The peroxisome proliferator-activated receptor γ is an inhibitor of ErbBs activity in human breast cancer cells

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SUMMARY

One of the most interesting recent developments in the nuclear receptor field has been the identification of natural and synthetic agonists of the peroxisome proliferator-activated receptor (PPAR) family, coupled with a growing recognition that the γ isoform (PPARγ) affects pathways important in a variety of human diseases. Here we show that the activation of PPARγ through the 15-deoxy-Δ-12,14-prostaglandin J_2 (PG-J_2) ligand causes a dramatic inhibition of ErbB-2 and ErbB-3 tyrosine phosphorylation caused by neuregulin 1 (NRG1) and neuregulin 2 (NRG2) in MCF-7 cells. This effect is accompanied by a very efficient blocking of ErbBs effects upon proliferation, differentiation and cell death in these cells. Preincubation of MCF-7 cells with PG-J_2 before addition of NRG1 and NRG2 had a dramatic growth-suppressive effect accompanied by accumulation of cells in the G0/G1 compartment of the cell cycle, and a marked increase in apoptosis. NRG1 and NRG2 induce G1 progression, which was associated with stimulation of the phosphatidylinositol-3 kinase (PI 3-K) pathway, whereas survival was dependent on ERK1/ERK2 activation. Both pathways were inhibited by PG-J_2. Furthermore, PG-J_2 can abolish the NRG1 and NRG2-induced increase in anchorage-independent growth of these cells. PG-J_2 also blocks phosphorylation of other receptor tyrosine kinases, such as IGF-IR, in MCF-7 cells, and suppress proliferation of other breast cancer cell lines. In summary, our data show a specific inhibitory action of PG-J_2 on the activity of the ErbB receptors in breast cancer cells.

Key words: Breast cancer, ErbBs, Phosphorylation, PPARγ, Transformation

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor gene superfamily (Mangelsdorf and Evans, 1995). The regulation of gene expression is exerted by the receptors upon heterodimerization with the 9-cis retinoic acid receptor and binding to specific response elements termed peroxisome proliferator-response elements (PPREs). Most PPREs identified to date reside in genes involved in lipid metabolism (Schoonjans et al., 1997). There are three members of the PPAR subfamily of nuclear receptors: α, δ and γ (Tontonoz et al., 1994a; Dreyer et al., 1992; Kliewer et al., 1994). PPARγ is abundant in adipose tissue where it triggers adipocyte differentiation and lipid storage by regulating the expression of genes critical for adipogenesis (Tontonoz et al., 1994a; Tontonoz et al., 1994b). PPARs function as transcription factors regulating gene transcription in response to the binding of their ligands (Michalik and Wahli, 1999). There are several known ligands for PPARγ, including the natural prostaglandin 15-deoxy-Δ-12,14-prostaglandin J2 (PG-J_2, a prostaglandin D_2 metabolite), the synthetic antidiabetic thiazolidinediones (Lehmann et al., 1995) and certain polyunsaturated fatty acids. One of the thiazolidenediones, troglitazone (TGZ), is currently used in some countries for the treatment of type II diabetes (Johnson et al., 1998).

Although PPARγ is primarily expressed in adipose tissue, it is also expressed in many other tissues and cell types although its role is still poorly understood. Recent studies indicate that PPARγ is expressed in cells of the monocyte/macrophage lineage and that ligand activation of this receptor powerfully regulates several aspects of monocyte biology such as the development of monocytes along the macrophage lineage, in particular in the conversion of monocytes to foam cells (cholesterol-engorged macrophages) (Spiegelman, 1998). Sarraf et al. have demonstrated that human colonic epithelium and colon cancer cell lines express PPARγ and that growth of the cell lines is inhibited by diverse PPARγ agonists, whereas an inactive metabolite of troglitazone and a selective PPARα agonist have no effect (Sarraf et al., 1998). In addition, Mueller et al. have shown that PPARγ is expressed at significant levels in human primary and metastatic breast adenocarcinomas (Mueller et al., 1998). Ligand activation of this receptor in cultured breast cancer cells caused extensive lipid accumulation, changes in breast epithelial gene expression associated with a more differentiated, less malignant, state. Some effects upon breast cancer cells have also being observed by other groups (Elstner et al., 1998; Kilgore et al., 1997).

Control of protein phosphorylation at tyrosine residues is a fundamental regulatory mechanism in signal transduction pathways involved in transformation and growth of breast cancer cells (Nguyen et al., 1995). Overexpression of type 1

receptor tyrosine kinases has been associated with several types of human cancers, including breast cancer and glioblastoma (Slamon et al., 1989; Kraus et al., 1987; Walker, 1998; Krisst and Yarden, 1996). This family of proteins consists of the epidermal growth factor receptor (EGFR/ErbB1), neu (ErbB2), ErbB3 and ErbB4 (Olayioye et al., 2000). Several studies have demonstrated that ErbB2 is amplified and overexpressed in 20-30% of primary breast cancers, a finding that correlates with poor patient prognosis (Paterson, 1991; Andrulis, 1998) and a more aggressive disease. An ErbB2-positive status may predict the likelihood of resistance to some conventional therapies. Furthermore, blockade and functional inhibition of c-erbB2 by monoclonal antibodies inhibits the growth of tumors that overexpress c-erbB2 (Drebin et al., 1986). Breast tumor progression is also associated with elevated levels of ErbB3, and a survey of primary human breast tumors revealed frequent co-expression of both ErbB2 and ErbB3 transcripts (Siegel et al., 1999). The incidence of amplification of the neu and ErbB3 oncogene-encoded protein tyrosine kinases in human breast cancer strongly supports the concept that protein tyrosine phosphorylation and dephosphorylation are key regulatory mechanisms in the proliferation, differentiation and neoplastic transformation of breast epithelial cells. In view of all the evidence commented above, the ErbB2 and ErbB3 receptor proteins have become very important targets for novel and specific anticancer treatment.

The neuregulins (NRGs) are a family of proteins that serve as ErbB ligands. They contain a region structurally related to EGF, the EGF-like domain, that can bind to and induce ErbB autophosphorylation. In addition to the NRGs and EGF, other molecules that contain an EGF-like domain and that can activate one or more ErbB receptors include transforming growth factor α (TGFα), heparin-binding EGF (HB-EGF), amphiregulin, betacellulin, epiregulin and cripto (Riese and Stern, 1998; Alroy and Yarden, 1997). Four distinct neuregulin genes (*NRG1*, *NRG2*, *NRG3* and *NRG4*) have been described (Carraway et al., 1997; Zhang et al., 1997; Harari et al., 1999). NRG1 was first cloned as neu differentiation factor (NDF) and heregulin (Burden and Yarden, 1997; Lemke, 1996), but is best known for its roles as the acetylcholine receptor inducing activity (ARIA), and as the potent Schwann cell mitogen, glial growth factor (GGF). Mice that lack NRG1 die at E10.5 from a heart defect and have virtually no Schwann cell precursors. NRG1 has been implicated in multiple cellular processes, including proliferation, differentiation, survival and migration (Burden and Yarden, 1997; Lemke, 1996). The neuregulin 1 gene encodes multiple isoforms that each contain an EGF-like domain. Splicing appears to give rise to alternative extracellular regions including Ig-domain-containing forms, which are believed to serve in the function described as ARIA, and a cysteine-rich domain (CRD)-containing form, which is thought to serve as GGF. Recombinant neuregulin protein forms that contain the EGF-like domain have been shown to induce receptor activation in vitro (Olayioye et al., 2000).

We have investigated the ability of PG-J_2, a natural specific ligand of PPARγ, to affect the induction by NRG1 and NRG2 of tyrosine phosphorylation/activation of ErbB2 and ErbB3 receptors expressed in MCF-7 cells, as well as its capacity to overcome the cellular responses elicited by the activation of these receptors. Our results provide evidence that PPARγ effectively blocks ErbB phosphorylation and interferes with ErbB signaling pathways. It therefore appears to play a suppressive regulatory role in the tumor growth of human breast carcinoma cells that express c-erbB2/neu and ErbB3 protein tyrosine kinases.

MATERIALS AND METHODS

Cell culture and purification of recombinant NRGs

MCF-7 cells were propagated and maintained in RPMI medium (Life Technologies) containing 10% fetal bovine serum (FBS), 1000 IU penicillin-streptomycin and 2 mM glutamine at 37°C and 5% CO_2. T47D and SKBR3 cells were cultured in DMEM medium (Life Technologies) containing 10% FBS. Experimental cultures were usually grown in serum-starved RPMI or, in some experiments, with 5% dextran-charcoal stripped FBS. After 24 hours of growth in these conditions, cells were stimulated with the appropriate ligand as indicated. Some cultures were pre-incubated with freshly dissolved Na_3VO_4 at 100 μM for 60 minutes. Specific kinase inhibitors were usually added 1 hour prior to treatment. These included LY294002 (Calbiochem, Nottingham, UK), a reversible inhibitor of PI 3-K used at 4 μM, and PD98059 (Calbiochem), an inhibitor of MEK1 and -2 used at 40 μM. Glutathione-S-transferase fusion proteins encoding the EGF-like domains of mouse NRG1β (heregulin-β1 residues 176-246) and NRG2β (NRG2β residues 246-314) were produced in High Five cells and purified by glutathione affinity as described previously (Carraway et al., 1997).

Immunoprecipitation and immunoblotting

For immunoprecipitation assays, cells were seeded at a density of 20,000/cm^2 and grown for 24 hours in complete medium, switched to the serum-treated medium and exposed for 10 hours to 10 μM PG-J_2 (Calbiochem-Novabiochem Corp.). Cells were then stimulated with the growth factors NRG-1, NRG-2, EGF (30 nM) or IGF-I (100 nM) for 5 minutes. Cells were washed twice with ice-cold TD buffer (20 mM Tris-HCL pH 7.4, 130 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4), lysed in 500 μl of PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and protease and phosphatase inhibitors, and spun at 12,000 ***g*** for 20 minutes. Supernatants were precleared with pansorbin (Calbiochem-Novabiochem Corp.) and incubated for 12 hours at 4°C with the appropriate antibodies. Protein-A-Sepharose was added to each sample and additionally incubated for 5 hours at 4°C. Beads were collected by microcentrifugation and washed five times with lysis buffer.

The immunocomplexes were eluted by boiling for 3 minutes in SDS sample buffer (100 mM Tris, pH 6.8, 36% glycerol, 4% SDS, 0.01% bromophenol blue and 200 mM DTT) and subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membranes. The blots were blocked with 3% BSA in TBST buffer (20 mM Tris-HCL pH 7.6, 130 mM NaCl, 0.1% Tween-20) for 1 hour at room temperature and incubated with the horseradish peroxidase-coupled antiphosphotyrosine antibody RC-20 (Transduction Laboratories, Lexington, KY) used at 1:2500 dilution in TBST with 3% BSA for 2 hours. After several washes, immunoreactive bands were visualized using Amersham's ECL detection kit according to the manufacturer's instructions. Membranes were stripped and incubated with the corresponding antibodies for loading control.

MAPK activation was determined with an antiphospho-MAPK (p42 and p44)-specific mouse monoclonal antibody (New England Biolabs, Beverly, MA), and activated Akt was measured using a polyclonal antibody specific to phosphoserine 473 (New England Biolabs). After stripping, the membranes were incubated with anti-MAPK and anti-Akt polyclonal antibodies (Santa Cruz Biotechnology) for loading control.

Proliferation assay and cell cycle studies

To monitor proliferation, cells were seeded in triplicate onto 96-well plates at a density of 7000 cells/well. After 24 hours of growth in normal medium, the cells were switched to serum-free medium and stimulated with NRG1 or NRG2 for 24 hours in the presence or absence of PG-J_2. Radiolabeled [^{3}H]thymidine (0.5 μCi) was then added and the cells were grown for an additional 24 hours. Cells were harvested and [^{3}H] radioactivity was measured in a solid scintillation counter. For cell cycle analysis, cells were treated with the NRGs for 24 hours and some of the cultures were preincubated for 10 hours with PG-J_2. Cells were then fixed in 70% ethanol/PBS, pelleted and resuspended in buffer containing 100 μg/ml RNAse A and 0.01 mg/ml propidium iodide. Cell cycle distribution was determined by cytofluorometry using the ModFit LT program.

Soft agar colony assays

Anchorage-independent growth was determined by first suspending 10,000 cells in 0.3% agar in tissue culture medium containing 5% stripped serum in 60 mm plates over a bottom layer of 0.5% agar in medium. The cells were allowed to grow for 20 days with weekly refeeding. NRGs and/or PG-J_2 were added every 3-4 days. Colonies were stained with p-iodotetrazolium violet. Experiments were carried out three times in duplicate.

Nile red staining

Nile red staining was performed essentially as described (Greenspan et al., 1985). Briefly, cells were seeded at in a 6-well plate and, after a 48 hour incubation with PG-J_2 and/or NRGs, directly stained with 1 ml of 0.1 μg/ml final concentration of the fluoresccent stain Nile red (Sigma Chemical Co.) in TD (prepared by dilution of a stock solution which was 0.1 mg/ml in acetone) for 5 minutes. Sample observation was carried out immediately after its preparation. Nile red-stained cells were then examined with a Zeiss Axiophot microscope.

Apoptosis assay

To calculate the extent of cell death, 0.5×10^6 cells were cultured in 10-cm diameter tissue culture plates and grown in RPMI for 24 hours before switching to serum-free medium containing the factors indicated in the figure legends for an additional 72 hours. For analyses, both the floating cells in the supernatant and in the PBS wash were collected from each plate. Apoptotic cells were assayed by analyzing annexin V conjugated to fluoresceinisothiocyanate (annexin-V-FITC) (Bender MedSystems, Vienna, Austria) to determine the translocation of phosphatidylserine from the inside to the outside of the plasma membrane. Cell staining was performed according to the manufacturer's instructions.

RESULTS

Effects of PPARγ activation on ErbB2/ErbB3 protein levels and phosphorylation

We first examined the ability of NRG1 and NRG2 to activate ErbB2 and ErbB3 in MCF-7 cells. Cells were treated with factor, lysed and ErbB receptors were immunoprecipitated.

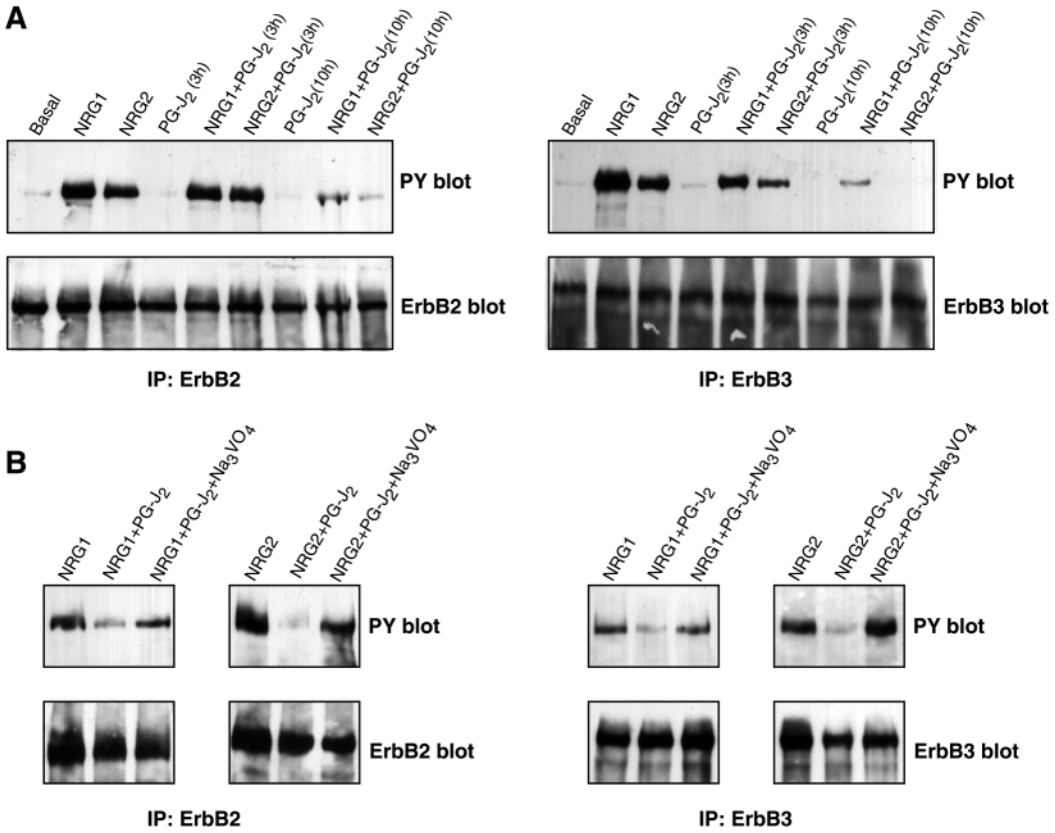


Fig. 1. Effect of PG-J_2 treatment on the phosphorylation state of ErbB2 and ErbB3. (A) Subconfluent MCF-7 cells were cultivated for 3 or 10 hours in the presence or absence of 10 μM PG-J_2 as indicated. Thereafter the cells were stimulated with NRG1 or NRG2 for 5 minutes and the lysates were used for immunoprecipitation (IP) with anti-ErbB2 or anti-ErbB3 polyclonal antibodies. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies. (B) Same experiment as in A; however, the cells were treated with 1 mM ortovanadate prior to PG-J_2 treatment.

These proteins were size-fractionated on acrylamide gels, transferred to nitrocellulose membranes and phosphorylated receptors were visualized with antiphosphotyrosine antibodies. As can be seen in Fig. 1A, the level of tyrosine phosphorylation of both ErbB2 and ErbB3 in MCF-7 cells was markedly increased by treatment with either growth factor and the extent of receptor tyrosine phosphorylation was similar for both neuregulins, although NRG1 was slightly more efficient in stimulating phosphorylation than NRG2.

To assess the effect of activation of PPARγ upon phosphorylation of ErbB2 and ErbB3, PG-J_2 ligand was applied to MCF-7 cells 3 and 10 hours before the addition of neuregulin factor, which was applied for 5 minutes prior to cell harvesting. As shown in Fig. 1A, the addition of PG-J_2 virtually eliminated the observed phosphorylation of both ErbBs. This effect was only observed when MCF-7 cultures were preincubated for 10 hours. After stripping, the blots were reprobed with either anti-ErbB2 or anti-ErbB3 and it was determined that treatment of MCF-7 cells with PG-J_2 did not alter the level of ErbB protein. When transcription was inhibited with actinomycin D, the dephosphorylation effect of PG-J_2 was almost completely abolished (data not shown). These data imply that the novo transcription is required for PG-J_2 to alter ErbB2/3 phosphorylation in MCF-7 cells.

To ascertain the possible involvement of PTPases in this reduction of receptor phosphorylation by PG-J_2, MCF-7 cells were treated with 1 mM pervanadate prior to PG-J_2 stimulation. We determined that the dephosphorylation of the ErbB2 and ErbB3 receptors was almost completely blocked by the addition of pervanadate to the culture medium (Fig. 1B). These data suggest that the reduction in phosphotyrosine content of the ErbB receptors induced by PG-J_2 is mediated by PTPases.

To determine whether PG-J_2 effects were restricted to ErbB2 and ErbB3 phosphorylation, the effect of this prostaglandin on phosphorylation of two other receptor tyrosine kinases: epidermal growth factor receptor (EGF-R) and insulin-like growth factor I receptor (IGF-IR) was examined. As shown in Fig. 2, the level of tyrosine phosphorylation of EGF-R and IGF-IR in MCF-7 cells was significantly increased by treatment with EGF and IGF-I, respectively. The addition of PG-J_2 markedly decreased the observed phosphorylation of IGF-IR without affecting the phosphotyrosine content of EGF-R.

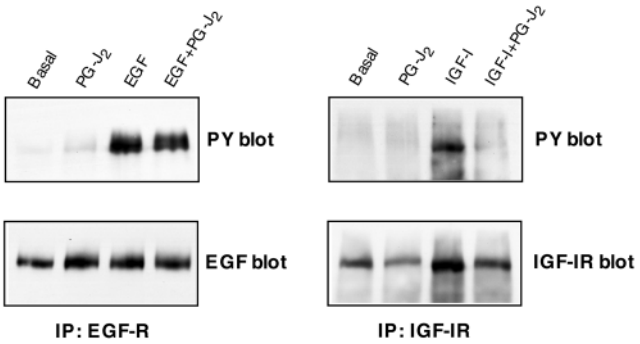


Fig. 2. Effect of PG-J_2 treatment on the phosphorylation state of EGF-R and IGF-IR. Subconfluent MCF-7 cells were cultivated for 10 hours in the presence or absence of 10 μM PG-J_2 as indicated. Thereafter the cells were stimulated with EGF or IGF-I for 5 minutes and the lysates were used for immunoprecipitation (IP) with anti-EGF-R or anti-IGF-IR polyclonal antibodies. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies.

Effects of NRGs and PPARγ activation on cell growth and transformation

The effects of ligand activation of PPARγ on cell growth was assessed by monitoring cell proliferation in a [^{3}H]thymidine incorporation assay. Treatment of MCF-7 cells with PG-J_2 for 24 hours resulted in a 90% inhibition of thymidine incorporation (Fig. 3A). By contrast, addition of either NRG1 or NRG2 caused a twofold increase in growth rate. However, when NRG1 or NRG2 were added to the culture medium together with PG-J_2, the prostaglandin completely abolished the ability of either neuregulin to stimulate thymidine incorporation (Fig. 3A). These results reveal that the activation of PPARγ blocks the pathway activated by the neuregulins to promote cell proliferation.

To better understand the nature of the growth retardation associated with PG-J_2 treatment, MCF-7 cells were synchronized in G0/G1 by serum starvation and their progression through the cell cycle was followed for 24 hours upon release into normal medium or medium supplemented with the neuregulins and/or PG-J_2. The proportion of cells at specific stages of the cell cycle was determined by flow cytometry. It was observed (Fig. 3B) that the addition of NRG1 or NRG2 induced a significant increase in the number of cells in the S phase of the cell cycle (51% and 44%, respectively). By contrast, the cell cycle profiles of MCF-7 cells preincubated with PG-J_2 for 10 hours before the addition of NRG1 or NRG2 were significantly altered, with a complete block at the G0/G1 boundary and almost no cells progressing through the S phase. In randomly proliferating MCF-7 cells PG-J_2 causes a G2/M arrest (data not shown), suggesting that the prostaglandin can be acting at different stages of the cell cycle.

To identify intracellular signal transduction pathways linked to NRG1 and NRG2-induced cell cycle progression, we treated MCF-7 cells with specific kinase inhibitors. Treatment of MCF-7 cells with LY294002, an inhibitor of PI 3-K, prevented NRG1 and NRG2-induced G1 progression (Fig. 3B). However, PD98509, an inhibitor of the mitogen-activated protein kinase pathway that selectively inhibits the MAPK activating enzyme, MAP kinase kinase (MEK), did not prevent NRG1 and NRG2-induced cell cycle progression. These data suggest that it is the PI 3-K pathway and not the MAPK pathway that is required for neuregulin-induced DNA synthesis.

To better understand the regulation of the PI 3-K signaling pathway by neuregulins, and the possible involvement of PG-J_2, MCF-7 cells were preincubated with PG-J_2 and then treated with either NRG1 or NRG2. Phosphorylation of Akt was analyzed by using a phospho-specific antibody (which recognizes Akt only when phosphorylated at the Ser-473 residue). As shown in Fig. 4A, Akt phosphorylation was not present in unstimulated serum-starved MCF-7 cells. Stimulation with both NRG1 and NRG2 dramatically increased phosphorylation of Akt within 10 minutes of treatment. When cells were preincubated with prostaglandin, a significant suppression of Akt phosphorylation was detected.

One measure of cell transformation is the ability of cells to exhibit anchorage independent growth. MCF-7 cells treated either with NRG1 or NRG2 exhibit the ability to grow

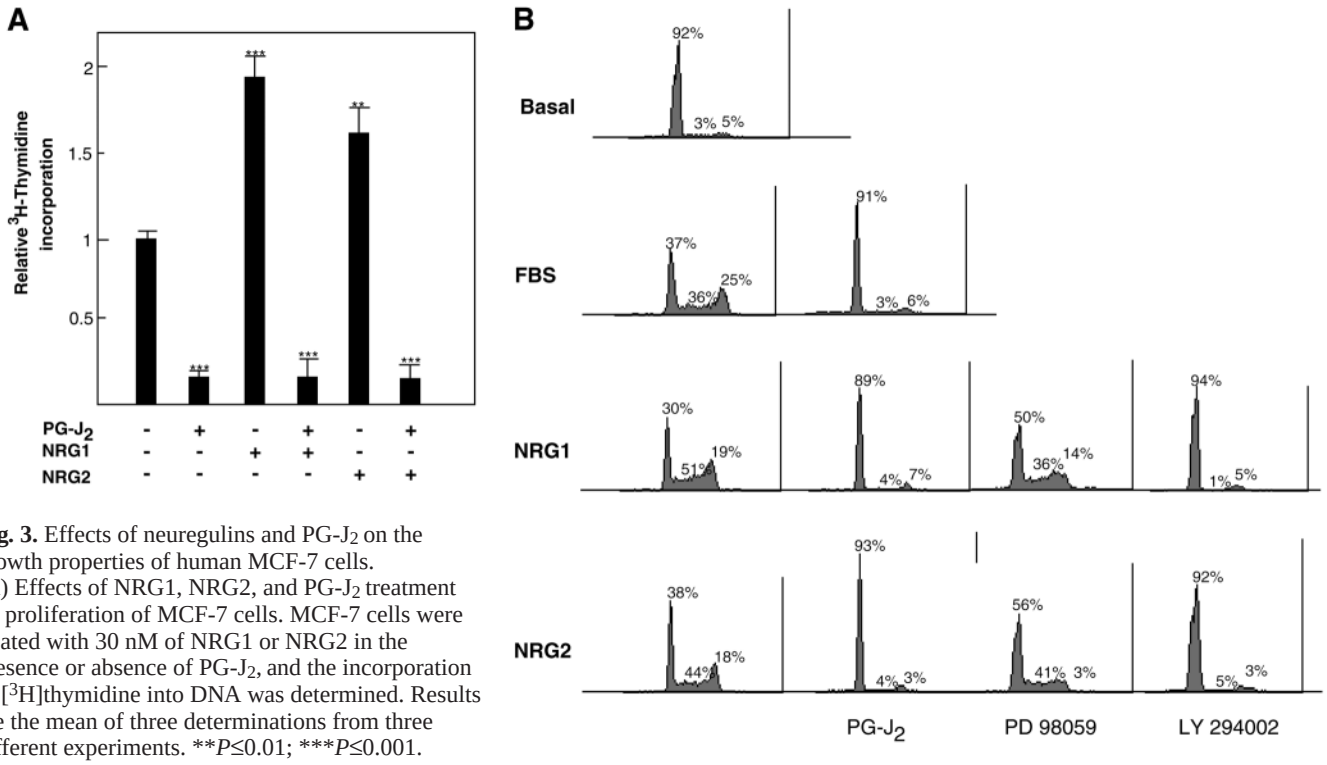
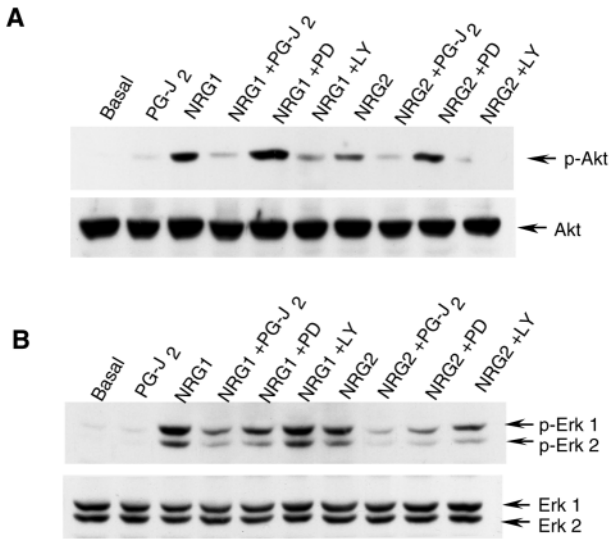


Fig. 3. Effects of neuregulins and PG-J_2 on the growth properties of human MCF-7 cells. (A) Effects of NRG1, NRG2, and PG-J_2 treatment on proliferation of MCF-7 cells. MCF-7 cells were treated with 30 nM of NRG1 or NRG2 in the presence or absence of PG-J_2, and the incorporation of [^{3}H]thymidine into DNA was determined. Results are the mean of three determinations from three different experiments. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. (B) Effects of PG-J_2 and kinase inhibitors on NRG-1 and NRG-2 effects on cell cycle progression. Cells were grown for 24 hours in the presence or absence of NRG1 or NRG2 preincubated or not for 10 hours with PG-J_2, and analyzed by PI staining and FACS analysis. Inhibitors of MEK1 and 2 (40 μM PD98059) or PI 3-K (4 μM LY294002) were added 1 hour before treatment with neuregulins. Curves modeling the G0/G1, S, and G2/M compartments, derived by using the ModFit program, are shown. Cell number is plotted on the *y* axes, and DNA content is plotted on the *x* axes.

numerous colonies in soft agar (Fig. 5). After 20 days in culture, these colonies were large enough to be visible upon inspection, even when unstained. Cells treated with PG-J_2 did not exhibit anchorage-independent growth. These results suggest that PG-J_2 treatment can restore normal cellular growth characteristics and abrogate the transforming effects of the neuregulins on MCF-7 cells, although the observed effects could be due to the potent anti-mitogenic activity of this prostaglandin.



Effects of NRGs and PPARγ activation on differentiation and programmed cell death

To determine the effects of PPARγ activation on MCF-7 cell differentiation, PG-J_2 was applied to MCF-7 cells. As shown in Fig. 6, when the cells were treated with 10 μM PG-J_2 for 48 hours, they underwent a dramatic morphological change where they became filled with neutral lipids that stained with Nile red. Only a very small number of stained cells were observed in the control condition and after incubation with NRG1 or NRG2. Addition of PG-J_2 to the culture medium along with the neuregulins resulted in approximately the same percentage of stained cells as when prostaglandin was added alone. Thus activation of PPARγ can stimulate a distinct change in the appearance of these breast cancer cells even in the presence of ErbB ligands.

Fig. 4. Activation of MAPK and PI 3-K by NRG1 and NRG2. Cells were stimulated with NRG1 or NRG2 and some cultures were preincubated for 1 hour with PD98059 (40 μM) or LY294002 (4 μM), or for 10 hours with PG-J2. (A) PI 3-K activation was determined by measuring the extent of Akt phosphorylation by western blotting using an anti-phospho-Akt-specific goat polyclonal antibody. Total Akt protein was determined by stripping and reprobing the blot with an anti-Akt polyclonal antibody. (B) MAPK activation was determined by measuring the extent of MAPK phosphorylation by western blotting using an antiphospho-MAPK (p42 and p44)-specific mouse monoclonal antibody. Total MAPK protein content was determined by stripping and reprobing the blot using an anti-MAPK-p42 and an anti-MAPK-p44 polyclonal antibody.

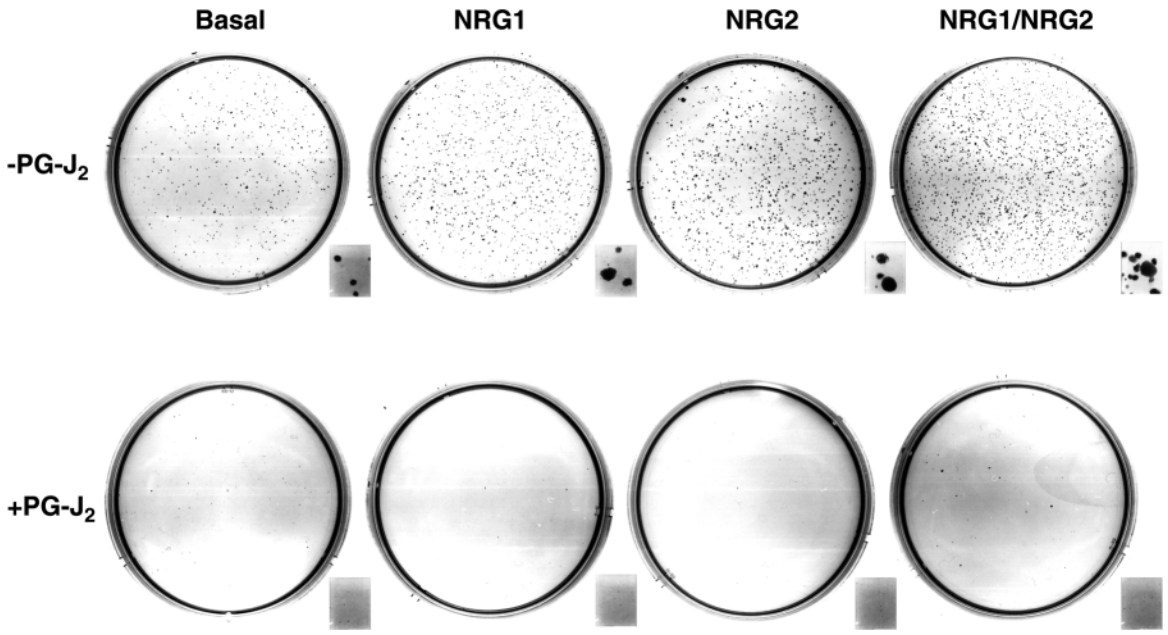


Fig. 5. Effects of NRG1, NRG2 and/or PG-J_2 on the clonogenicity of MCF-7 cells. After 24 hours of seeding, cells were treated as indicated in Materials and Methods. Representative plates and microphotographs (25-fold magnification) of clones are shown.

To determine if NRG1 and NRG2 were able to affect programmed cell death and whether the addition of PG-J_2 could overcome this effect, we added NRG1 or NRG2, either alone or in combination with PG-J_2, and measured the number of apoptotic cells 72 hours after treatment. Apoptotic cells were assessed by staining with annexin-V-FITC and quantified by flow cytometry. As shown in Fig. 7, untreated cells incubated in the absence of serum yielded approximately 10% annexin-V-FITC$^+$ apoptotic cells. Under these conditions both NRG1 and NRG2 induced a significant increase in cell survival, whereas activation of PPARγ completely abolished this effect and instead led to a significant increase in the apoptotic population.

We next tested which signaling pathways were essential for the survival effect induced by neuregulins in these cells. Fig. 7 shows that NRG1 and NRG2-induced survival could be largely repressed by adding the MAPK inhibitor PD98059, but only weakly repressed by the PI 3-K inhibitor LY294002, suggesting that neuregulin-induced survival in MCF-7 cells is primarily mediated through the MAPK pathway. Exposure of cells to NRG1 or NRG2 results in a significant ERK activation, whereas MCF-7 cells not treated with the neuregulins show only a very small level of kinase activity (Fig. 4B). However, when MCF-7 cells are preincubated with PG-J_2 no activation of ERK was observed, suggesting a

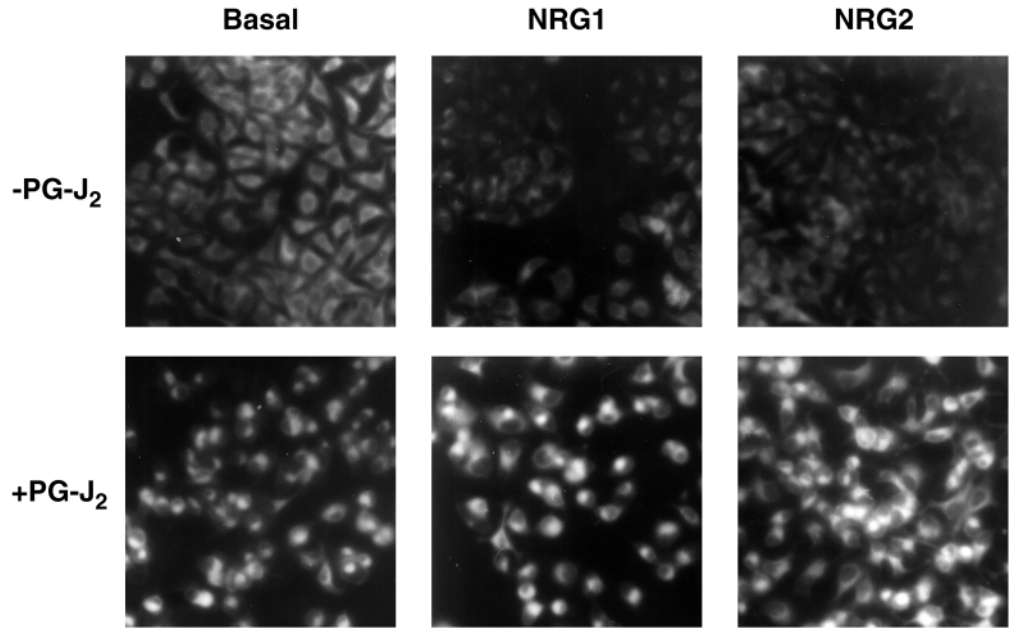


Fig. 6. Fluorescence microphotographs (100-fold magnification) of MCF-7 cells stained with 0.1 μg/ml of Nile red and viewed at yellow-gold fluorescence.

block of the MAP kinase cascade induced by these neuregulins.

Finally, to address the question of whether the dephosphorylation/deactivation effect of PG-J_2 was restricted to MCF-7 cells, we next examined the effects of PG-J_2 in two other human mammary epithelial cell lines: T47D and SKBR3, the latter having a high basal ErbB2 phosphotyrosine content. We first analyzed the phosphorylation state of ErbB2 and ErbB3 in cells stimulated with NRG1 or NRG2 and preincubated or not with PG-J_2. As shown in Fig. 8A, NRG1-induced phosphorylation of ErbB2 and ErbB3 in T47D was completely abolished by PG-J_2 preincubation. The same results were obtained with NRG2 (data not shown). In SKBR3 cells, as expected, ErbB2 was already highly phosphorylated in basal conditions and no further effect of the NRGs was observed. By contrast, ErbB3 phosphorylation was clearly induced by NRGs (only NRG1 data are shown). Preincubation with PG-J_2 dramatically blocked the basal phosphorylation levels of ErbB2. NRG1-induced phosphorylation of ErbB3 was also significantly inhibited by this prostaglandin (Fig. 8A). Next, we examined the effect of ligand activation of PPARγ on cell growth of T47D and SKBR3 by analyzing thymidine incorporation (Fig. 8B). Consistent with its effects on ErbB2 and ErbB3 phosphorylation, PG-J_2 also caused a dramatic decrease in basal and neuregulin-induced T47D and SKBR3 cell proliferation.

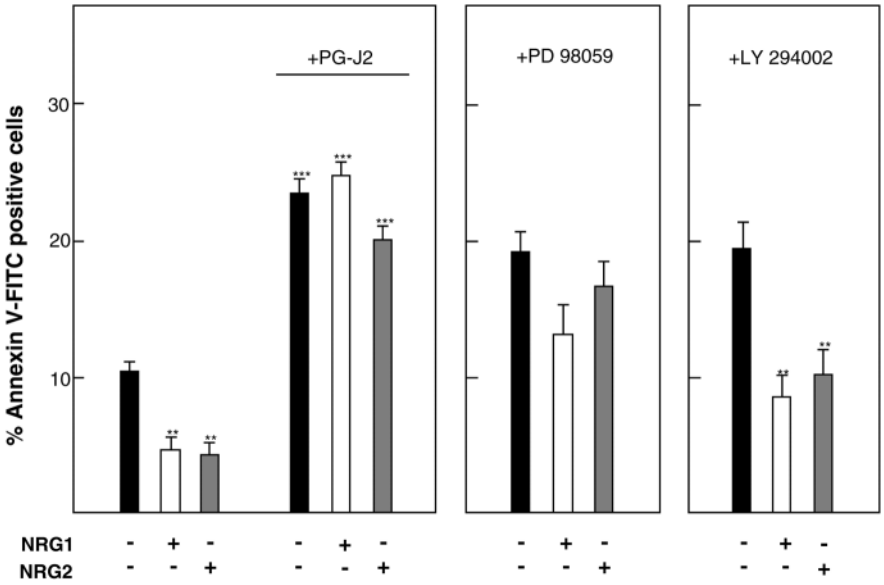


Fig. 7. Effects of NRG1, NRG2 and/or PG-J_2 on apoptosis of MCF-7 cells. Cells were cultured for 72 hours in serum-free medium containing the indicated factors, and apoptotic cells were detected by measuring annexin V-FITC binding by flow cytometry. Inhibitors of MEK1 and -2 (40 μM PD98059) or PI 3-K (4 μM LY294002) were added 1 hour before treatment with the neuregulins. Results are the mean of three determinations from three different experiments. **$P \leq 0.01$; ***$P \leq 0.001$.

DISCUSSION

Upon neuregulin stimulation, the ErbB2 and ErbB3 receptors are phosphorylated on multiple tyrosine residues that function as docking sites for cytoplasmic signaling proteins that contain a phosphotyrosine binding motif (SH2 or PTB domains) (Olayioye et al., 2000). In this paper, we show that activation of ErbB2 and ErbB3 in MCF-7 cells results in a number of changes including an increase in cell proliferation and enhanced cell survival. Moreover, results from soft agar assays reveal that NRG1 and NRG2 promote anchorage-independent growth of these cells. Importantly, we demonstrate that treatment of MCF-7 cells with a natural ligand of PPARγ, PG-J_2, dramatically reduces ligand-induced ErbB phosphorylation. This is accompanied by a complete block of the effects of the NRGs on these cells, as the treatment of MCF-7 breast cancer cells with the prostaglandin PG-J_2 inhibits growth and tumorigenicity induced by ErbB activation. Inhibition of ErbB2 and ErbB3 phosphorylation by PG-J_2 leads to G1 arrest and induction of apoptosis in MCF-7 cells. These observations provide a possible mechanism for the observed effects of prostaglandins on breast cancer cells.

Although PPARγ was initially described in adipocytes and hepatocytes, recently it was shown that this receptor is expressed in macrophages and in human mammary epithelial cell lines. Elstner et al. have recently found that breast cancer cells express high levels of this receptor, whereas normal human breast epithelial cells lining the mammary ducts express only a low level of PPARγ protein (Elstner et al., 1998). These authors have also shown that some ligands of PPARγ, such as troglitazone (TGZ), inhibited the clonal growth of several breast cancer cell lines and slightly increased the levels of the apoptotic population. Also, an anti-tumor effect of PPARγ ligands was observed in mice injected with prostate tumor cells and it has been shown that troglitazone promotes terminal differentiation of human liposarcoma cells in vitro and in patients suffering from advanced liposarcoma (Demetri, 1999). These data, along with the findings of others (Kilgore et al., 1997; Gimble, 1998; Mueller et al., 1998) suggest a possible role for ligand-activated PPARγ as an anti-tumor agent in breast cancer. The evidence presented here not only supports this hypothesis but also indicates a possible mechanism by which prostaglandins could affect the proliferation, differentiation and death of breast cancer cells.

Our studies provide a link between PPARγ and ErbB signaling, with PPARγ being able to suppress ErbB activation dramatically. The negative effects of PPARγ on ErbB signaling appear to block the cell's ability to mount proliferative and anti-apoptotic responses, thereby ensuring a non-proliferative outcome regardless of the presence of activating ErbB ligands. Based on the relatively long period of time (10 hours) required to achieve its effect, and its sensitivity to actinomycin D, it appears that the effects of PG-J_2 are directly mediated through PPARγ activation. The NRGs have been implicated in a wide variety of physiological and developmental processes including cardiac development, the proliferation and differentiation of oligodendroglial and Schwann cell precursors, the formation of the neuromuscular synapse,

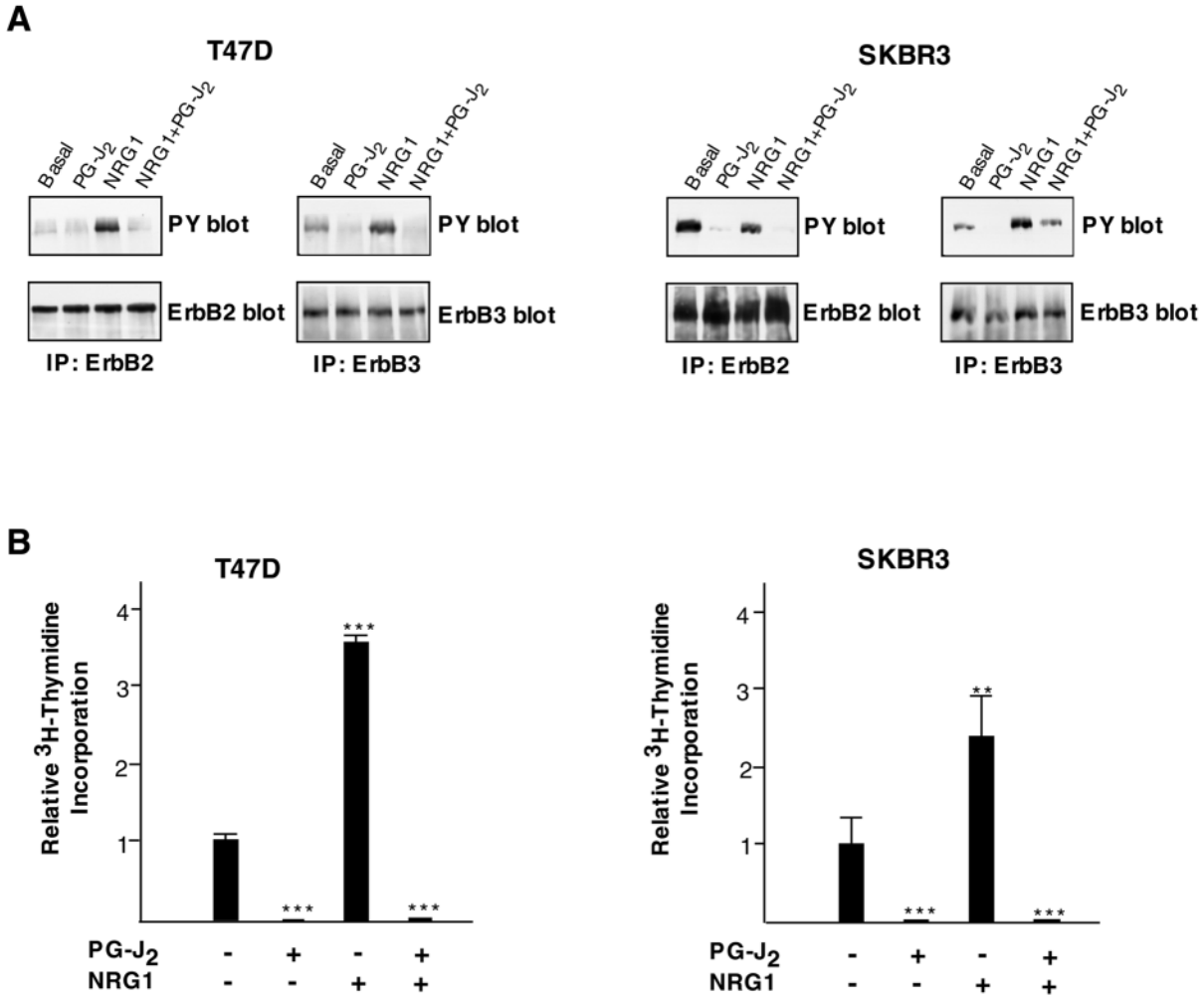


Fig. 8. Effect of PG-J_2 on T47D and SKBR3 breast cancer cell lines. (A) Effect of PG-J_2 treatment on the phosphorylation state of ErbB2 and ErbB3. Subconfluent cells were cultivated for 10 hours in the presence or absence of 10 μM PG-J_2 as indicated. Thereafter the cells were stimulated with NRG1 for 5 minutes and the lysates were used for immunoprecipitation (IP) with anti-ErbB2 or anti-ErbB3 polyclonal antibodies. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies. (B) Effects of NRG1 and PG-J_2 treatment on cell proliferation. Both cell lines were treated with 30 nM of NRG1 in the presence or absence of PG-J_2, and the incorporation of [^{3}H]thymidine into DNA was determined. Results are the mean of three determinations from two different experiments. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

epithelial morphogenesis, as well as in pathological states (Burden and Yarden, 1997). The discovery of additional NRG genes has increased the potential signaling complexity of the NRG/ErbB network. NRGs have been shown to be potent mitogens for Schwann cells and astrocytes in cell culture as well as inhibitors of apoptosis in cardiac myocytes (Lemke, 1996; Zhao et al., 1998). However, several reports show that, in addition to promoting proliferation, NRGs can induce apoptosis (Daly et al., 1997; Kirchhoff and Hauser, 1999; Daly et al., 1999). Our results further support a role for the NRGs in promoting the proliferation of breast cancer cells, but differ from the pro-apoptotic effects reported by other authors, as we have observed that NRG1 and NRG2 have a significant anti-apoptotic effect in MCF-7 cells.

In this work we have also examined the signaling pathways involved in neuregulin-induced cell proliferation and survival. Our results suggest that, in MCF-7 cells, G1 progression is associated with the phosphatidylinositol 3-kinase pathway, whereas the anti-apoptotic effects were dependent on ERK1/2 activation. The inhibition of ERK1 and ERK2 by the MEK inhibitor PD98059 largely blocks the survival effects of NRG1 and NRG2 but has little effect on cell cycle progression. These results are noteworthy as, in most systems, activation of the MAPK signaling pathway has been associated with cell proliferation (Pages et al., 1993; Dhnasekaran and Reddy, 1998). The PI 3-K inhibitor LY294002 blocks Akt phosphorylation in MCF-7 cells, a finding that correlates with the abolishment of NRG1 and NRG-2-induced proliferation. This inhibitor has little effect on the reduction of apoptosis. These results are in agreement with previous data showing an induction of G1 progression in the breast cancer cell line SKBR3 through the PI 3-K pathway (Daly et al., 1999). However, studies with cells of the oligodendrocyte lineage show that activation of Akt by heregulin plays a major role in cell survival but has no effect upon proliferation (Flores et al., 2000). The role of Akt in the regulation of the cell cycle has

received significantly less attention than its role in the regulation of apoptosis. Cell cycle regulation by Akt was first observed by Ahmed et al. (Ahmed et al., 1997) and was later confirmed by Brennan et al. who showed that Akt transduces PI-3K-dependent IL-2 signals leading to the phosphorylation of Rb and promoting the activation of E2F (Brennan et al., 1997).

The present data demonstrate that PG-J_2, a specific ligand of PPARγ, is a robust inhibitor of ErbB signaling pathways in the MCF-7 breast cancer cell line and is capable of completely blocking the transforming capacity of ErbB2 and ErbB3. These findings indicate that prostaglandins inhibit mammary epithelial proliferation and induce apoptosis, at least in part by antagonizing the actions of the neuregulins via receptor dephosphorylation. The strong blocking effect of PG-J_2 on cell cycle progression induced by serum (Fig. 3B) suggests that other growth factor signaling pathways may be the target of the action of this prostaglandin. In fact, we have also demonstrated that, in addition to blocking the ErbB signaling pathway, PG-J_2 caused a significant decrease in the phosphotyrosine content of IGF-IR, without modifying the phosphorylation levels of EGF-R. These results suggest that, although not all signaling pathways are affected, PG-J_2 blocks other tyrosine kinase systems in breast cancer cells. Interestingly, the effects of PG-J_2 seem to be quite general, since the ErbB tyrosine phosphorylation is also completely abolished in other breast cancer cell lines, such as T47D and SKBR3.

In conclusion, these observations suggest that PPARγ and locally produced prostaglandin D2 metabolites may be involved in the regulation of cancer cell growth and development. These findings raise the possibility that synthetic PPARγ ligands may be of therapeutic value in human diseases, such as breast cancer, in which activated ErbB receptors play prominent pathogenic roles.

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