treating serum-deprived K562 cells with a cholinergic agonist, CCh, a choline ester, led to a significant increase in DNA synthesis, implying the roles of cholinergic receptors in cell growth [9]. On the other hand, CCh produced a decrease in DNA synthesis in

K562 cells supplemented with 1% fetal bovine serum after starvation. We also demonstrated that phospholipase C and intracellular calcium were involved in CCh-mediated inhibition of proliferation in K562 cells [29]. These findings lend support to the hypothesis that ACh or CCh can generate intracellular effects through their action on cholinergic receptors. Our previous study demonstrated that CCh also enhanced NO production in K562 cells [9, 28]. The current study showed that treatment with CCh for 48h decreased the cell number, indicating that CCh had a very fast and irreversible effect to promote cells to necrotic cell death. Supporting this hypothesis, decrease of cell number was not reversed by atropine treatment. Exposing K562 cells to 100 μM CCh for 24 hours decreased the number of apoptotic cells, possibly because of its promoting effect on necrosis. Treatment with 100 μM CCh for 48 hours increased the number of necrotic cells whereas apoptotic cells were decreased compared to control cells (Figure 2). The increase in the necrotic cell number in 48 hours appeared to be compatible with decrease in cell proliferation.

Kodaira and his colleagues show that in gastric cancer, ERK signaling is observed following muscarinic receptor activation. However, the failure of ERK signaling to stimulate gastric cancer cell proliferation raises questions regarding the importance of this observation [30]. Expression of muscarinic receptors, ChAT (choline acetyltransferase), and CrAT (carnitine acetyltransferase), and ACh production, have been reported in human leukemia cell lines [31,32]. Shah and his colleagues show that muscarinic receptors and ligands play a major role in cancer, and then suggest that these possible carcinogens are an additional matter to investigate and environmental effects need to be addressed [33]. CCh affects cell proliferation via cholinergic receptors through ERK signaling [34]. In our experiments we demonstrated that ERK expression increased overall in response to CCh treatment, however, phospho-ERK expression was reduced when compared to control (Figure 4). Therefore, we suggest that increase in ERK indicates a cellular survival reflex of K562 cells, whereas CCh reduces the phosphorylation of ERK, which would otherwise promote proliferation.

In conclusion, our results support the notion that the cholinergic agonist CCh may have roles in cell death and affect cell proliferation by activating not only muscarinic receptors but also other signaling pathways. Future studies addressing a possible role for nicotinic acetylcholine receptors merit investigation.

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