

Enhanced proliferation of SNU-407 human colon cancer cells by muscarinic acetylcholine receptors

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We investigated the expression of muscarinic acetylcholine receptors (mAChRs) and their possible involvement in the regulation of cell proliferation in four colon cancer cell lines (SNU-61, SNU-81, SNU-407, and SNU-1033) derived from Korean colon carcinoma patients. A ligand binding assay showed that all four cell lines expressed mAChRs. Treatment of the four cell lines with the cholinergic agonist carbachol led to the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2). In SNU-407 cells, carbachol significantly stimulated cell proliferation, which could be abolished by the muscarinic antagonist atropine and the ERK1/2 kinase inhibitor PD98059. These results indicate that mAChRs specifically mediate the proliferation of SNU-407 colon cancer cells via the ERK1/2 pathway. [BMB reports 2008; 41(11): 803-807]

INTRODUCTION

Colon cancer, one of the leading causes of cancer death, results from multiple genetic alterations. The sequential mutations of *adenomatous polyposis coli* (*APC*), *K-ras*, and *p53* genes are generally believed to be important in the initiation and progression of colon tumors. However, it was reported that only 6.6% of colon tumors contain mutations in all three genes and suggested that diverse genetic pathways exist for colon carcinogenesis (1). Thus, studies on various colon cancer cell lines with different genetic backgrounds are necessary to better understand the molecular mechanism of colon carcinogenesis.

Muscarinic acetylcholine receptors (mAChRs) mediate various nervous functions (2). Besides these functions, mAChRs have been implicated in cell growth regulation in many cell types (3-6). Among the five subtypes of mAChRs that have been identified (M1-M5) (7, 8), M1 and M3, which couple to phospholipase C activation, effectively induce cell proliferation (9).

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Received 11 July 2008, Accepted 12 September 2008

Keywords: Cell proliferation, Colon cancer cell line, ERK1/2, Muscarinic acetylcholine receptors

Many human colon cancer cell lines express mAChRs (3, 10, 11) and M3 mAChRs are overexpressed in 60% of colon cancers examined (12), implying the importance of mAChRs in colon carcinogenesis. In fact, mAChRs stimulate the proliferation of NCI-H508 (13) and T84 (14) colon cancer cells. In this work, we report that mAChRs enhance SNU-407 colon cancer cell proliferation.

RESULTS AND DISCUSSION

A ligand binding assay was utilized to examine the expression of mAChRs in the SNU-61, SNU-81, SNU-407, and SNU-1033 colon cancer cell lines. The assay used 0.2 nM [³H]NMS, which falls within the range of reported values of K_d for M3 mAChRs in colon cancer cells (3, 11). As shown in Table 1, cells from all four cell lines bound [³H]NMS and the receptor levels were calculated to be 4-20 fmol/mg protein.

The ERK1/2 pathway has been implicated in regulating cell proliferation in various cell types (16), including NCI-H508 and T84 colon cancer cells (13, 14). We thus investigated whether mAChRs activate ERK1/2 in the four colon cancer cell lines. As shown in Fig. 1, a 5 min exposure to 1 mM carbachol stimulated ERK1/2 activation in the four cell lines. This carbachol-stimulated ERK1/2 activation was significantly inhibited by the muscarinic antagonist atropine at a concentration of 10 μM, consistent with the response being specifically mediated by mAChRs. ERK1/2 activation by mAChRs was strong in SNU-81 and SNU-407, intermediate in SNU-1033 cells, and relatively weak in SNU-61 cells. Since SNU-61 cells express a low level of mAChRs, the results support the suggestion that

Table 1. Expression of mAChRs in colon cancer cell lines

Cell line ^a	[³ H]NMS binding (fmol/mg protein) ^b	Origin
SNU-61	4.0 ± 0.6	Rectum
SNU-81	20.2 ± 2.0	Colon
SNU-407	12.9 ± 2.0	Colon
SNU-1033	18.4 ± 3.3	Rectum

^a Characteristics of each cell line have been described (15).

^b Ligand binding assay was performed using 0.2 nM [³H]NMS. Values are mean ± SEM of four to seven separate experiments.

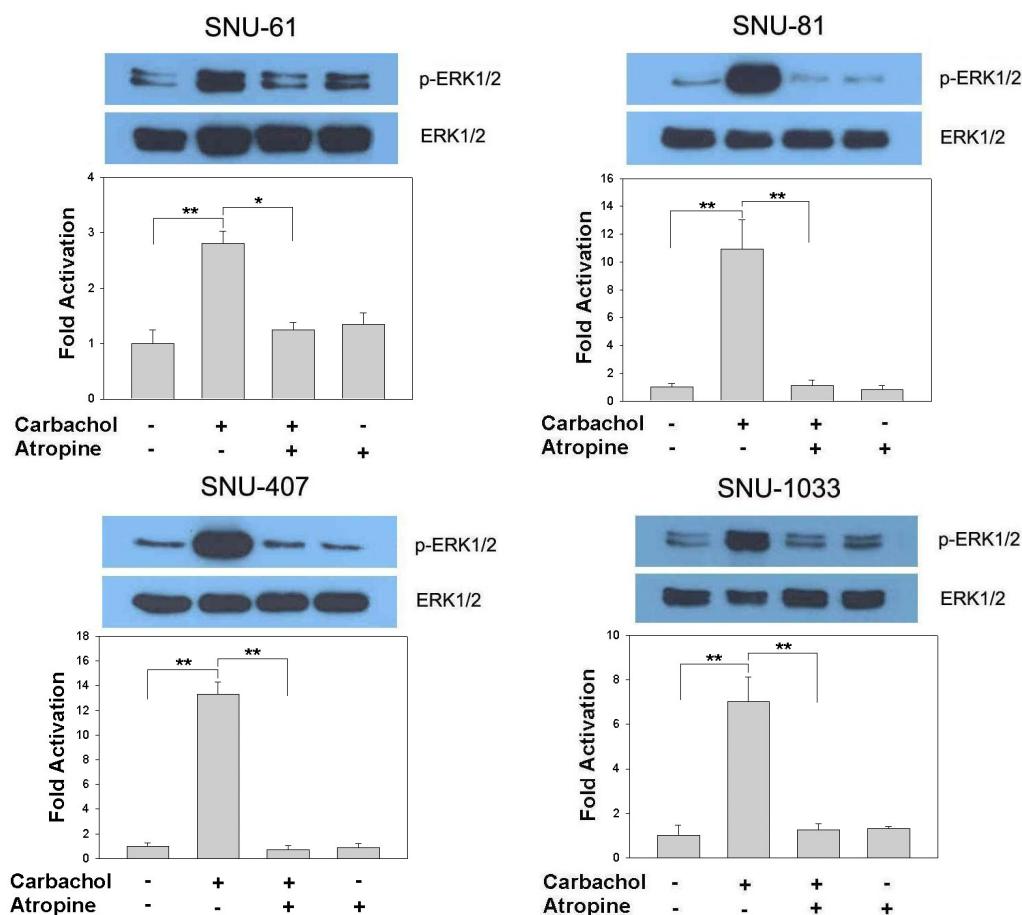


Fig. 1. mAChRs mediate ERK1/2 activation in colon cancer cell lines. Cells were serum-starved for 18-24 h and treated with 1 mM carbachol for 5 min in the absence or presence of 10 μ M atropine. Cell lysates were immunoblotted with anti-phospho-ERK1/2 antibody and anti-ERK1/2 antibody for activated ERK1/2 (p-ERK1/2) and total ERK1/2 (ERK1/2), respectively. A representative Western blot is shown in the upper panel and data for ERK1/2 activation (expressed as fold activation compared with untreated control, mean \pm SEM) from three to five separate experiments are shown in the lower panel. * $P < 0.05$ and ** $P < 0.01$ by paired *t*-test.

the magnitude of ERK1/2 activation may partly depend on the expression level of the receptors.

We next examined whether mAChRs mediated cell proliferation in the four colon cancer cell lines using a well-established assay of enzyme activity in viable cells that detects the colorimetric conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan product. Treatment of SNU-407 cells with 1 mM carbachol for 48 h resulted in an approximately 36% increase in cell proliferation (Fig. 2A). This effect was almost completely blocked by pretreatment of the cells with atropine, indicating the involvement of mAChRs in cell proliferation (Fig. 2B). SNU-81 cells showed a small but consistent increase in cell proliferation. However, the effect was so weak that we did not analyze this cell line any further. On the other hand, SNU-61 and SNU-1033 cells exhibited little, if any, change in cell proliferation in response to carbachol.

To determine whether mAChR-mediated SNU-407 cell proliferation occurred via ERK1/2 activation, we tested the effect of PD98059, an ERK1/2 kinase inhibitor, on carbachol-stimulated ERK1/2 activation and cell proliferation. A 30 min exposure to 20 μ M PD98059 abolished ERK1/2 activation (Fig. 3A) and cell proliferation (Fig. 3B) suggesting that mAChRs mediate cell proliferation through the ERK1/2 pathway.

SNU-407 cells treated with PD98059 alone exhibited lower ERK1/2 activity and cell proliferation than untreated control cells. SNU-407 cells contain a mutation in the *K-ras* gene (15), which leads to constitutive ERK1/2 activation and cell proliferation. Thus, it is likely that PD98059 treatment blocked the basal level of ERK1/2 activation and cell proliferation to some extent by interfering with the K-Ras-ERK1/2 pathway.

Although mAChRs stimulated ERK1/2 activation in the four colon cancer cell lines, cell proliferation was evident only in SNU-407 cells, which showed relatively strong ERK1/2 activa-

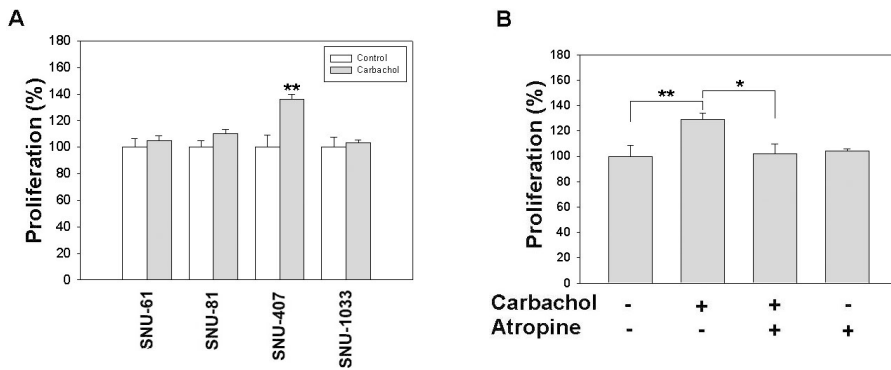


Fig. 2. mAChRs stimulate SNU-407 colon cancer cell proliferation. (A) Cells were serum-starved for 18-24 h and treated with 1 mM carbachol for 48 h. (B) SNU-407 cells were serum-starved for 18-24 h and treated with 1 mM carbachol for 48 h in the absence or presence of 10 μ M atropine. Cell proliferation was determined using the MTT assay. Data from four to seven separate experiments are expressed as a percentage of untreated control cells (mean \pm SEM). * $P < 0.05$ and ** $P < 0.01$ by paired t-test.

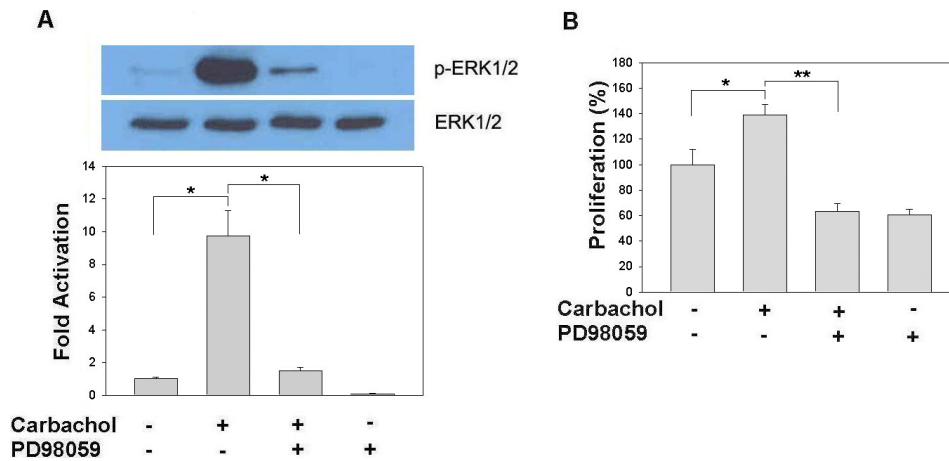


Fig. 3. mAChR-mediated SNU-407 colon cancer cell proliferation depends on ERK1/2 activation. (A) Cells were serum-starved for 18-24 h and treated with 1 mM carbachol for 5 min in the absence or presence of 20 μ M PD98059. Cell lysates were immunoblotted with anti-phospho-ERK1/2 antibody and anti-ERK1/2 antibody for activated ERK1/2 (p-ERK1/2) and total ERK1/2 (ERK1/2), respectively. A representative Western blot is shown in the upper panel and data for ERK1/2 activation (expressed as fold activation compared with untreated control, mean \pm SEM) from three separate experiments are shown in the lower panel. (B) Cells were serum-starved for 18-24 h and treated with 1 mM carbachol for 48 h in the absence or presence of 20 μ M PD98059. Cell proliferation was determined using the MTT assay and data from four separate experiments are expressed as a percentage of untreated control cells (mean \pm SEM). * $P < 0.05$ and ** $P < 0.01$ by paired t-test.

tion. These results imply that the magnitude of ERK1/2 activation is critical for cell proliferation. Previous studies have shown that the duration as well as the magnitude of ERK1/2 activation is important for regulating cell proliferation (17, 18). Consistent with this view, we observed that mAChR-induced ERK1/2 activation reached a maximum at 5 min and declined to a lower level that was sustained for at least 4 h in SNU-407 cells (data not shown).

While the molecular mechanism underlying mAChR-mediated proliferation of colon cancer cells remains to be elucidated, it is likely that the different genetic background of each cell line is the basis for cell line-specific proliferation by mAChRs. In fact, the four colon cancer cell lines used in this study possess distinct genetic alterations (15). For example, SNU-61 and SNU-1033 cells contain mutations in the *APC*, *p53*, and *K-ras* genes, whereas SNU-81 and SNU-407 cells

contain a mutation in the *APC* gene and the *K-ras* gene, respectively. SNU-407 cells have additional mutations in DNA repair (*hMSH2*) and *β -catenin* genes. Further studies are needed to investigate how these genetic changes might influence mAChR-mediated cell proliferation.

In summary, we have shown that the colon cancer cell lines SNU-61, SNU-81, SNU-407, and SNU-1033 express mAChRs that stimulate ERK1/2 activation, and that the expression of mAChRs enhances the proliferation of SNU-407 cells via the ERK1/2 pathway.

MATERIALS AND METHODS

Cell culture

Colon cancer cell lines (SNU-61, SNU-81, SNU-407, and SNU-1033) were obtained from the Korean Cell Line Bank

(Seoul, Korea). The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B) at 37 °C in an atmosphere of 5% CO₂.

Ligand binding assay

Cell homogenates prepared from each colon cancer cell line were incubated with 0.2 nM [³H]N-methyl scopolamine (82.0 Ci/mmol; New England Nuclear, Waltham, MA) at room temperature for 30 min. After the reaction mixtures were filtered through GF/C glass fiber filters (Whatman, Buckinghamshire, UK), the filters were dried, and the bound radioactivity was measured by liquid scintillation spectrophotometry. Nonspecific binding was determined in the presence of 10 µM atropine.

ERK1/2 assay

Colon cancer cells were grown in 12-well plates for 20-24 h, serum-starved for 18-24 h, and treated with 1 mM carbachol in serum-free RPMI 1640 for 5 min at 37°C. In some experiments, cells were pretreated with atropine or PD98059 for 5 or 30 min, respectively, prior to the carbachol treatment. The cells were washed twice with ice-cold phosphate buffered saline, lysed in lysis buffer (0.1 M KCl, 20 mM HEPES (pH 7.0), 10 mM EDTA, 0.3 M NaCl, 0.1% Nonidet P-40) for 1 h at 4°C, and centrifuged at 15,000 g for 15 min at 4°C. Proteins in the supernatant were boiled for 5 min in sodium dodecyl sulfate sample buffer, separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Duplicate membranes were incubated for 1 h in blocking buffer (20 mM Tris-HCl (pH 7.6), 140 mM NaCl, 0.1% Tween-20, 5% non-fat dried milk) at room temperature and treated with anti-phospho-ERK1/2 monoclonal antibody or anti-ERK1/2 polyclonal antibody (Cell Signaling Technology, Danvers, MA) diluted 1:2,000 in blocking buffer overnight at 4°C. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) diluted 1:2,000 in blocking buffer for 1 h at room temperature and immunoreactive bands were visualized using the WEST-ZOL plus detection system (iNtRON, Seoul, Korea). The densities of the 44 kDa ERK1 and 42 kDa ERK2 bands were analyzed by densitometry. ERK1/2 phosphorylation was normalized by total ERK1/2.

Cell proliferation assay

Cell proliferation was monitored by the MTT assay. Cells were seeded in 96-well plates at a density of 1-2 × 10⁴ cells/well, allowed to grow overnight, and serum-starved for 18-24 h. The cells were treated with 1 mM carbachol for 48 h in 100 µl serum-free RPMI 1640. In some experiments, cells were pretreated for 30 min with atropine or PD98059 prior to the carbachol treatment. Following the treatment, 10 µl of MTT solution (5 mg/ml) was added to each well and the plates were incubated for 3 h at 37°C. After the medium was removed, the formazan crystals formed were solubilized in 100 µl dimethyl

sulfoxide and the absorbance at 540 nm was measured using a microplate reader (Molecular Devices, Sunnyvale, CA). Each assay was performed in triplicate.

Acknowledgments

This work was supported by the Korea Research Foundation grant (KRF-2005-005-J15001) funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) and by the research grant of the Chungbuk National University in 2007.

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