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Cholinergic receptor up-regulates COX-2 expression and prostaglandin E2 production in colon cancer cells

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The M3 muscarinic cholinergic receptor has important physiological functions on normal colonic cells. It is frequently expressed on human colon cancer cells and is biologically active. Although it is mitogenic in certain cell models, the importance of this receptor on colon carcinogenesis is unknown. In the present study we have determined expression of the M3 receptor on human colon cancer tissue compared with matched normal tissue and examined the downstream effect of receptor activation in the HT-29 human colon carcinoma cell line. Using reverse transcription–PCR, M3 receptor RNA expression was detected in all matched colon carcinoma and normal specimens from eight patients. Five of the eight (62%) patients showed an up to 8-fold greater level of M3 receptor expression in cancer compared with the matched normal tissue. Exposure of HT-29 cells to carbachol, a stable receptor agonist, results in a 10-fold increase in cyclooxygenase-2 (COX-2) protein. This induction of COX-2 protein was dose dependent and was inhibited by the cholinergic receptor antagonist N-methylscopolamine (NMS). Carbachol caused a dose-dependent increase in prostaglandin E2 (PGE2), the main product of cyclooxygenase activity. The maximum stimulatory effect (40-fold increase) was noted with 1 mM carbachol. The increase in PGE2 was completely abolished by NMS and by the COX-2 selective inhibitor NS398. This suggests that the M3 receptor mediates PGE2 production by a mechanism involving COX-2. As COX-2 and PGE2 are known promoters of gastrointestinal cancer, these data suggest that M3 receptor activation may facilitate progression of colon carcinoma, in part by a COX-2-mediated cellular mechanism.

Cyclooxygenases (COXs) are rate-limiting enzymes in the synthesis of prostaglandins (PGs) and thromboxanes, which are involved in numerous normal and pathophysiological events (1). At present, two distinct isoforms of COX have been described. COX-1 is constitutively expressed in many mammalian tissues and is thought to be important in the housekeeping function of PGs. In contrast, COX-2 is an early response gene that, like c-fos and c-jun, is rapidly induced in response to growth factors, cytokines, oncogenes, phorbol esters and carcinogens (2,3). Although COX-2 expression is usually suppressed in most tissues, various types of tumors and transformed cells constitutively overexpress COX-2 (4–6). A growing body of evidence suggests that COX-2 expression is associated with colon cancer formation and progression. A null mutation of COX-2 markedly reduces the number and size of intestinal polyps in ApcΔ716 (adenomatous polyposis coli) knockout mice, a model for human familial adenomatous polyposis (7). In addition, overexpression of COX-2 inhibits apoptosis and increases the invasive potential of malignant cells (8,9). Studies investigating the role of COX-2 in human disease have suggested that chronic activation of this enzyme results in colon cell pathology. An understanding of the factors affecting COX-2 expression would therefore provide insight into colon cancer etiology.

We have previously shown that the M3 subtype of the muscarinic cholinergic receptor is expressed on 60% of colon cancer cell lines analyzed (10). However, little is known about the effect that activation of this G-protein-coupled muscarinic cholinergic receptor has on colon cancer biology, progression or formation. M3 receptor activation is coupled to the phospholipase C (PLC) signaling cascade (11,12) and the PLC-activated protein kinase C (PKC) pathway is a prototypic inducer of COX-2 (13,14).

We first established that M3 receptors are relevant on human colon cancers by determining that all normal colon and cancer tissues studied express the receptor. In 62% of patients the receptor is overexpressed on colon cancers. We therefore undertook an investigation to determine whether M3 receptor activation affects COX-2 expression and PGE2 synthesis in colon cancer cell line HT-29, which is known to express functional M3 receptors (11). We show that stimulation of the M3 receptor up-regulates COX-2 protein expression, which subsequently increases the release of PGE2. These findings suggest that activation of the M3 receptor has a role in colon carcinogenesis, in part through a COX-2 and PG pathway.

Colon cancer cell line HT-29 was obtained from the American Type Culture Collection and cultured in McCoy’s 5A medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. Cultures were maintained in a humidified atmosphere of 95% air, 5% CO2 at 37°C.

All colon carcinoma and matched paired normal tissues were obtained from patients undergoing surgical resection at the Fox Chase Cancer Center. This process was approved by the Institutional Review Board of the Fox Chase Cancer Center. Tissue samples were snap frozen in liquid nitrogen immediately upon surgical removal and maintained at −80°C until the tissue was utilized for RNA extraction. To extract RNA for reverse transcription–PCR (RT–PCR), frozen tissue was pulverized with a tissue gun on dry ice and total RNA was isolated with an RNA extraction kit (RNAzol; Gibco.
BRL, Gaithersburg, MD). First strand cDNA synthesis was performed on 1 µg total RNA using the random priming extension method. The reaction condition was as follows: 10 min at room temperature, 30 min at 42°C, 5 min at 95°C and 5 min at 4°C.

PCR primers were designed according to the published human cDNA sequences. The primer sequences were: M3 receptor (sense) 5′-CGAGACAGAGCATCTACTGTT-3′; M3 receptor (antisense) 5′-GACCAGGCATCTCTTCGCG-3′; β-actin (sense) 5′-GGAGCATGAGAAATCTGGG-3′; β-actin (antisense) 5′-GGAATGCAAATCATTGCT-3′.

The predicted size of the PCR products for each of the primer pairs was 376 (M3) and 171 (β-actin) bp. PCR was initiated by adding 2 µl of the RT reaction product into PCR reagent mix consisting of 20 mM Tris-KCl, 1.5 mM MgCl2, 0.5 µl of the RT reaction product into PCR reagent mix consisting of 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 0.5 µM each primer, 2.5 U Taq DNA polymerase. PCR cycle conditions included denaturation for 30 s at 95°C, annealing for 30 s at 65°C for M3 or 60°C for actin and extension for 30 s at 72°C. Each sample was subjected to PCR at multiple cDNA concentrations for 20–30 cycles to determine the range of linearity in amplification. The PCR products were compared at the same cDNA concentration and number of cycles within the linear range. Amplification of β-actin was used to normalize M3 expression for RT efficiency and RNA loading.

Cells were lysed for western blotting with RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris, pH 7.4, 100 µM sodium orthovanadate, 50 mM sodium fluoride) containing protease inhibitor (Complete; Boehringer Mannheim, Indianapolis, IN). Lysates were frozen at −80°C for 15 min and centrifuged at 10,000 g for 10 min to remove the particulate sediment material. Protein concentration in the supernatant was measured using the Bio-Rad protein determination protocol. Total cellular protein (50–60 µg) was separated by electrophoresis on a 7.5% SDS–polyacrylamide gel and electrotransferred onto PVDF membrane. Membranes were incubated with anti-human COX-2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI) followed by horseradish peroxidase-labeled secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The signal was revealed using a chemiluminescence detection system (Kirkegaard & Perry Laboratories).

To determine PGE2 production, cells (5 × 10⁵) were plated in 60 mm dishes and grown to 60% confluence. The culture medium was collected to determine the amount of PGE2 secreted by the cells. PGE2 production was determined by enzyme immunoassay (Cayman Chemical).

We determined the expression of M3 receptor RNA in normal human colon epithelium and in colon cancer tissue from the same patient. Colon cancers in various stages of progression were utilized, including Dukes stage B–D. M3 receptor RNA expression was detected in the cancer and matched normal specimens from all of the patients (Figure 1). Five of eight patients (62%) showed an up to 8-fold increase in M3 receptor expression in cancer tissue compared with the matched adjacent normal tissue. Three of these patients exhibited twice the receptor expression in tumors compared with normal tissue (1.8, 2 and 2; Figure 1) and two patients exhibited a >5-fold increase in receptor expression in tumor compared with normal tissue (5.6 and 8.2; Figure 1). Increased levels of M3 cholinergic receptor expression in colon cancer tissue suggests that the M3 receptor is important in colon cancer pathogenesis. This provided the basis for subsequent study of the role of the M3 receptor in human colon cancer.

HT-29 colon carcinoma cells contain a pure population of M3 receptors, which are coupled to the PLC signaling cascade (11). We used this well-defined model system to investigate the role of the M3 receptor in human colon cancer. As COX-2 is important in colon carcinogenesis and can be induced by a PLC-activated PKC pathway, we examined the effect of M3 receptor activation on COX-2 expression.

To determine the effect of M3 receptor activation on COX-2 protein expression, HT-29 cells were exposed to increasing concentrations of carbachol (0.1 µM–1 mM) for 6 h. These doses of carbachol have previously been shown to bind to the M3 receptor (10). No cellular toxicity was noted at these agonist concentrations. Carbachol increased COX-2 protein expression in a concentration-dependent manner (Figure 2A). An increase in COX-2 was detected at a dose of 0.1 µM carbachol and the maximal increase in COX-2 was obtained at 1 mM carbachol. The COX-2 response to carbachol was inhibited by the muscarinic cholinergic antagonist N-methyl-scopolamine (NMS) in a dose-dependent fashion (Figure 2B). Induction of COX-2 by carbachol was time dependent. Treatment of HT-29 cells with 1 mM carbachol resulted in an increase in COX-2 with a peak response (10-fold increase) detected at 3 h (Figure 2C).

PGE2 is the main product of COX activity. We therefore sought to determine whether induction of COX-2 protein is associated with an increase in the release of PGE2 by HT-29 cells. HT-29 cells were incubated with (1 mM) or without carbachol for the indicated period of time. Carbachol stimulation resulted in a 40-fold increase in PGE2 production after 3 h treatment (Figure 3). The level of increase gradually returned to baseline, but a 10-fold increase in PGE2 was still evident 24 h after treatment.

Carbachol-induced PGE2 production was dose dependent at...
Carbachol up-regulates COX-2 expression

Fig. 4. Dose-dependent increase in PGE\(_2\) production induced by carbachol. HT-29 cells were treated for 3 h with carbachol at concentrations ranging from 10 \(\mu\)M to 1 mM. The amount of PGE\(_2\) secreted into the culture medium was measured by enzyme immunoassay as described in Materials and methods. The data represent the means \pm SD of three experiments.

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Fig. 5. NMS and NS398 block carbachol-induced PGE\(_2\) production. HT-29 cells were treated with either NMS (1 \(\mu\)M) or NS398 (5 \(\mu\)M) in the presence of 1 mM carbachol for 3 h. The amount of PGE\(_2\) secreted into the culture medium was measured by enzyme immunoassay as described in Materials and methods. The data represent the means \pm SD of three experiments.

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concentrations ranging from 10 \(\mu\)M to 1 mM (Figure 4). A 10-fold increase was noted at a concentration of 10 \(\mu\)M and the maximum stimulatory effect (40-fold) was reached at a concentration of 1 mM. Carbachol-induced PGE\(_2\) production was completely abolished by NMS (1 \(\mu\)M), a competitive receptor antagonist (Figure 5). In addition, the COX-2-specific inhibitor NS398 completely inhibited PGE\(_2\) production at a concentration known not to inhibit COX-1 (Figure 5). This suggests that the carbachol-stimulated increase in PGE\(_2\) production is a result of M\(_3\) receptor–ligand binding which mediates its effect through COX-2 expression.

We have previously shown that the human colon cancer cell line NCI-H508 contains functional M\(_3\) receptors and that ligand binding of the receptor leads to an increase in cell growth (10,15). Cholinergic receptor activation has also been shown to increase tumorigenic potential of several cell models (16–19). To determine whether COX-2 is involved in carbachol-induced cell growth in NCI-H508 cells, we first studied the effect of carbachol on COX-2 expression in these cells as low levels of COX-2 RNA are detected using RT–PCR. Treatment of NCI-H508 cells with carbachol did not result in induction of COX-2 (data not shown). Although carbachol elicits a mitogenic effect in NCI-H508 cells independent of COX-2 induction, it does not stimulate the growth of HT-29 cells in which COX-2 levels are enhanced by receptor activation (data not shown). These data suggest that carbachol-induced COX-2 expression is not necessary for human colon cancer growth and that it is not sufficient to induce colon cancer growth.

The gastrointestinal tract is the largest endocrine organ in the body. Physiological growth and function are regulated by
peptide growth hormones and neurotransmitters. Most of these biological regulators bind to specific receptors belonging to a superfamily of seven transmembrane domain G-protein-coupled receptors. It is well established that the colon is innervated by cholinergic nerve endings and is constantly exposed to the neurotransmitter acetylcholine (20). We have previously shown that human colon cancer cell lines frequently possess functional muscarinic cholinergic receptors of the M2 subtype (10). The naturally occurring ligand for this receptor is the neurotransmitter acetylcholine. In the present study we have demonstrated that the M3 receptor is expressed in all human colon tissues analyzed and that the receptor is frequently overexpressed on cancer tissue compared with normal adjacent tissue. Previous study has shown that M3 receptor activation can induce neoplastic transformation (16) and changes in adhesion activity in some cell systems (17). It is possible that M3 receptor expression confers an advantage to human colon cancer cells by decreasing apoptosis or facilitating metastatic spread by affecting tissue adhesion.

We show that activation of the M3 receptor by carbachol causes a significant increase in COX-2 protein and its downstream product PGE2. As is the case for other immediate early genes, induction of COX-2 is detected as early as 30 min after carbachol treatment. Tsuji et al. found that COX-2-expressing Caco-2 cells have identical growth rates when compared with control cells (9). The described difference in growth effects and in COX-2 inducibility between colon cancer cell lines in response to M3 receptor activation is not yet clear. Given the heterogeneous nature of colonic neoplasia (21), carbachol may elicit distinct responses in colon cancer cell signaling based on the genetic background of the individual cancer. A recent study has suggested that constitutive overexpression of COX-2 resulted from the absence of wild-type p53 (22). It is therefore possible that cholinergic induction of COX-2 in colon cancer cells may only occur in cells lacking p53. This notion is supported by the fact that HT-29 cells do not express functional p53 (23,24).

Increased expression of COX-2 and its major downstream product PGE2 play an important role in the pathogenesis of colon cancer by a number of mechanisms (7–9): COX-2 is involved in activation and formation of carcinogens (25); overexpression of COX-2 inhibits apoptosis (8), increases the production of angiogenic factors (26) and enhances the metastatic potential of colon cancer cells (9); elimination of COX-2 activity, either through gene knockout or by selective inhibitor, reduces the number and size of intestinal polyps in ApcΔ716 knockout mice (7). We observed a profound and sustained increase in COX-2 protein and PGE2 induced by carbachol in HT-29 human colon cancer cells. As COX-2 overexpression has been shown to increase the metastatic potential of colon cancer cells, the results of this study suggest that M3 receptor activation may increase the metastatic potential of colon cancer by enhancing COX-2 activity. The present results, together with the previously shown growth-stimulating effect of carbachol on NCI-H508 cells (15), suggest that M3 receptor stimulation may result in distinct biological effects on individual colon cancers. The results of these studies lead to the notion that cholinergic activation in colon cancer cells favors the progression of colon cancer.

An important aspect of this study is the demonstration that in addition to the growth factor receptors possessing intrinsic tyrosine kinase activity, the G-protein-coupled receptors may also play a role in the pathogenesis of colon cancer. This is the first time, to our knowledge, that the mechanism by which a G-protein-coupled cholinergic receptor involved in colon carcinogenesis has been delineated.

In conclusion, we demonstrate that M3 receptors are expressed in all human colon tissues analyzed and are frequently overexpressed in human colon cancer compared with normal colon tissue. Activation of this M3 receptor up-regulates COX-2 and results in PGE2 production. This suggests that M3 receptors may contribute to the malignant progression of human colon carcinoma and warrants further study.

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References

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