Estrogen Up-regulates Neuropeptide Y Y1 Receptor Expression in a Human Breast Cancer Cell Line

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Abstract

Normal breast tissue mainly expresses the neuropeptide Y (NPY) Y2 receptor whereas primary human breast carcinomas express the Y1 receptor (Y1R) subtype. We hypothesized that activation of estrogen signaling systems plays a role in the induction of Y1R. To investigate this possibility, we used estrogen receptor–positive (ER+) human breast carcinoma cell line, MCF-7, and examined the effect of estrogen on Y1R gene expression and its signaling pathways. Saturation binding studies revealed that MCF-7 cells express high-affinity NPY receptor. NPY inhibited forskolin-stimulated adenosine 3’5’-cyclic monophosphate (cAMP) accumulation and mobilized intracellular Ca2+ in MCF-7 cells. Chronic estrogen treatment enhanced NPY-mediated inhibition of cAMP accumulation by 4-fold and caused a significant increase in Y1R mRNA expression through ERα. Similarly, estrogen increased Y1R mRNA expression in T-47D (ER+) but not in MDA-MB231 or MDA-MB468 (ER−) cell lines. Cycloheximide decreased basal Y1R mRNA expression; however, it did not affect its increase by estrogen. Moreover, estrogen treatment of MCF-7 cells did not increase Y1R mRNA stability. The up-regulation of Y1R expression by estrogen is prevented by hydroxycurea but not by nocodazole or IB-MECA (cell cycle inhibitors). Lastly, NPY inhibited estrogen-induced cell proliferation through Y1R. In conclusion, MCF-7 cells express a functional Y1R coupled to both Ca2+ and cAMP pathways. Estrogen up-regulates Y1R expression through ERα. This effect is independent of increased Y1R mRNA stability or new protein synthesis, and likely occurs during S phase completion of the cell cycle. Estrogen plays an important role in the up-regulation of Y1R, which in turn regulates estrogen-induced cell proliferation in breast cancer cells. (Cancer Res 2006; 66(7): 3706-14)

Introduction

Neuropeptide Y (NPY) is a 36 amino acid peptide amidated at the COOH terminus (1). Structural studies suggest that NPY belongs to the pancreatic polypeptide family and exhibits 70% amino acid sequence homology with peptide YY (PYY) and 50% with pancreatic polypeptide. NPY is the most abundant neuropeptide in the mammalian brain (2) and modulates several functions that include appetite, anxiety, circadian rhythm, memory, and blood pressure (3). NPY is a potent vasoconstrictor peptide whose actions are mediated by at least five different types of receptors (Y1-Y5) that are coupled to G proteins (4, 5). Recent findings have shown that NPY Y1 receptor (Y1R) subtype is expressed in 85% of the primary human breast carcinomas (6). In contrast, normal breast tissue expresses the Y2 receptor (Y2R) subtype. The factors causing the high incidence of Y1R expression in human breast carcinoma remain unknown.

The Y1R was the first NPY receptor subtype to be cloned and characterized (7). An interaction between estrogen, NPY and its receptors has been proposed to explain the concerted action of estrogen and progesterone on increased NPY level and an associated increase in luteinizing hormone release (8–10). Estrogen treatment of rats has been shown to increase Y1R mRNA in hypothalamus as detected by competitive reverse transcription-PCR method (11). Although several lines of evidence suggest the interaction of estrogen and NPY in brain, the physiologic interaction of estrogen and NPY/NPY receptor system has not been reported in the periphery.

The high incidence of Y1R expression in human breast tumors suggests a role for Y1R in the pathophysiology of breast malignancy (6). In a differential display study using MCF-7 cells, it was shown that the expression of Y1R mRNA was increased in response to estrogen treatment (12). However, whether Y1R mRNA translates into a functional protein that binds to NPY or its ligands, and is coupled to second messenger systems, remains to be investigated.

The purpose of the present study is to characterize the functional status of Y1R and to determine the cellular mechanisms mediating its regulation by estrogen in MCF-7 cells. Toward this end, we used saturation receptor binding assay and displacement studies to monitor the receptor affinity, density, and ligand specificity. Additional studies were carried out to determine the coupling of Y1R to adenosine 3’5’-cyclic monophosphate (cAMP) and calcium. The mechanism of increased Y1R mRNA expression by estrogen was determined using cell transcription and translation, and cell cycle inhibitors. We further determined whether the induction of Y1R mRNA expression traced back to ER+ cells, and whether the induction of Y1R affects the cellular response to estrogen.

Materials and Methods

Materials. MCF-7, SK-N-MC, and SK-N-BE2 cells were purchased from American Type Culture Collection (Rockville, MD). T-47D, MDA-MB231, and MDA-MB468 were obtained from Drs. S. Khan and E.M. Bahassi (Department of Anatomy and Cell Biology, University of Cincinnati). Porcine NPY, NPY-related peptides, and BIBP3226 were obtained from American Peptide Company, Inc. (Sunnyvale, CA). ICI 182,780, genistein (Tocris Biosciences, Ellisville, MO), porcine 125I-PYY, α25P-dCTP, and 125I-cAMP RIA kit were obtained from Perkin-Elmer Life Sciences, Inc. (Boston, MA). Nocodazole, IB-MECA, hydroxyurea, cycloheximide, 5,6-dichloro-1-ß-ribofuranosylbenzimidazole (DRB) were purchased from Sigma (St. Louis, MO). DMEM, phenol red–free DMEM (PR-free DMEM), fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Dextran charcoal-stripped FBS (CSS) was purchased from Cocalico Biologicals, Inc., (Beamstown, PA). Tri-Reagent and formazol were obtained from Molecular Research Center, Inc. (Cincinnati, OH). All other chemicals and reagents were the highest grade available and obtained commercially from Sigma and Invitrogen.
Cell culture. MCF-7 cells were grown in high-glucose DMEM containing 10% FBS, in the presence of penicillin/streptomycin. For estrogen treatment studies, cells were incubated either in serum-free DMEM containing 0.1% bovine serum albumin (BSA), or in PR-free DMEM containing 5% CSS. This last medium was used to avoid the estrogenic activity of serum and phenol red.

Receptor binding studies. Membrane fractions were isolated from MCF-7 cells as previously described (15). The binding assay was done with modifications as previously reported by our laboratory (14). Membrane fractions (125-150 μg protein) were incubated in the binding buffer [50 mmol/L HEPES (pH 7.3), 0.1% BSA, 2.5 mmol/L CaCl2, 1 mmol/L MgCl2, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)] containing 125I-PYY, unlabeled PYY or other related peptides as needed. In saturation binding studies, 125I-PYY was mixed with unabeled PYY and used at 0.5 to 40 nmol/L concentration. One thousand-fold excess of unlabeled PYY was used to determine the nonspecific binding. In displacement studies, experiments were carried out as described for saturation binding, with a constant amount of 125I-PYY (40 pmol/L) and increasing concentrations of competing unlabeled peptides at 0.01 to 1.000 nmol/L concentration. At the end of incubation, the assay was terminated by adding 1 mL of ice-cold PBS and centrifuged for 15 minutes at 10,000 g. 4°C. Peptide bound to the membrane was counted in a Packard gamma counter for 1 minute.

cAMP accumulation studies. cAMP accumulation in MCF-7 cells was determined as previously described for AR-5 cells in our laboratory (15). Cells were grown in 24-well plates for 3 days. When cells reached 80% confluence, the growth medium was replaced with serum-free DMEM high-glucose medium containing 0.1% BSA, 5 mmol/L theophylline, and 0.1 mmol/L PMSF, and incubated for 1 hour at 37°C. Cells were then treated with estrogen (100 mmol/L), forskolin (10 μmol/L) NPY agonists (10 μmol/L), or NPY at various concentrations (0.1-1.000 nmol/L) for 30 minutes. The cells were then lysed by adding 50 mmol/L acetic acid buffer (pH 6.2) containing 2% Triton X-100, and the lysate was centrifuged at 1.250 × g for 15 minutes at 4°C. The supernatant was directly used (5-100 μL) for cAMP measurement with a RIA kit. Protein content in the supernatant was determined using a bicinchoninic acid kit (Pierce, Rockford, IL) and was used to normalize for cAMP levels; the results are expressed as pmol/mg of protein.

Intracellular Ca++ measurement. Intracellular Ca++ concentration ([Ca++]/i) was estimated using Ca++-sensitive fluorescent probe fura-2 as previously described in our laboratory (16). The emission fluorescence intensity was recorded at a 495-nm wavelength and the fluorescence ratio at excitation wavelengths of 340 and 380 nm was used to estimate the changes in intracellular calcium.

Total RNA isolation and Northern hybridization. Total cellular RNA was extracted from MCF-7 cells as previously described and used in our laboratory (17, 18). The coding sequence of human Y1R was used to generate Y1R-specific probes by reverse transcription-PCR using total RNA obtained from MCF-7 cells. The primers 5'-ATAATTGGACACTGGTTCTTG-3' (forward) and 5'-TTAAGATGTTAAGAGGAGGCA-3' (reverse) corresponding to bases 499 to 1,044 were used in the PCR reaction (11).

Roles of estrogen receptor α and estrogen receptor β subtypes in estrogen-induced Y1R mRNA expression. In these experiments we investigated the role of estrogen receptor (ER) subtypes in the induction of Y1R mRNA by estrogen. MCF-7 cells were switched to 0.1% BSA containing serum-free media at 70% confluency. Cells were then treated with estrogen (100 nmol/L), forskolin (10 μmol/L) or their vehicles. Total RNA was isolated from these cells and examined for Y1R mRNA expression by Northern hybridization.

Role of Y1R mRNA stability in the regulation of its expression by estrogen. To determine the role of Y1R mRNA stability in estrogen-increased Y1R expression in MCF-7 cells, the cells were treated with 100 nmol/L estrogen or its vehicle and incubated for 16 hours. Immediately after this incubation (time 0), total RNA was isolated and saved from a set of cells subjected to vehicle and estrogen treatments. Another set of cells from both groups was washed and incubated in fresh medium in the presence of 65 μmol/L DRB, a gene transcription blocker (21), in the presence of estrogen and its vehicle. The experiment was terminated after 3 and 6 hours of DRB treatment. The cells from time 0, 3, and 6 hours were then used for total RNA isolation and examined for Y1R mRNA expression by Northern hybridization.

Role of cell cycle inhibitors in the up-regulation of Y1R mRNA by estrogen in MCF-7 cells. Estrogen is known to stimulate MCF-7 cell proliferation and growth. We therefore investigated whether estrogen-mediated up-regulation of Y1R is due to cell cycle progression. Toward this end, MCF-7 cells were grown in PR-free DMEM containing 5% CSS. At 70% confluence, cells were treated with either 100 nmol/L IB-MECA, G1-S phase blocker (22), 50 ng/mL nocodazole, G2-M phase blocker (23), or 0.5 mmol/L hydroxyurea, S phase blocker (24) in the presence or absence of 100 nmol/L estrogen or their vehicles. Total RNA was isolated from these cells and examined for Y1R mRNA expression by Northern hybridization.

Cell proliferation assay. MCF-7 cell proliferation was measured using the protocol previously described (25) with minor modifications. Briefly, MCF-7 cells were grown in PR-free DMEM containing 5% CSS for 3 days. Cells were then seeded in 96-well plates at a concentration of 3,000 cells per well. After 2 days, cells were switched to 0.25% CSS in PR-free DMEM for 24 hours. Cells were then treated with estrogen and NPY, in the presence or absence of IBIBP 3226 (Y1R-specific blockade) for 48 hours, and cell proliferation was determined using MTS assay kit (Promega, Madison, WI).

Statistical analysis. The Kd, Bmax, and IC50 values were determined using prism software (GraphPad Software, Inc., San Diego, CA). The quantitative data were expressed as mean ± SE. Data were analyzed by ANOVA followed by Tukey-Kramer's multiple comparisons post test or direct comparisons (Instat program, GraphPad Software) by Student’s t test. P < 0.05 was accepted as statistically significant.

Results

Saturation binding. We selected 125I-PYY for NPY receptor binding studies as it has been shown to have low nonspecific binding and similar affinity as NPY to NPY receptor subtypes (26). Receptor saturation binding studies showed that 125I-PYY binding to MCF-7 cell membrane was specific and saturable. Total and nonspecific binding increased linearly with increasing concentrations of 125I-PYY, whereas specific binding was saturated at a concentration of 2.5 to 5.0 nmol/L of 125I-PYY (Fig. 1A). The nonspecific binding was 35% to 40% of the total 125I-PYY bound to the cell membrane at 1 to 2.5 nmol/L concentration of 125I-PYY (data not shown). Analysis of binding data using Prism software showed that Bmax or receptor density was 1,015 ± 31 fmol/mg protein with an apparent Kd value of 0.9 ± 0.2 nmol/L (Fig. 1A, inset).

Displacement studies. Specific binding of 125I-PYY accounted for 76 ± 2% of the total binding. We determined the relative affinities of NPY receptor using Y1 subtype-specific ligand [Leu]3 Pro34[NPY], or Y2 subtype–specific ligands, PYY(3-36), NPY(13-36), and NPY(16-36), or receptor nonsensitive ligands, PYY and NPY to displace 125I-PYY binding to MCF-7 cell membranes. The Y1 selective agonist and receptor nonsensitive peptides exhibited high-affinity binding, whereas Y2 receptor subtype–specific ligands showed weaker affinity to displace 125I-PYY binding (Fig. 1B). Furthermore, increasing concentrations of Y1R- and Y2R-specific ligands displace 125I-PYY binding with varying affinities (Fig. 1B). The IC50 values for the tested peptides were as follows:
[Leu31, Pro34]NPY 0.01 ± 0.001 nmol/L, PYY 0.03 ± 0.002 nmol/L, NPY 0.06 ± 0.004 nmol/L, PY(Y3-36) 72 ± 3 nmol/L, NPY(13-36) 110 ± 10 nmol/L, and NPY(16-36) 377 ± 6 nmol/L.

Effects of NPY and estrogen on cAMP accumulation. Forskolin-treatment increased cAMP accumulation in MCF-7 cells by ~100-fold from 28 ± 4 to 2,865 ± 305 pmol/mg protein (compare Fig. 2A and C). This effect is significantly inhibited by [Leu31, Pro34]NPY and NPY (Fig. 2A). However, NPY(13-36) showed no significant effect on forskolin-induced cAMP accumulation (Fig. 2A). These peptides (at 10 nmol/L) reduced forskolin-stimulated cAMP accumulation level from 2,865 ± 305 to 1,420 ± 58 (P < 0.05), 1,862 ± 122 (P < 0.05), and to 2,431 ± 69 pmol/mg protein (P > 0.05) by [Leu31, Pro34]NPY, NPY, and NPY(13-36), respectively (Fig. 2A).

To study the regulation of NPY Y1R by estrogen, we first examined whether estrogen treatment alters cAMP response to NPY. Accordingly, MCF-7 cells were incubated with estrogen for 16 hours and then exposed to different doses of NPY for 30 minutes under forskolin-stimulated state. The results indicate that estrogen enhanced the dose-response inhibitory effect of NPY on cAMP accumulation in MCF-7 cells (Fig. 2B). Indeed, the NPY dose-response curve shifted to the left with IC50 decreasing from 0.2 ± 0.02 to 0.05 ± 0.002 nmol/L (Fig. 2B). This increase in affinity for NPY by estrogen treatment may be due to the increase in Y1R expression. Interestingly, we observed that estrogen treatment of MCF-7 cells for 16 hours significantly reduced both basal (Fig. 2C) and forskolin-stimulated (Fig. 2D) cAMP level as compared with vehicle-treated cells.

Effect of NPY on intracellular Ca2+ mobilization. As shown in Fig. 2E, treatment of MCF-7 cells with 100 nmol/L NPY caused a rapid increase in intracellular calcium, which peaked within 24 seconds, and then returned toward baseline level (Fig. 2E). This Ca2+ spike likely originated from NPY-induced Ca2+ mobilization from its intracellular stores. No effect on intracellular Ca2+ release was observed when fura-2-loaded MCF-7 cells were treated with NPY(13-36) or NPY(16-36) at 100 nmol/L concentration (data not shown).

Regulation of NPY Y1R mRNA expression by estrogen. In the first set of experiments, we examined the specificity of Y1R probe using cell lines known to express both Y1 and Y2 receptors. The results indicate that a single band corresponding to Y1R is detected in MCF-7 as well as cells known to express Y1R (SK-N-MC), but not in SK-N-BE2 that is known to express Y2R (Fig. 3A). Furthermore, we examined the effects of estrogen on the mRNA expression levels of Y1R in MCF-7 cells by Northern analysis. The results indicate that the treatment of MCF-7 cells with 10 and 100 nmol/L of estrogen for 16 hours increased Y1R mRNA expression by 72% (P < 0.001, n = 4; Fig. 3B) and 98% (P < 0.001, n = 4; Fig. 3B), respectively, as compared with vehicle (n = 4).

Interestingly, MCF-7 cells treated with 1,000 nmol/L of estrogen for 16 hours had no effect on NPY Y1R mRNA expression (P > 0.05, n = 4; Fig. 3B) as compared with vehicle. These results suggest that estrogen exhibits a dual effect on Y1R expression in MCF-7 cells. This likely involves two different and opposing pathways, a stimulatory pathway activated by lower doses of estrogen (10-100 nmol/L), and an inhibitory pathway stimulated by higher doses of estrogen (1,000 nmol/L). It should be noted that unlike estrogen, another steroid hormone such as dex-methasone, a synthetic glucocorticoid, did not affect NPY Y1R mRNA expression levels in MCF-7 cells (data not shown), suggesting the specificity of estrogen action.

Effect of estrogen on Y1R mRNA expression in ER+ and ER− breast cancer cell lines. We examined the baseline expression and the effects of estrogen on Y1R mRNA in three additional cell lines including T-47D (ER+), MDA-MB231 (ER−), and MDA-MB-468 (ER−) cells derived from human breast cancer. The results indicate a moderate expression of Y1R mRNA in both MCF-7 (Fig. 3B and C) and T-47D (Fig. 3C and D) cells, which is significantly up-regulated by estrogen compared with vehicle. No detectable Y1R mRNA was observed in MDA-MB231 cells (Fig. 3C), in the absence or presence of estrogen (Fig. 3C). However, MDA-MB 468 cells express low levels of Y1R mRNA but were not affected by estrogen treatment (Fig. 3C). These findings likely indicate that an association exists between increased Y1R expression and ER status of the breast cancer cells.

Estrogen-induced Y1R mRNA expression is inhibited by ERα blockade. In the next set of experiments, we examined whether the up-regulation of NPY Y1R by estrogen is mediated through the activation of ERα or ERβ subtypes. Accordingly, MCF-7 cells were incubated in the presence of ICI alone (ERα-specific blockade) at different concentrations (0.1, 1, or 10 ICI μmol/L), or ICI + 100 nmol/L estrogen, or a mixture of their vehicles as a control. After 16 hours of treatment, total RNA was isolated and used for Northern analysis. The results depicted in Fig. 4A indicate that ICI treatment...
alone significantly decreased the baseline expression NPY Y1 receptor (89% for 100 nmol/L, \(P<0.0001\); Fig. 4A). Interestingly, estrogen treatment reversed the effects of 0.1 and 1 \(\mu\)mol/L ICI (Fig. 4), but failed to increase the expression of NPY Y1 receptor in the presence of 10 \(\mu\)mol/L ICI (Fig. 4A, c and Fig. 4B, c). In similar experiments, we used genistein to block the ER\(\beta\) subtype, and the results showed that this inhibitor did not affect the basal or estrogen-induced NPY Y1R mRNA expression (data not shown).

Because ICI decreased the baseline expression of Y1R mRNA, which could result from the estrogenic activity of phenol red, we reexamined the effect of estrogen on Y1R mRNA in the absence or presence of ICI. As shown in Fig. 4C, Y1R mRNA expression increased by 4-fold in response to estrogen \((P<0.05; \text{Fig. 4C and D})\) as compared with vehicle. ICI did not affect the baseline expression of Y1R mRNA, but prevented its up-regulation by estrogen (Fig. 4C and D). These results indicate that the up-regulation of Y1R by estrogen is likely mediated through ER\(\alpha\). These studies further suggest that the decrease in baseline expression of Y1R observed in the presence of phenol red in the medium (Fig. 4A and B) is due to the estrogenic activity of phenol red.

**Estrogen-induced Y1R mRNA expression is independent of protein synthesis.** In these experiments, we determined whether the increase in Y1R mRNA expression by estrogen requires newly
synthesized proteins. Accordingly, MCF-7 cells were incubated with cycloheximide alone, or first incubated with cycloheximide for 30 minutes and then treated with estrogen (100 nmol/L), or treated with a mixture of diluted DMSO and ethanol, vehicles for cycloheximide and estrogens, respectively. After 16 hours of treatment, total RNA was isolated for Y1R mRNA analysis. The result was that cycloheximide alone caused a significant decrease in baseline mRNA expression of Y1R \( (P < 0.001, n = 3; \text{Fig. 5}A\text{ and }B) \), but did not prevent estrogen from causing a significant increase in Y1R mRNA expression \( (P < 0.01, n = 3; \text{Fig. 5}A\text{ and }B) \) compared with vehicle. These findings suggest that the stimulation of Y1R mRNA expression in MCF-7 cells by estrogen does not require newly synthesized proteins.

**Effect of estrogen treatment on Y1R mRNA stability.** The objective of these experiments is to determine whether the increased expression of Y1R mRNA by lower doses of estrogen in MCF-7 cells results from enhanced stability of its mRNA. Toward this end, MCF-7 cells were incubated with 100 nmol/L estrogen or its vehicle in serum-free DMEM. After 16 hours, a set of vehicle- and estrogen-treated cells were immediately used for RNA isolation (time 0), whereas another set of both vehicle- and estrogen-treated cells were incubated with 65 μmol/L of DRB (inhibitor of gene transcription) for another 3 to 6 hours. Total RNA was then isolated from these cells and examined for Y1R expression by Northern analysis (Fig. 5C and D). As expected, the results indicate that estrogen treatment significantly increased the mRNA expression of Y1R as compared with vehicle \( (P < 0.001, n = 3; \text{Fig. 5}E) \). Interestingly, the rate of Y1R mRNA decay is actually slightly higher in estrogen- versus vehicle-treated cells as indicated by the slopes of the curves \( (\text{Fig. 5}E) \). These results indicate that the increase in Y1R mRNA expression levels by estrogen is not mediated through enhanced mRNA stability and likely involves an increase in Y1R gene transcription.

**Effect of cell cycle blockers on basal expression and estrogen-mediated up-regulation of Y1R mRNA in MCF-7 cells.** As shown in Fig. 6A, none of the three cell cycle blockers affected the baseline expression of Y1R mRNA, as compared with their respective vehicles \( (\text{Fig. 6}A) \). Furthermore, the up-regulation of Y1R mRNA by estrogen was not affected by IB-MECA or nocodazole treatments \( (\text{Fig. 6}A) \) as compared with the inhibitors alone. Interestingly, hydroxyurea completely prevented the stimulatory effect of estrogen on Y1R mRNA \( (\text{Fig. 6}A) \).

**Figure 3.** Cell-specific expression of Y1R and its regulation by estrogen. A, representative Northern analysis of Y1R mRNA and 28S rRNA in total RNA isolated from MCF-7, SK-N-MC, and SK-N-BE2 cells. As shown, Y1R mRNA is detected in MCF-7 and SK-N-MC but not in SK-N-BE2 cells. B, representative Northern analysis showing the mRNA expression of Y1R in MCF-7 cells incubated with different concentrations of estrogen or its vehicle for 16 hours (top), and corresponding densitometric analysis showing the mean expression of Y1R mRNA-to-28S rRNA expressed as the percentage of controls (bottom). Estrogen up-regulates Y1R mRNA at lower (10-100 nmol/L) but not at higher (1,000 nmol/L) doses; \( **, P < 0.001 (n = 4-5\text{ determinations}) \). C, representative Northern analysis of Y1R mRNA and 28S rRNA in total RNA isolated from MCF-7, T47-D, MDA-MB231, and MDA-MB468 cell lines treated with 100 nmol/L estrogen or its vehicle for 16 hours. D, Northern hybridization of NPY Y1R mRNA in the presence of 100 nmol/L estrogen or its vehicle in T47-D cells (top), and corresponding densitometric analysis showing the mean expression of Y1R mRNA-to-28S rRNA expressed the percentage of controls (bottom). Thirty micrograms of total RNA from different samples were loaded per lane in all blots.
Effect of NPY on estrogen-induced cell proliferation in MCF-7 cells. To determine whether Y1R affects the proliferative effect of estrogen on MCF-7 cells, we examined the effects of NPY on estrogen-induced cell proliferation. The results shown in Fig. 6 indicate that NPY decreased estrogen-induced cell proliferation in a dose-dependent manner with an IC50 value of 32 pmol/L (Fig. 6B). The results depicted in Fig. 6C, show that estrogen treatment increased cell proliferation by 3-fold (Fig. 6C). This effect is significantly reduced in the presence of NPY (P < 0.05; Fig. 6C). The presence of 1 μmol/L BIBP3226, an Y1R blockade (5), completely attenuated the NPY-induced inhibition of the proliferative effect of estrogen (P < 0.05; Fig. 6C). BIBP3226 alone did not affect estrogen-induced cell proliferation.

Discussion
In the present studies, binding assays using 125I-PYY showed that MCF-7 cells contain high-affinity NPY receptor that is saturable and reversible (Fig. 1). Scatchard analysis of the binding data (Fig. 1A) revealed that the affinity (Kd) of the receptor is in the physiologic range (27). Earlier studies have shown that SK-N-MC (7, 27) HEL cells (28) and several other neuroblastoma cell lines express high-affinity NPY receptors in the nanomolar range (29). Our present studies showed that NPY receptor subtype–specific peptide ligands inhibited 125I-PYY binding to MCF-7 membranes in a dose-dependent manner (Fig. 1B). The affinities of these ligands to NPY receptor were in the following rank order [Leu31, Pro34]NPY ≈ PYY > PYY(3-36) > NPY(13-36) > NPY(16-36). Similar pharmacologic profiles have been reported in various Y1R-expressing cell lines or cell lines transfected with Y1R cDNA (5). Moreover, our displacement studies (Fig. 1B) show that this receptor exhibits selectivity towards Y1R ligands, suggesting that NPY receptor expressed in the MCF-7 cell line is a Y1R subtype. The effects of NPY on cAMP accumulation and Ca2+ mobilization (Fig. 2) in MCF-7 cells further confirm this hypothesis. In addition to this functional data, Northern hybridization studies (Fig. 3A) showed that MCF-7 cells express a single transcript detected by a Y1R-specific probe similar to that previously reported in the SK-N-MC cell line (7). Y1R mRNA is absent in SK-N-BE2 cells, which express the NPY Y2 receptor subtype (Fig. 3A), indicating the specificity of the probe used to detect Y1R.

In addition to the pharmacologic characterization of Y1R, we examined the effect of estrogen on NPY-mediated cAMP inhibition in MCF-7 cells. Our results show that estrogen treatment for 16 hours significantly decreased the IC50 value of NPY (Fig. 2B). This effect could be mediated through a dual mechanism that includes a direct effect of estrogen on cAMP synthesis and/or through an increase in Y1 receptor expression. When investigating these possibilities, we observed that in the absence of NPY, estrogen (100 nmol/L) treatment of MCF-7 cells for 16 hours significantly inhibited basal (Fig. 2C) and forskolin-stimulated (Fig. 2D) cAMP accumulation. This observation suggests a direct action of estrogen on cAMP synthesis and supports the possibility that estrogen receptors are negatively coupled to the cAMP system in MCF-7 cells. It is not clear which subtype of estrogen receptor is mediating the inhibitory effect on cAMP synthesis in MCF-7 cells. An earlier
study has shown the biphasic effect of estrogen on cAMP accumulation in GT1-7, a neuronal cell line (30). This inhibitory effect of estrogen is sensitive to ER blocker and pertussis toxin treatment (30), suggesting that plasma membrane–associated ER is modulating cAMP levels through the activation of Gi protein.

We next investigated the possibility that estrogen could induce Y1R mRNA expression in MCF-7 cells. Northern analysis indicates that estrogen regulates Y1R mRNA expression in a dose-dependent manner (Fig. 3B). This effect is cell-specific as estrogen treatment of SK-N-MC cells, a human neuroblastoma cell line expressing Y1R but lacking estrogen receptor (31), had no effect on Y1R mRNA expression (data not shown). Moreover, our earlier studies have shown that forskolin, thapsigargin, and NPY treatments of SK-N-MC cells up-regulate Y1R mRNA (17). The stimulation of Y1R mRNA by estrogen is observed only in ER+ breast cancer cell lines (Fig. 3C and D). In some subsets of ER− cell lines, the baseline expression of Y1R is detectable (MDA-MB468), although not up-regulated by estrogen. This may suggest that in some cells, Y1R expression is likely mediated by other factors. These findings suggest that Y1R expression is independent of the ER status of the cell, however, the stimulatory effect of estrogen on Y1R tracks to ER+ cells. The stimulatory effect of estrogen on Y1R mRNA expression is likely mediated through estrogen receptors. In fact, we showed that the presence of ICI 182-780, an ERα blocker (19), inhibited estrogen-induced Y1R mRNA expression in a dose-dependent manner (Fig. 4). ICI also decreased the baseline expression of Y1R; however, this effect is due to the estrogenic activity of phenol red (Fig. 4C and D). Genistein, an ERβ inhibitor (20), did not prevent the stimulation of Y1R expression by estrogen (data not shown). It should be noted that estrogen has a dual effect on Y1R expression in MCF-7 cells (Fig. 3B). Studies in NG-108-15 cells transfected with ERα and/or ERβ showed that the presence of ERβ attenuates basal as well as EBox-induced Y1R gene promoter activity (32). These findings are consistent with earlier reports that activation of ERβ can inhibit EBox-mediated activity in certain cell lines (33). Based on these and our present findings, we propose that the loss of the stimulatory effect of estrogen at a higher dose on Y1R mRNA in MCF-7 cells is likely due to the activation of ERβ. However, this hypothesis needs to be investigated in detail.

With respect to the mechanism involved in the effect of estrogen on Y1R expression, our results indicate that the up-regulation of Y1R mRNA is independent of new protein synthesis. However, the
decrease in baseline Y1R mRNA expression by cycloheximide points to a mechanism in which newly synthesized proteins may be required for maintaining basal Y1R mRNA (Fig. 5A). We further examined whether the estrogen-induced increase in Y1R mRNA is a consequence of increased transcription or increased stability of gene transcript. Our results indicate that the decay of Y1R mRNA, if any, was actually slightly higher in estrogen versus vehicle-treated cells (Fig. 5C and D). These findings suggest that the up-regulation of Y1R mRNA expression is likely mediated through an increase in Y1R gene transcription rate. In support of these findings, two earlier studies have shown that mouse (32) and rat (34) Y1R gene promoter contains consensus sequences for several transcription factor binding sites, including estrogen receptor element (ERE). Human genomic cloning studies revealed that three different promoters regulate the Y1R gene in a tissue-specific manner (35). A chimeric plasmid containing mouse Y1R promoter linked to luciferase reporter gene activity was increased 2-fold by estrogen (32) in transfected NG-108-15 cells. In the same study, introducing mutations in both ERE sites abolished the estrogen-induced gene activity (32). These findings support the hypothesis that estrogen-induced up-regulation of Y1R in MCF-7 cells likely involves the activation of ERα and its binding to ERE sites present in the promoter region. Our results on cell cycle suggest that the up-regulation of Y1R by estrogen is independent of G1-S or G2-M phases, and S phase completion is required for this function (Fig. 6A). Alternatively, the down-regulation of ER by hydroxyurea (36) could contribute to the loss of estrogen effect on Y1R mRNA expression. These findings support the hypothesis that the stimulatory effect of estrogen on Y1R is not simply due to cell cycle progression and is rather specific to estrogenic stimulus. NPY Y1R expression, affinity, and second messenger coupling in the MCF-7 cell line are similar to the previously characterized Y1R system in other cell types. However, our findings that estrogen up-regulates Y1R mRNA is of pathophysiologic importance in breast cancer. PYY-induced cAMP inhibition has been reported to be responsible for the inhibition of MCF-7 cell proliferation in vivo and in vitro (37). In contrast, overexpression of the regulatory subunits of protein kinase A in malignant breast tissue is associated with the induction of epithelial cell proliferation (38). It remains to be investigated whether inhibition of cAMP by PYY plays a significant role in the antiproliferative effect of this peptide. Up-regulation of Y1R in the majority (85%) of breast cancers (6) suggests that this receptor subtype may play a significant role in neoplastic transformation of epithelial cells. Our findings support the hypothesis that estrogen is likely to be one of the mediators responsible for the induction of Y1R. It has

Figure 6. Effects of cell cycle inhibitors on estrogen-induced Y1R expression (A) and effect of NPY on estrogen-induced cell proliferation in MCF-7 cells (B and C). A, MCF-7 cells were incubated in PR-free DMEM and first treated with either 100 μmol/L IB-MECA, 50 ng/mL nocodazole, or 0.5 mmol/L hydroxyurea alone for 16 hours, and then 100 nmol/L estrogen was added to the cells and the incubation continued for another 16 hours. Y1R mRNA expression and 28S rRNA were then examined by Northern hybridization (n = 2-3 determinations). Thirty micrograms of total RNA from different samples were loaded per lane. B, dose-response effect of NPY on estrogen-induced cell proliferation in MCF-7 cells. Cells were maintained in 0.25% CSS and PR-free DMEM for 24 hours, and then treated with 100 nmol/L of estrogen. Fifteen minutes later, different doses of NPY (0.1-1,000 nmol/L) were added to the cells and incubation continued for another 48 hours before cell proliferation assay. The results are expressed as the percentage of estrogen-treated cells. C, MCF-7 cells were treated with either 100 nmol/L estrogen alone, estrogen + 1 nmol/L NPY, estrogen + 1 μmol/L BIBP3226 + NPY, or estrogen + BIBP3226. These cells were then used for cell proliferation assay. The results are expressed as the percentage of vehicle (control; n = 4 determinations in each group). *, P < 0.05 versus estrogen and **, P < 0.05 versus estrogen + NPY.
been shown that NPY plays a role in cell proliferation through Y1R in neuronal precursor cells (39). Recent studies showed that NPY stimulates neuroblastoma cell proliferation by activating Y2 and Y5 receptor subtypes, whereas it inhibits tumor growth in Ewing’s sarcoma family of tumors via Y1 and Y5 receptor (25), suggesting differential functions for Y1R in cell proliferation. In the present studies, we showed that NPY significantly inhibited estrogen-induced cell proliferation through Y1R in MCF-7 cells (Fig. 6B and C). These findings likely suggest that the up-regulation of Y1R by estrogen may have a beneficial effect by reducing the proliferative activity of estrogen in breast cancer.

In conclusion, breast carcinoma cell line MCF-7 expresses a functional Y1R that is coupled to both CAMP and calcium signaling pathways. In these cells, estrogen up-regulates Y1R expression and function through ERα. This stimulatory effect is independent of new protein synthesis and Y1R mRNA stability, and likely results from increased gene transcription rate. Estrogen-mediated up-regulation of Y1R is expected to be confined to ERα-positive breast cancer cells and likely occurs during S phase completion of cell cycle. Synthesizing Y1R-specific compounds and developing a novel strategy in tumor-specific delivery of these compounds will open new avenues in breast cancer research.

Acknowledgments


Grant support: University of Cincinnati Academic Development Fund (H. Amlal) and by NIH grant DK 53548 (S. Sheriff) and GM-47122 (A. Balasubramaniam). Portions of this work were presented at the 34th Annual meeting, Society for Neuroscience, October 2004, San Diego, CA.

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The authors thank Shruti Narayana and Ayesha Yahya for their technical help with protein and cell proliferation assays.

References

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