Neuropeptide Y Y5 Receptor Promotes Cell Growth through Extracellular Signal-Regulated Kinase Signaling and Cyclic AMP Inhibition in a Human Breast Cancer Cell Line

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Abstract

Overexpression of neuropeptide Y (NPY) and its receptor system has been reported in various types of cancers. NPY Y5 receptor (Y5R) has been implicated in cell growth and angiogenesis. However, the role of Y5R in breast cancer is unknown. To identify the role of Y5R in breast cancer, we screened several breast cancer cell lines to examine the expression of Y5R and its function in breast cancer. All screened cell lines express both Y1 receptor and Y5R except BT-549, which expresses mainly Y5R. Binding studies showed that NPY, Y5R-selective agonist peptide, and Y5R-selective antagonist (CGP71683A) displaced 125I-PYY binding in BT-549 cell membranes in a dose-dependent manner. The displacement studies revealed the presence of two binding sites in Y5R with IC50 values of 29 pmol/L and 531 nmol/L. NPY inhibited forskolin-stimulated cyclic AMP accumulation with an IC50 value of 52 pmol/L. NPY treatment of BT-549 cells induced extracellular signal-regulated kinase phosphorylation but did not alter intracellular calcium. Y5R activation stimulates BT-549 cell growth, which is inhibited by CGP71683A, pertussis toxin, and extracellular signal-regulated kinase blockade. CGP71683A alone induced cell death in a time- and dose-dependent manner in Y5R-expressing cells. The stimulation of MDA MB-231 cell migration by NPY is inhibited by CGP71683A. Together, our results suggest that Y5R plays an important role in cancer cell growth and migration and could be a novel therapeutic target for breast cancer.

Introduction

Neuropeptide Y (NPY) is a 36–amino acid peptide amidated at the COOH terminus. Structural studies suggest that NPY belongs to the pancreatic polypeptide (PP) family and exhibits 70% amino acid sequence homology with peptide YY (PYY) and 50% to PP. NPY is produced by the neurons and vascular endothelial cells (1, 2). NPY mRNA translates into 98–amino acid prepropeptide. Proteolytic processing of the NPY precursor peptide results in a 28–amino acid signal peptide, a 36–amino acid functional NPY, and a carboxy 30–amino acid peptide (reviewed in ref. 1). NPY is the most abundant neuropeptide reported to date in the mammalian brain. In the periphery, NPY is costored and coreleased with norepinephrine in the sympathetic nerve endings (1). NPY exerts potent biological effects on many target areas in the brain and in the periphery. NPY plays an important role in the regulation of cardiovascular system, lung function, feeding behavior, angiogenesis, and the release of hypothalamic and pituitary hormones (1-5). NPY actions are mediated by at least six different types of receptors (Y1R-Y6R) that are coupled to G proteins. Among the six NPY receptor subtypes, Y3R subtype has not been cloned; Y4R subtype belongs to PP; and Y6R is a nonfunctional receptor in rat and human (1). Therefore, it seems that Y1R, Y2R, and Y5R are the three major subtypes of NPY receptors that mediate the biological functions of NPY and PYY in human and rat. Interestingly, these three major NPY receptor subtypes are localized to human chromosome 4q31-q32 region (6, 7).

The Y1R subtype was the first NPY receptor subtype to be cloned and characterized (8). Activation of Y1R subtype requires entire NPY or PYY molecule (9). Y1R mediates NPY-induced appetite signaling, vasoconstriction, and proliferation of neuronal precursor cells and vascular smooth muscle cells (9-12). Expression of Y1R mRNA has been reported in neuroblastoma cell lines (13, 14). Activation of Y1R in the SK-N-MC cell line significantly reduced the cell proliferation in vitro and in vivo (14). Recent findings suggest that normal breast tissue expresses Y2R subtype, whereas 85% of human breast carcinoma expresses Y1R subtype (15). PYY treatment has been shown to inhibit the growth of human MCF-7 breast cancer in vitro and in vivo (16).
Our recent findings showed that human breast cancer cell line MCF-7 expresses functional Y1R, which mobilizes intracellular Ca\(^{2+}\) and decreases cyclic AMP (cAMP) accumulation in response to NPY stimulation (17). Further, our studies showed that the activation of Y1R attenuates estrogen-induced cell proliferation in MCF-7 (17).

The Y2R subtype has been shown to mediate several biological functions of NPY. Y2R subtype can be activated by NPY, PYY, or COOH-terminal fragment of these peptides such as NPY 3-36 or PYY 3-36. Activation of Y2R leads to the inhibition of neurotransmitter release (18). In addition, studies have shown that Y2R promotes angiogenesis by stimulating proliferation, migration, and capillary tube formation in endothelial cells (2) and high level expression of Y2R subtype has been reported in glioblastoma and neuroblastoma tumors (19, 20). An earlier study reported that the activation of Y2R subtype by NPY led to an increase in tumor vascularization in neuroblastoma and Ewing’s sarcoma (14).

Studies have shown that Y5R plays an important role in the hypothalamic regulation of feeding (21). Similar to Y2R, human Y5R can be activated by NPY, PYY, NPY 3-36, and PYY 3-36. The pharmacologic distinction between these two receptors is that Y5R has higher affinity for human PP (1, 21). The findings from Herzog et al. (7) suggest that the human Y1R and Y5R are in close proximity and transcribed in opposite directions from a common promoter region on chromosome 4q31-q32.

Previous studies showed that Y5R is the primary NPY receptor subtype in mouse cardiac myocytes and its activation leads to increased PKC activity and to mitogen-activated protein kinase phosphorylation and activity (22). These signaling pathways have been implicated in NPY-induced cardiac hypertrophy through Y5R activation (22). In neuroblastoma cell lines, NPY acts as an autocrine growth factor by activating Y2R and Y5R (14). In these cell lines, activation of Y5R increases extracellular signal-regulated kinase (ERK)1/2 activity and cell proliferation, whereas its inhibition attenuates cell growth (14). However, whether Y5R plays a role in the control of growth, proliferation, and/or migration of breast cancer cells is still unknown.

The objective of the present studies was to determine whether Y5R is expressed in human breast cancer cell lines and whether it regulates cell proliferation and cell migration. Toward this goal, we screened six different human breast carcinoma cell lines using PCR method. Most of the cells showed coexpression of Y5R and Y1R, except for BT-549 cell line, which expressed mainly Y5R. In BT-549 cell line, Y5R mRNA translates into functional protein and its activation inhibits cAMP accumulation, increases ERK phosphorylation, and promotes cell growth, whereas its inhibition promotes cell death.

Materials and Methods

Materials

BT-549 and HS-578T cells were obtained from Dr. S. Waltz (Department of Surgery, University of Cincinnati, Cincinnati, OH). T47D, MDA MB-231, and MDA MB-468 cells were obtained from Drs. S. Khan and E.M. Bahassi (Department of Anatomy and Cell Biology, University of Cincinnati, Cincinnati, OH). MCF-7 was purchased from the American Type Culture Collection. Porcine NPY, BIBP 3226 was obtained from the American Peptide Company, Inc. Y5R-selective agonist peptide (Y5R-SAP; cPP1-7, NPY 19-23, Ala31, Aib32, and Gln34)-human PP and Y5R-selective antagonist CGP71683A (CGP) were obtained from Tocris Biosciences. 125I-PYY and 125I-cAMP RIA kits were obtained from Perkin-Elmer Life Sciences, Inc. Goat anti-human Y5R antibody was purchased from Everest Biotech. Horseradish peroxidase–conjugated anti-goat antibody and radioimmunoprecipitation assay buffer were obtained from Santa Cruz Biotechnology, Inc. NPY, Y1R, Y2R, and Y5R primers used in real-time PCR were obtained from Super Array, Inc. All other chemicals and reagents were the highest grade available and were obtained from Sigma.

Cell Culture

BT-549, MDA MB-231, and MDA MB-468 cells were grown in high-glucose DMEM-F12 medium, whereas MCF-7, T47D, and HS578T cells were grown in DMEM high-glucose medium. Both media were supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin/fungizone. Cells were subcultured at 90% confluency.

Real-time Reverse Transcription-PCR

Total RNA was isolated using Tri reagent as previously described (23). Five micrograms of total RNA were used for cDNA synthesis using oligo (dT), SuperScript reverse transcriptase, and RNase inhibitor (Invitrogen Life Technologies). Two microliters of cDNA were used to examine the expression of NPY, Y1R, Y2R, and Y5R primers used in real-time PCR were obtained from Super Array, Inc. All other chemicals and reagents were the highest grade available and were obtained from Sigma.

Cellular Protein Isolation and Immunoblotting

BT-549, MCF-7, T47D, and MDA-MB468 cells were grown to 90% confluency in growth media. Cells were scraped in radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors, and the suspension was passed through a 1-ml syringe with a 21-gauge needle thrice. The resulting material was centrifuged for 15 min at 10,000 × g at 4°C. The protein...
concentration of the supernatant was then determined using a bicinchoninic acid (BCA) kit from Pierce (Pierce). The protein samples were mixed with Laemmli buffer and stored at -20°C. BT-549, and other breast cancer cell proteins were separated by SDS-PAGE 4% to 20% in Criterion gradient gel (Bio-Rad). The proteins were transferred to polyvinylidene difluoride membranes and blocked in 5% dry milk in PBS supplemented with 0.2% Tween 20. Membranes were then probed with goat anti-Y5R antibody (1:2,000) overnight at 4°C. After three washes in PBS supplemented with 0.2% Tween 20 buffer, membranes were incubated with secondary antibody (1:3,000) for 1 h at room temperature. Enhanced chemiluminescence detection (GE Health Care) system was used to detect Y5R protein. To ensure equal loading of the proteins between groups, membranes were probed with anti-β-actin antibody and the intensity of Y5R signal was normalized with the β-actin signal.

Receptor Binding Studies in BT-549 Cells
Crude membrane fractions were isolated from BT-549 cells as previously described (25, 26). Membrane fractions were incubated in the binding buffer as described in our previous report (17). In brief, 50 to 75 μg membrane protein were incubated in a final volume of 0.25 mL of HEPES buffer [50 mmol/L HEPES (pH 7.3), 2.5 mmol/L CaCl2, 1 mmol/L MgCl2, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 0.1% bovine serum albumin (BSA)] containing 40 pmol/L of 125I-PYY and unlabeled peptides or compounds as needed and incubated at room temperature at various time points. We chose to use 125I-PYY as radioactive ligand for the detection of Y5R because PYY and NPY bind with equal potency to Y1R, Y2R, and Y5R subtypes. Moreover, 125I-PYY exhibits minimal nonspecific binding compared with 125I-NPY (17). In standardized competition binding studies, a time dependency was determined and 2-h incubation at room temperature was found to be optimal. Assay was terminated by adding 1 mL of ice-cold PBS in each tube and centrifuged for 15 min at 10,000 × g at 4°C. Unbound ligands were separated by aspirating the supernatant. Nonspecific binding was determined in the presence of 10 μmol/L PYY. 125I-PYY bound to the membrane fraction was determined by counting the radioactivity in a Packard γ counter for 1 min.

cAMP Accumulation Studies in BT-549 Cells
cAMP accumulation was determined in BT-549 cells as we previously described for MCF-7 cells (17). In brief, cells were grown in 24-well plates for 2 to 3 d. At 80% confluency, cells were incubated with serum-free DMEM-F12 medium containing 0.1% BSA, and 5 mmol/L theophylline for 1 h at 37°C. The cells were then incubated with forskolin (5 μmol/L) in the presence or absence of varying concentrations of NPY or other compounds for 30 min. The assay was terminated by aspirating the medium and lysing the cells with 50 mmol/L acetic acid buffer containing 2% Triton X-100. Lysate was centrifuged at 1,250 × g for 15 min at 4°C and the supernatant was used for cAMP measurement. The protein content of the samples was determined using a bicinchoninic acid kit (Pierce) and was used to normalize for cAMP levels.

Intracellular Ca2+ Mobilization in BT-549 Cells
Intracellular Ca2+ mobilization in BT-549 cells was determined using a Ca2+-sensitive fluorescent probe (fura-2AM) as described in our laboratory (17). Briefly, the emission fluorescence intensity was recorded at 495 nm and the fluorescence ratio at excitation wavelength of 340 and 380 nm was used to estimate the intracellular calcium. The influx of extracellular calcium into BT-549 cells was determined by injecting ionomycin (10 μmol/L) into the cuvette.

ERK Phosphorylation in BT-549 Cells
Cells were serum-starved for 3 h by incubating the cells in serum-free, 0.1% BSA–containing medium. Three hours later, the medium was renewed and incubated with 30 nmol/L NPY for 5 min. Cells were harvested and total protein was extracted and analyzed for phospho-ERK (pERK) expression using pERK-specific antibody (Cell Signaling Technology, Inc.) by Western blot.

Cell Growth Assay
BT-549 and MCF-7 cell growth was monitored using modified crystal violet assay as previously described (27). Briefly, BT-549 cells grown in 10% FBS containing DMEM-F12 medium cells were trypsinized and seeded (3,000 cells per well) in a 24-well plate. The next day, cells were switched to 0.25% charcoal-stripped FBS (CSS)–containing medium for 24 h. The following day, cells were treated with vehicle, NPY or Y5R-selective blocker (CGP) in 0.25% CSS-containing medium (28, 29). Two days after the treatment, cells were fixed in 4% paraformaldehyde for 20 min and stained in 0.25% crystal violet for 30 min. The cells were then washed under running tap water, air dried, and extracted with 2% triton X-100 for 30 min. The absorbance of the triton X-100 extract was determined at 570 nm using a microplate reader. In growth inhibition studies, 5,000 cells were seeded in 10% FBS–containing medium. Two days later, cells were switched to 0.25% CSS–containing medium and the treatment was continued every other day, and the cell growth determined for 4 to 10 d. CGP, pertussis toxin (PT) and ERK inhibitor (U0126) were added 15 min before the addition of NPY to determine their effects on NPY-induced cell growth.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay
Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay was performed on BT-549 cells treated with vehicle or CGP using the In-Situ Cell Death detection kit (Roche, Inc.) as previously described (30).
Quantitative Analysis of Apoptosis
We have used the Cell Death Detection ELISA assay kit for the quantitative determination of apoptosis in BT-549 cells (Roche Diagnostics). In this assay, 18 × 10^3 cells in 0.25% serum containing DMEM:F12 medium were seeded into a 24-well plate. The next day, cells were treated with vehicle (0.0015% DMSO) or increasing concentrations CGP (0.25-3 μmol/L) for 16 h. The DNA fragments generated in the cells were determined using anti-histone and anti-DNA-peroxidase antibodies as described in the supplier's protocol.

Cell Migration Assay
The migration of MDA MB-231 cells was determined in a transwell Boyden Chamber (Costar) containing a polycarbonate filter (8-μm pores) in the upper chamber (31). Cells were suspended in a 0.1% BSA–containing DMEM medium and treated with NPY, CGP, or both for 15 min and loaded onto the upper chamber (3 × 10^4 cells/well in 0.25 mL). Serum (10%) added to the lower chamber (0.5 mL) and incubated for 24 h. The cells on the top chamber were removed with cotton swab. The cells that migrated to the other side of the chamber were fixed in 4% paraformaldehyde and stained with crystal violet. The number of migrated cells in five different fields was counted using a phase-contrast microscope.

Statistical Analysis
Data are expressed as ± SEM. All results were analyzed for statistical significance by Student’s unpaired t test or one-way ANOVA followed by Tukey-Kramer multiple comparisons test for intergroup comparisons. The IC_{50} values for the binding, cAMP, and cell growth assays were determined by GraphPad Prism. A P value of <0.05 was considered statistically significant.

Results
Differential Expression of Y1R and Y5R mRNA in Human Breast Cancer Cell Lines
The results shown in Table 1 indicate that MCF-7 cells showed a higher level of Y1R mRNA whereas MDA MB-231 had the lower amount of Y1R. BT-549 cells showed the least amount of Y1R mRNA. All the other cell lines expressed varying Ct values for Y1R transcript. In contrast to Y1R, Y5R mRNA transcript level was higher and expressed in all breast cell lines tested in this study. NPY and Y2R mRNA were not detected in any of these cell lines (Table 1).

Y5R Protein Expression Levels in Human Breast Cancer Cell Lines
We next determined the abundance of Y5R protein in these breast cancer cell lines. Immunoblotting studies depicted in Fig. 1 indicate the presence of Y5R protein (~50 kDa) in BT-549, MCF-7, T47D, and MDA MB-468 cell lines (Fig. 1A). MDA MB-231 cells also exhibited significant levels of Y5R protein (data not shown). Normalization of the intensity of Y5R protein with β-actin shows that MCF-7 and T47D had a higher level of Y5R protein than BT-549 (P < 0.05; Fig. 1B).

Binding Studies of NPY and Y5R-SAP to Y5R in BT-549 Cells
We used ^125^I-PYY as a ligand for Y5R binding studies because this peptide shows lower nonspecific binding and has similar affinity to Y5R as NPY. Specific binding of ^125^I-PYY to BT-549 cell membranes was 60% to 66% of the total binding. As shown in Fig. 1C, the displacement data indicate the presence of two binding sites in Y5R protein in BT-549 cells. NPY binds to a high-affinity site with an IC_{50} value of 29 ± 2 pmol/L and to a low-affinity site with an IC_{50} value of 531 ± 47 nmol/L. Similarly, Y5R-SAP showed a high-affinity binding of 43 ± 1 pmol/L and a low-affinity binding of 2,100 ± 50 nmol/L to BT-549 cell membrane. Moreover, Y5R-selective antagonist also exhibited two binding sites with an IC_{50} value of 78 ± 7 pmol/L and 7,100 ± 1,100 nmol/L for high- and low-affinity sites, respectively. Y5R-selective blocker BIBP had no effect in displacing ^125^I-PYY bound to BT-549 cell membrane up to 10 μmol/L used in this study (Fig. 1C).

Y5R Signaling Pathways in BT-549 Cells
Effect of Y5R activation on cAMP accumulation in BT-549 cells. Forskolin treatment significantly elevated basal levels of cellular cAMP content in BT-549 cells (Fig. 1D). BIBP 3226, a Y1R blocker, had no significant effect on forskolin-induced cAMP accumulation. NPY treatment attenuated forskolin-induced increase in cell cAMP content, which is not affected by Y1R blockade (P < 0.01; Fig. 1D). Y5R blocker alone had no significant effect on forskolin-induced cAMP accumulation in BT-549 cells.

Table 1. Real-time RT-PCR of Y1R, Y2R, Y5R, and NPY in human breast cancer cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Target genes expression (Ct values)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Y1R</td>
</tr>
<tr>
<td>MCF-7</td>
<td>26.7 ± 0.4 &gt;36.0</td>
</tr>
<tr>
<td>T47D</td>
<td>30.1 ± 0.8 &gt;36.0</td>
</tr>
<tr>
<td>MDA MB-231</td>
<td>32.6 ± 0.4 &gt;36.0</td>
</tr>
<tr>
<td>MDA MB-468</td>
<td>30.0 ± 0.2 &gt;36.0</td>
</tr>
<tr>
<td>HS578T</td>
<td>29.2 ± 0.1 &gt;36.0</td>
</tr>
<tr>
<td>BT-549</td>
<td>&gt;36.0</td>
</tr>
</tbody>
</table>

NOTE: Equal amount of RNA was loaded in each well as determined by the GAPDH gene expression from all the samples. The Ct value for the target genes was determined by the software program built into the MX-3000p machine (Stratagene). There is an inverse relationship between Ct values and mRNA abundance. Samples with higher Ct values have lesser abundance of target mRNA. Ct values of >36 were considered as least detectable amounts of mRNA for the target genes.
effect on forskolin-induced cAMP accumulation. However, Y5R blocker completely antagonized the inhibitory effect of NPY on forskolin-induced cAMP accumulation in BT-549 cells (*P < 0.01; Fig. 1D).**

**Dose-response effect of NPY or Y5R-SAP on cAMP accumulation in BT-549 cells.** NPY inhibited forskolin-induced cAMP accumulation in a dose-dependent manner with an IC50 of 52 ± 4 pmol/L (Fig. 2A). Similarly, the treatment of BT-549 cell with Y5R-SAP caused a significant inhibition of forskolin-induced cAMP accumulation in a dose-dependent manner with an IC50 of 107 ± 19 pmol/L (Fig. 2B).

**Effect of NPY on intracellular calcium mobilization in BT-549 cells.** We next examined the effect of Y5R stimulation on intracellular calcium (Ca2+) mobilization in BT-549 cells. As shown in Fig. 2C, the addition of either NPY or Y5R-SAP did not affect intracellular Ca2+ levels, whereas addition of 1 μmol/L ionomycin (Ca2+ ionophore) caused a sharp increase in intracellular Ca2+ concentration as indicated by changes in the ratio of fura-2 fluorescence (F340/380; Fig. 2C).

**Effect of NPY on ERK1/2 phosphorylation in BT-549 cells.** It has been suggested that activation of Y5R promotes cardiac hypertrophy by activating ERK1/2 signaling pathways in cardiac myocytes (22). In the following experiments, we examined the time-dependent effects of NPY (30 nmol/L) on ERK phosphorylation in BT-549 cells. Cells were treated with NPY at various indicated time points. The results shown in Fig. 2D indicate that NPY treatment induced the phosphorylation of ERK 1/2 (42- to 44-kDa proteins) as early as 2.5 minutes and reached a peak at 5 minutes before returning to a lower level at 30 minutes after NPY addition (Fig. 2E).

**Role of Y5R in BT-549 Cell Growth**

**Effect of Y5R activation on cell growth.** We sought to determine the role of Y5R in cell growth and apoptosis in...
BT-549 cells. Toward this end, we tested the effect of NPY in the absence or presence of CGP on BT-549 cell growth. The results shown in Fig. 3A clearly indicate that NPY increased cell growth by 19% (\( P < 0.01 \)) at 30 pmol/L and by 25% (\( P < 0.001 \)) at 30 nmol/L (Fig. 3A). This effect is prevented by CGP (Fig. 3A). CGP alone had no effect on basal cell growth (Fig. 3A).

Role of ERK1/2 and cAMP signaling in Y5R-mediated cell growth. To investigate the roles of cAMP and ERK pathways in NPY-induced increased BT-549 cell growth, cells were pretreated with PT or U0126 or vehicle before the addition of NPY and cell growth was determined after 48 hours of treatment. As shown in Fig. 3B, cell growth was significantly stimulated by NPY (127 ± 3% versus vehicle 100 ± 5%; \( P < 0.01 \); Fig. 3B) but was not affected by PT or U0126 (\( P > 0.05 \) versus vehicle; Fig. 3B). However, NPY-induced cell growth was attenuated in the presence of U0126 (89 ± 8%; \( P < 0.001 \); Fig. 3B) or PT (100 ± 8; \( P < 0.01 \); Fig. 3B), compared with NPY alone.

Effects of CGP Alone on Cell Growth and Apoptosis

The rationale behind these studies was to determine whether pharmacologic inhibition of Y5R using CGP can be used to reduce breast cancer cell growth. As shown in Fig. 3B, the presence of CGP alone caused a significant inhibition of cell growth in a dose- and time-dependent manner (Fig. 3C). The IC50 values for the blocker for 48- and 96-hour treatments were 506 and 139 nmol/L, respectively. This effect is supported by the morphology of the cells depicted in Fig. 3D. In additional studies, the lower doses of CGP (10 and 100 nmol/L) did not affect BT-549 cell growth (data not shown).

To determine whether the apoptotic effect of CGP in BT-549 cells is due to nonspecific or toxic effects of the drug (32), we examined its effect on cell growth using another human epithelial cell line lacking Y5R expression (i.e., HEK-293 cells). The results shown in Fig. 4A indicate that CGP did not alter HEK cell growth at any of the doses tested for both 48- and 96-hour treatments (Fig. 4A).

FIGURE 2. Y5R signaling pathways in BT-549 cell line. A, effects of NPY on forskolin-induced cAMP accumulation in BT-549 cells. Cells were exposed to 5 μmol/L forskolin in the presence or absence of NPY for 30 min and then cellular content of cAMP was determined by RIA. **, \( P < 0.01 \); *, \( P < 0.05 \) versus the forskolin-treated group. B, same as A except that Y5R-SAP was used instead of NPY. *, \( P < 0.05 \) versus the forskolin-treated group. C, effects of NPY, or Y5R-SAP, on intracellular calcium mobilization in BT-549 cells. This is a representative figure of four different experiments. Fura-2-loaded cells were injected with either Y5R-SAP or NPY. Ionomycin was injected 5 min after the peptide injection. D, effect of NPY on ERK phosphorylation in BT-549 cells. Cells were serum starved for 3 h in serum-free 0.1% BSA medium and then exposed to 30 nmol/L NPY for various time points. Total protein was harvested from the cells and the phosphorylation of ERK protein was determined by Western blot using pERK-specific antibody (top). Protein loading (100 μg) was determined by reprobing the membrane with β-actin antibody (bottom). E, densitometric analysis of pERK (42 + 44 kDa) normalized to β-actin signal. Points, mean of four data points (n = 4); bars, SEM. Although no bands were seen at the 0’ time point in the figure, higher exposure time revealed the presence of two faint bands. We chose this optimal exposure time to show the differences in the PERK phosphorylation intensities between 5 and 30 min. **, \( P < 0.01 \); *, \( P < 0.05 \) compared with the 0 time point. F, effect of CGP on NPY-induced ERK phosphorylation in BT-549 cells. Experimental conditions were same as D, except that CGP (0.25 μmol/L) or 0.0125% DMSO (control) was added 15 min before the addition of NPY (30 nmol/L). Cells were exposed for additional 5 min after the addition of NPY. Total cellular protein was harvested and 100 μg proteins from each sample were analyzed for pERK and β-actin expression as described in D. Three determinations were used (n = 3) for each treatment.
We further tested whether CGP treatment affects apoptosis of BT-549 cells. Toward this end, we used terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay to determine the number of apoptotic cells and 4′,6′-diamidino-2-phenylindole staining was used to stain the nuclei. As shown in Fig. 4B, CGP treatment of cells for 16 hours increased apoptosis by 4- to 5-fold compared with vehicle treatment ($P < 0.05$).

Further, we tested the dose-response effect of CGP on apoptosis in BT-549 cells using quantitative apoptotic ELISA assay. As shown in Fig. 4C, neither the vehicle nor CGP (0.25 μmol/L) had any effect on apoptosis. However, further increase in CGP doses resulted in an increased apoptotic signal in BT-549 cells ($P < 0.05$; Fig. 4C). In addition to BT-549 cells, we also examined the effect of CGP on cell growth in MCF-7 cells in the same conditions as described above for BT-549 cells. The results indicate that MCF-7 cells exhibit a sharp and time-dependent increase in cell growth in the absence of CGP (vehicle). However, the presence of 1 μmol/L CGP completely blocked cell growth of MCF-7 cells (Fig. 4D). This effect is clearly shown by the changes in cell morphology depicted in Fig. 4E. We next examined whether CGP treatment has a toxic effect on MCF-7 cells. Toward this end, we tested the dose-response effect of CGP on MCF-7 cell growth by increasing the cell density. As shown in Fig. 4F, 0.25 μmol/L CGP had no effect on cell growth. However, further increase in CGP concentration attenuated...
serum-induced cell growth in a dose-dependent manner, both at 24 and 48 hours ($P < 0.01$; Fig. 4F).

**Effect of CGP on Cell Migration**

To examine the effect of CGP on cell migration, we used the MDA MB-231 cell line, which expresses Y5R (Table 1) and is known for its invasive and metastatic property (31). These cells were treated with NPY in the absence or presence of CGP and cell migration was measured 24 hours after treatment. As shown in Fig. 5, NPY treatment increased cell migration by $>2$-fold ($P < 0.001$) compared with vehicle. This effect is blocked in the presence of CGP (Fig. 5; $P < 0.01$) compared with vehicle. CGP alone had no effect on cell migration compared with vehicle (Fig. 5; $P > 0.05$).

**Discussion**

Our present study revealed that functional NPY Y5R subtype is expressed in several human breast carcinoma cell lines. There is a varying degree of Y5R mRNA expression in these cell lines. Based on the Ct values from the PCR study, it seems that the relative abundance of Y5R mRNA expression is high in MCF-7, modest in BT-549, and low in MDA MB-231 cell line. Although the significance of the differential expression of Y5R in human breast carcinoma...
The presence of Y5R both in estrogen receptor–positive and estrogen receptor–negative cell lines suggests that Y5R may play a role in both estrogen receptor–positive and estrogen receptor–negative human breast carcinoma.

The lack of NPY mRNA expression in these cell lines rules out the possibility of Y5R activation by autocrine mechanism. The displacement studies indicate that Y5R has two binding sites to NPY and Y5R-SAP, suggesting that this receptor binds its ligands with high and low affinity. In humans, the circulating levels of NPY (33), PYY 3-36 (34), and PP peptides (35) are within 5 to 19, 10, and 18 to 23 pmol/L, respectively. Interestingly, our data showed that activation of Y5R in BT-549 with NPY and Y5R-SAP inhibited forskolin-induced cAMP accumulation with an IC50 value of 52 + 4 and 107 + 19 pmol/L, respectively. This picomolar range of IC50 value represents the activation of Y5R through the binding of the ligand to the high-affinity site. The lower affinity binding site in Y5R is likely to be activated by the elevated levels of Y5R ligands under pathophysiologic conditions. However, the function and the signaling pathways associated with the activation of the low-affinity site in Y5R remain unknown. Nonetheless, our data clearly show for the first time that breast cancer cells express Y5R protein that can be activated by all of its circulating ligands (NPY, NPY 3-36, PYY, PYY 3-36, or PP). A modest but significant increase in cell growth induced by 30 pmol/L of NPY in the present findings suggest that circulating levels of NPY or PYY and high-affinity Y5R present in the breast epithelial cells are likely involved in the cell growth of breast carcinoma.

Analysis of signaling pathways in BT-549 cells revealed that Y5R activation is coupled to cAMP inhibition but not to intracellular Ca2+ signaling pathway. Previous studies have shown that activation of Y5R in transfected HEC-1B cells had no effect on intracellular Ca2+ mobilization but inhibited forskolin-induced cAMP accumulation (36). Further, activation of Y5R increases mitogen-activated protein kinase and protein kinase C activities in a calcium-independent manner in cardiac myocytes (22). In rat aortic smooth muscle cells, activation of Y5R had no effect on intracellular Ca2+ mobilization but increased cell growth (37). The lack of Ca2+ mobilization by Y5R in BT-549 cells is consistent with the above findings albeit in different cellular systems.

The effect of cAMP on breast epithelial cell growth is complex. There are reports indicating that cAMP promotes the growth of some cell types and inhibits the growth of others (reviewed in ref. 38). In normal breast epithelial cells, cAMP stimulates cell growth in vitro (39), whereas an inhibitory effect of cAMP has been shown in breast cancer cell lines as well as in primary culture (40). 8-Cl-cAMP, a cAMP analogue that selectively downregulates type 1 protein kinase A, has been reported to inhibit the growth of a wide variety of cancer cell types (41). The dose-dependent decrease in forskolin-induced cAMP accumulation correlates with an increase in cell growth in BT-549 cells treated with NPY. These data indicate that the growth-stimulating effect of Y5R activation is likely coupled to cAMP inhibition. This argument is supported by the findings from PT treatment to the cells. PT treatment of cells causes ADP ribosylation of α subunit of the inhibitory G protein (Gi) and prevents its interaction with G protein–coupled receptor (42). Our data showed that the presence of PT prevented NPY-induced cell growth, indicating that cAMP inhibition is required for NPY-induced cell proliferation in BT-549 cells. The role of other signaling pathways cannot be ruled out in Y5R-induced cell growth. An inhibitory effect of cAMP on ERK1/2 activity has been reported in several cell lines (43, 44). In contrast, Lowe et al. (38) have shown that forskolin (cAMP–elevating agent) had no effect on insulin-like growth factor I– or epidermal growth factor–induced ERK activity in a human breast cancer cell line. These findings indicate that the interaction between cAMP and ERK1/2 exists in some but not all cell types. However, whether the effect of Y5R activation on cell growth involves an interaction between cAMP and ERK1/2 in BT-549 cells is not known.

ERK is a member of the mitogen-activated protein kinase family and is involved in the regulation of cell growth (45, 46). Earlier studies have shown that activation of Y5R by NPY increased ERK1/2 activity in a neuroblastoma cell line (14). We therefore tested whether the activation of Y5R in BT-549 cells affects ERK1/2 phosphorylation. Our results showed that activation of Y5R with NPY increased the phosphorylation (activation) of ERK1/2 in BT-549 cells. Others have shown that Y5R stimulation leads to

![Graph](image_url)
the activation of the ERK pathway in a calcium-independent manner in cardiac myocytes (22). In the present study, we used the ERK inhibitor U0126 (47) to determine the role of ERK on Y5R-mediated cell growth. Our results showed that NPY-induced cell growth was attenuated in the presence of U0126, suggesting that the activation of ERK1/2 pathway by NPY is involved in BT-549 cell growth.

CGP is a nonpeptide Y5R antagonist and used as an antiobesity agent in rats (29). With regards to the effects of CGP on the growth/proliferation of cancer cells, our data showed that CGP significantly attenuates the growth of BT-549 and MCF-7 cells. Although this effect is common to both cell lines, BT-549 cells express mainly Y5R, whereas MCF-7 cells coexpress both Y1R and Y5R. In agreement with our findings, others have shown that CGP inhibited the NPY-induced proliferation of Y2R/Y5R-positive SK-N-BE2 neuroblastoma cell line (14). In contrast, CGP treatment reversed NPY-induced apoptosis and augmented the cell proliferation in Y1R/Y5R-positive Ewing’s sarcoma SK-N-MC cell line (14, 48). It is not clear at present whether these differences in CGP response are attributed to the coexpression of Y1R or Y2R with Y5R. To rule out the possibility of toxic or nonspecific effect of CGP on cell growth, we tested the effect of CGP on HEK-293 cells, a human kidney embryonic carcinoma epithelial cell line (32). This cell line is negative for Y5R expression (49). Consistently, CGP treatment had no effect on cell growth of HEK-293 cell line, indicating that the effect of CGP on BT-549 and MCF-7 cell growth is rather mediated through Y5R blockade.

With regard to the relevance of Y5R to breast cancer, our previous studies showed that the activation of Y1R inhibited cell proliferation in breast cancer cells (MCF-7) and that its expression is upregulated in response to estrogen (17). Others have shown that normal breast tissues expressed Y2R, whereas neoplastic breast tissues exhibited high levels of Y1R (15). In our present study, Y2R mRNA was not detected in any of the cancer cell lines tested. This finding is consistent with other studies showing that Y2R expression is low or absent, whereas Y1R expression is induced or upregulated in breast cancer tissues (15). Based on current and published data (15, 17), we propose that the induction of Y1R in neoplastic breast tissues is an adaptive response to tumor formation in which the activation of Y1R attenuates cancer cell growth and proliferation. However, this protective function is prevented in vivo by the action of a serine protease enzyme, DPP IV, which is expressed in lymphocytes, endothelial, and epithelial cells and cleaves the circulating Y1R ligands (NPY and PYY) into Y2R/Y5R-selective ligands (NPY 3-36 and PYY 3-36). NPY 3-36 and PYY 3-36 can promote cell proliferation, tumor growth, and migration through the activation of Y5R (2, 14, 50). These cleaved peptides can equally activate Y2R present in the endothelial cells of the tumoral blood vessels and promote angiogenesis (14). These events are expected to facilitate breast tumor growth, angiogenesis, and metastasis.

CGP treatment of BT-549 or MDA MB-231 is associated with the attenuation of NPY-induced ERK phosphorylation, cell growth, and cell migration. In the absence of NPY, CGP inhibited cell growth and increased apoptosis in a dose- and time-dependent manner. Although the mechanisms of agonist-independent effects of CGP were not examined in the present study, we propose that CGP can be an important therapeutic agent for breast tumor growth.

In summary, for the first time, this work describes the presence of functional Y5R in human breast cancer cell lines. Its activation is coupled to the inhibition of cAMP and the phosphorylation/activation of ERK1/2 signaling pathways. The activation of Y5R by NPY promotes migration of MDA MB-231 cells and increases BT-549 cell growth through cAMP inhibition and ERK1/2 phosphorylation. These effects are specific to the activation of Y5R as they were prevented by CGP. These findings clearly suggest that Y5R could be a novel therapeutic target for breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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