Estrogen Receptor α Binds to Peroxisome Proliferator–Activated Receptor Response Element and Negatively Interferes with Peroxisome Proliferator–Activated Receptor γ Signaling in Breast Cancer Cells

Daniela Bonofiglio,[1] Sabrina Gabriele,[1] Saveria Aquila,[1] Stefania Catalano,[1] Mariaelena Gentile,[2] Emilia Middea,[1] Francesca Giordano,[2] and Sebastiano Andò[2,3]

Abstract

Purpose: The molecular mechanisms involved in the repressive effects exerted by estrogen receptors (ER) on peroxisome proliferator–activated receptor (PPAR) γ–mediated transcriptional activity remain to be elucidated. The aim of the present study was to provide new insight into the crosstalk between ERα and PPARγ pathways in breast cancer cells.

Experimental Design: Using MCF7 and HeLa cells as model systems, we did transient transfections and electrophoretic mobility shift assay and chromatin immunoprecipitation studies to evaluate the ability of ERα to influence PPAR response element–mediated transcription. A possible direct interaction between ERα and PPARγ was ascertained by coimmunoprecipitation assay, whereas their modulatory role in the phosphatidylinositol 3-kinase (PI3K)/AKT pathway was evaluated by determining PI3K activity and AKT phosphorylation. As a biological counterpart, we investigated the growth response to the cognate ligands of both receptors in hormone-dependent MCF7 breast cancer cells.

Results: Our data show for the first time that ERα binds to PPAR response element and represses its transactivation. Moreover, we have documented the physical and functional interactions of ERα and PPARγ, which also involve the p85 regulatory subunit of PI3K. Interestingly, ERα and PPARγ pathways have an opposite effect on the regulation of the PI3K/AKT transduction cascade, explaining, at least in part, the divergent response exerted by the cognate ligands 17β-estradiol and BRL49653 on MCF7 cell proliferation.

Conclusion: ERα physically associates with PPARγ and functionally interferes with PPARγ signaling. This crosstalk could be taken into account in setting new pharmacologic strategies for breast cancer disease.

Peroxisome proliferator–activated receptors (PPAR) are ligand-activated transcription factors belonging to the nuclear receptor superfamily (1). Activation of PPARs is a multistep process that involves ligand binding, heterodimerization with retinoic X receptor (RXR), interaction with cognate DNA sequences, and recruitment of coregulatory proteins (1). Three PPAR isoforms, α, β/δ, and γ, are expressed in multiple species in a tissue-specific manner (2–4). As for PPARγ, its involvement has been reported in several metabolic pathways, in adipocyte differentiation, and even in the growth inhibition of different cancer cell lines (5–14). In addition, PPARγ promoted terminal differentiation of malignant breast epithelial cells *in vitro* and induced morphologic changes associated with apoptosis and fibrosis in breast tumor cells injected in mice (15, 16).

A large body of evidence has shown that estrogen receptor (ER) α is involved in the development of breast cancer (17–20). On ligand binding, ERα undergoes a conformational change allowing chromatin interaction and the transcriptional regulation of target genes (21). It has also been reported that ERα binds to the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), leading to the activation of the protein kinase B/AKT pathway, which in turn regulates diverse processes like cell survival and proliferation (22, 23).

Recently, an increasing physiologic significance has been attributed to the crosstalk among nuclear receptors, which was observed at several levels of the signal transduction cascades (24–28). As it concerns the interaction between PPAR and ER pathways, the PPAR/RXR heterodimer has been shown to bind to estrogen response element (ERE)–related palindromic sequences; however, it cannot transactivate due to a nonpermissive natural promoter structure (26). On the other hand, ERs negatively interfere with PPAR response element (PPRE)–mediated transcriptional activity (29); however, the molecular mechanisms involved still remain to be elucidated.

Authors' Affiliations: Departments of [1]Pharmaco-Biology and [2]Cell Biology, and [3]Centro Sanitario, University of Calabria, Cosenza, Italy

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Requests for reprints: Sebastiano Andò, Department of Cell Biology, University of Calabria, Cosenza 87036, Italy. Phone: 39-0984-496201; Fax: 39-0984-496203; E-mail: sebastiano.ando@unical.it.

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Herein, we have shown for the first time, to our knowledge, that ERα binds to PPRE even in the context of the endogenous phosphatase and tensin homologue deleted on chromosome 10 (PTEN) promoter sequence, physically interacts with PPARγ, and generates a ternary complex involving the p85 regulatory subunit of PI3K. Moreover, ERα and PPARγ induce opposite effects on the regulation on the PI3K/AKT pathway eliciting consequently divergent growth responses on treatment with the respective cognate ligands 17β-estradiol and rosiglitazone (BRL49653) in hormone-dependent MCF7 breast cancer cells.

Materials and Methods

Reagents. BRL49653 was a kind gift from GlaxoSmithKline (West Sussex, United Kingdom). The irreversible PPARγ antagonist GW9662, 17β-estradiol, and hydroxytamoxifen were purchased by Sigma (Milan, Italy). ICI182780 was generously provided by Zeneca Pharmaceuticals (Cheshire, United Kingdom). All compounds were solubilized in DMSO or in ethanol (Sigma).

Plasmids. The pGL_3 vector containing three copies of a PPRE sequence upstream of the minimal thymidine kinase promoter ligated to a luciferase reporter gene (3XPPRE-TK-pGL_3) and the PPARγ expression plasmid were a gift from Dr. R. Evans (The Salk Institute, San Diego, CA). The expression vector of ERα and androgen receptor were previously described (30, 31). The ERβ expression vector was provided by Dr. J.A. Gustafsson (Karoliska Institute, Stockholm, Sweden). The constitutively active myristilated AKT mutant (myr-AKT) was kindly provided by Dr. T. Simoncini (University of Pisa, Pisa, Italy).

Cell cultures. Wild-type human breast cancer ERα-positive MCF7 cells (a gift from E. Surmacz, Sbarro Institute for Cancer Research and Molecular Medicine, Philadelphia, PA) were grown in DMEM-F12 containing 10% FCS, 1% L-glutamine, 1% Eagle's nonessential amino acids, and 1 mg/mL penicillin-streptomycin. The ER-negative HeLa cells were maintained with DMEM supplemented with 10% FCS, 1% L-glutamine, and 1 mg/mL penicillin-streptomycin.

Transfection assay. Transient transfection experiments were done using 3XPPRE-TK ligated to a luciferase reporter gene into the pGL_3 vector. Cells were transferred into 24-well plates with 500 μL of regular growth medium/well the day before transfection. The medium was replaced with DMEM or DMEM-F12 lacking phenol red and serum on the day of transfection, which was done using Fugene 6 reagent as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany) with a mixture containing 0.5 μg of reporter plasmid, 5 ng of pRL-CMV, and 0.1 μg of effector plasmid where applicable. Empty vectors were used to ensure that DNA concentrations were constant in each transfection. After 6 hours of transfection, the medium was changed and the cells were treated in serum-free DMEM or DMEM-F12 in the presence of 10 μmol/L BRL49653, 1 μmol/L ICI182780, 1 μmol/L hydroxytamoxifen, and 10 μmol/L GW9662 for 18 hours.

Firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Kit (Promega, Madison, WI). The firefly luciferase values of each sample were normalized by *Renilla* luciferase activity and data were reported as relative light units.

HeLa cells were plated in 10 cm dishes and then transfected with 5 μg ERα expression plasmid using Fugene 6 reagent to perform the electrophoretic mobility shift assay (EMSA). To determine PI3K activity, MCF7 cells were plated in 10 cm dishes and then transfected with 5 μg PPARγ and 5 μg ERα expression plasmids using FuGENE 6 reagent. To evaluate the role of PI3K/AKT pathway in MCF7 cell growth, 0.5 μg constitutively active myr-AKT was transfected using Fugene 6 reagent in six-well plates every 2 days where applicable.

Electrophoretic mobility shift assay. Nuclear extracts from MCF7 and HeLa cells were prepared as previously described (32). Briefly, MCF7 and HeLa cells plated into 10 cm dishes were scraped into 1.5 mL of cold PBS. Cells were pelleted for 10 seconds and resuspended in 400 μL cold buffer A [10 mmol/L HEPES-KOH (pH 7.9) at 4°C, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L leupeptin] by flicking the tube. The cells were allowed to swell on ice for 10 minutes and then vortexed for 10 seconds. Samples were then centrifuged for 10 seconds and the supernatant fraction discarded. The pellet was resuspended in 50 μL of cold buffer B [20 mmol/L HEPES-KOH (pH 7.9), 25% glycerol, 1.5 mmol/L $MgCl_2$, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L leupeptin] and incubated on ice for 20 minutes for high-salt extraction. Cellular debris were removed by centrifugation for 2 minutes at 4°C and the supernatant fraction (containing DNA binding proteins) was stored at −70°C. *In vitro* transcribed and translated PPARγ and ERα were synthesized using the T7 polymerase in the rabbit reticulocyte lysate system as directed by the manufacturer (Promega). The probe was generated by annealing single-stranded oligonucleotides and labeled with [γ-^{32}P]ATP (Amersham Pharmacia, Buckinghamshire, United Kingdom) and T4 polynucleotide kinase (Promega), and then purified using Sephadex G50 spin columns (Amersham Pharmacia).

A double-stranded PPRE was prepared by annealing the following sense and antisense-oligonucleotides: 5′-GGGACCAGGACAAAGGTCACGTT-3′ and 5′-GGGAACGTGACCTTTGTCCTGGTC-3′ (Sigma Genosys, Cambridge, United Kingdom). As control for nonspecific binding, a cold PPRE competitor was included. The protein binding reactions were carried out in 20 μL of buffer [20 mmol/L HEPES (pH 8), 1 mmol/L EDTA, 50 mmol/L KCl, 10 mmol/L DTT, 10% glycerol, 1 mg/mL bovine serum albumin, 50 μg/mL poly(deoxyinosinic-deoxycytidylic acid)] with 50,000 cpm of labeled probe, 5 μg of MCF7 and HeLa nuclear protein, or 2 μL of *in vitro* transcribed and translated PPARγ and ERα proteins, and 5 μg of poly(deoxyinosinic-deoxycytidylic acid). The mixtures were incubated at room temperature for 20 minutes in the presence or absence of unlabeled competitor oligonucleotides or *in vitro* transcribed and translated PPARγ and ERα proteins. For the experiments involving PPARγ and ERα (F-10 and D-12) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), the reaction mixture was incubated with these antibodies at 4°C for 30 minutes before addition of labeled probe. Under the conditions employed, cross-reactivity of the PPARγ and ERα antibodies was not observed in EMSA supershift assays (data not shown). The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25× Tris-borate-EDTA for 3 hours at 150 V. Gel was dried and subjected to autoradiography at −70°C.

Chromatin immunoprecipitation. According to the chromatin immunoprecipitation (ChIP) assay procedure previously described (33), MCF7 cells were grown in 10 cm dishes to 50% to 60% confluence, shifted to serum-free medium for 24 hours, and then treated with 10 μmol/L BRL49653, 10 μmol/L GW9662, 100 nmol/L 17β-estradiol, and 1 μmol/L ICI182780 for 1 hour. Thereafter, cells were washed twice with PBS and cross-linked with 1% formaldehyde at 37°C for 10 minutes. Next, cells were washed twice with PBS at 4°C, collected and resuspended in 200 μL of lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.1)], and left on ice for 10 minutes. Then, cells were sonicated four times for 10 seconds at 30% of maximal power (Sonics, Vibra Cell 500 W) and collected by centrifugation at 4°C for 10 minutes at 14,000 rpm. The supernatants were diluted in 1.3 mL of immunoprecipitation buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl (pH 8.1), 16.7 mmol/L NaCl] followed by immunoclearing with 80 μL of sonicated salmon sperm DNA/protein A agarose (UBI, Lake Placid, NY) for 1 hour at 4°C. The precleared chromatin was immunoprecipitated with specific antibodies anti-PPARγ (H-100, Santa Cruz Biotechnology) and anti-ERα (F-10, Santa Cruz Biotechnology). At this point, 60 μL of salmon sperm DNA/protein A agarose were added and precipitation was further continued for 2 hours at 4°C. After pelleting, precipitates were sequentially washed for 5 minutes with the following buffers: wash A [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.1), 150 mmol/L NaCl], wash B [0.1% SDS, 1% Triton X-100, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 8.1), 500 mmol/L NaCl], and

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wash C [0.25 mol/L LiCl, 1% NP40, 1% sodium deoxycholate, 1 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 8.1)], and then twice with Tris-EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA). The immunocomplexes were eluted with elution buffer (1% SDS, 0.1 mol/L $NaHCO_3$). The eluates were reverse cross-linked by heating at 65°C and digested with proteinase K (0.5 mg/mL) at 45°C for 1 hour. DNA was obtained by phenol/chloroform/isoamyl alcohol extraction. Two microliters of 10 mg/mL yeast tRNA (Sigma) were added to each sample and DNA was precipitated with 70% ethanol for 24 hours at −20°C, and then washed with 95% ethanol and resuspended in 20 μL of Tris-EDTA buffer. Five microliters of each sample were used for PCR amplification with the following primers flanking a PPRE sequence present in the *PTEN* promoter region: 5′-AGAGACTTATAACTGGGCAGG-3′ (forward) and 5′-CAAGTGATATCATATGTGATGCTG-3′ (reverse). The PCR conditions for PPRE in *PTEN* promoter fragment were 45 seconds at 94°C, 40 seconds at 57°C, and 90 seconds at 72°C. The amplification products obtained in 30 cycles were analyzed in 2% agarose gel and visualized by ethidium bromide staining. The negative control was provided by PCR amplification without DNA sample. The specificity of reactions was ensured using normal mouse and rabbit immunoglobulin G (Santa Cruz Biotechnology).

Reverse chromatin immunoprecipitation. According to the reverse ChIP procedure previously described (34), pellets obtained by immunoprecipitation of soluble chromatin with PPARγ antibody were eluted with 500 μL of reverse-ChIP buffer [0.5 mmol/L DTT, 1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 8.1)]. Next, the eluate from PPARγ immunoprecipitation was precipitated with anti-p85 (B-9, Santa Cruz Biotechnology) and anti-ERα (F-10) antibodies. The presence of PPRE in the *PTEN* promoter sequences in the resulting reverse-ChIP pellets was examined as described above for one-step ChIP.

Reverse transcription-PCR assay. MCF7 cells were grown in 10 cm dishes to 70% to 80% confluence and exposed to treatments for 24 hours in 1% charcoal-stripped FCS. Total cellular RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) as suggested by the manufacturer. The purity and integrity were checked spectroscopically and by gel electrophoresis before carrying out the analytic procedures. The evaluation of gene expression was done by semiquantitative reverse transcription-PCR method as previously described (35). For *PTEN* and the internal control gene *36B4*, the primers were 5′-CCACCACAGCTA-GAACTTATC-3′ (*PTEN* forward) and 5′-ATCTGCACGCTCTATACTGC-3′ (*PTEN* reverse), and 5′-CTCAACATCTCCCCCTTCTC-3′ (*36B4* forward) and 5′-CAAATCCCATATCCTCGTCC-3′ (*36B4* reverse) to yield products of 647 bp with 25 cycles and 408 bp with 12 cycles, respectively. The results obtained as absorbance arbitrary values were transformed to percentage of the control (percent control) taking the samples from untreated cells as 100%.

Immunoprecipitation and immunoblotting. MCF7 cells were grown in 10 cm dishes to 70% to 80% confluence and exposed to treatments for 1 hour or 24 hours in 1% charcoal-stripped FCS before lysis in 500 μL of lysis buffer >[50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, a mixture of protease inhibitors (aprotinin, phenylmethylsulfonyl fluoride, and Na-orthovanadate)]. Cell lysates were centrifuged at 12,000 × *g* for 5 minutes and 500 μg of total protein were incubated overnight with the anti-PPARγ antibody (1 μg; Santa Cruz Biotechnology) and 500 μL of HNTG (immunoprecipitation) buffer [50 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 0.1% Triton X-100, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 2 μg/mL pepstatin]. Immunocomplexes were recovered by incubation with protein A/G-agarose. The beads containing bound proteins were washed thrice by centrifugation in immunoprecipitation buffer, then denatured by boiling in Laemmli sample buffer and analyzed by Western blot to identify the coprecipitating effector proteins. Membranes were stripped of bound antibodies by incubation in 0.2 mol/L glycine (pH 7.6) for 30 minutes at room temperature. Before reprobing with different primary antibodies (anti-ERα and anti-p85; Santa Cruz Biotechnology), stripped membranes were extensively washed in Tween 20 in TBS (TTBS) and placed in blocking buffer (TTBS containing 5% milk) overnight.

Equal amounts of total protein were resolved on an 11% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, probed with rabbit polyclonal antiserum directed against PTEN, AKT, and phospho-AKT (Ser473) and with goat polyclonal antiserum directed against β-actin (all purchased from Santa Cruz Biotechnology). The antigen-antibody complex was detected by incubation of the membranes for 1 hour at room temperature with peroxidase-coupled goat anti-rabbit immunoglobulin G and revealed using the enhanced chemiluminescence system (Amersham Pharmacia). Blots were then exposed to film (Kodak film, Sigma).

p85-associated phosphatidylinositol 3-kinase activity. p85 was precipitated from 500 μg of MCF7 cell lysates. The negative control was done using a cell lysate where the p85 regulatory subunit of PI3K was previously removed by preincubation with the respective antibody (1 hour at room temperature) and subsequently immunoprecipitated with protein A/G-agarose. As a positive control, MCF7 cells were treated with 100 nmol/L insulin for 30 minutes before lysis and IRS-1 was precipitated from 500 μg of cell lysates. The immunoprecipitates were washed once with cold PBS, twice with 0.5 mol/L LiCl, 0.1 mol/L Tris (pH 7.4), and finally with 10 mmol/L Tris, 100 mmol/L NaCl, 1 mmol/L EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing

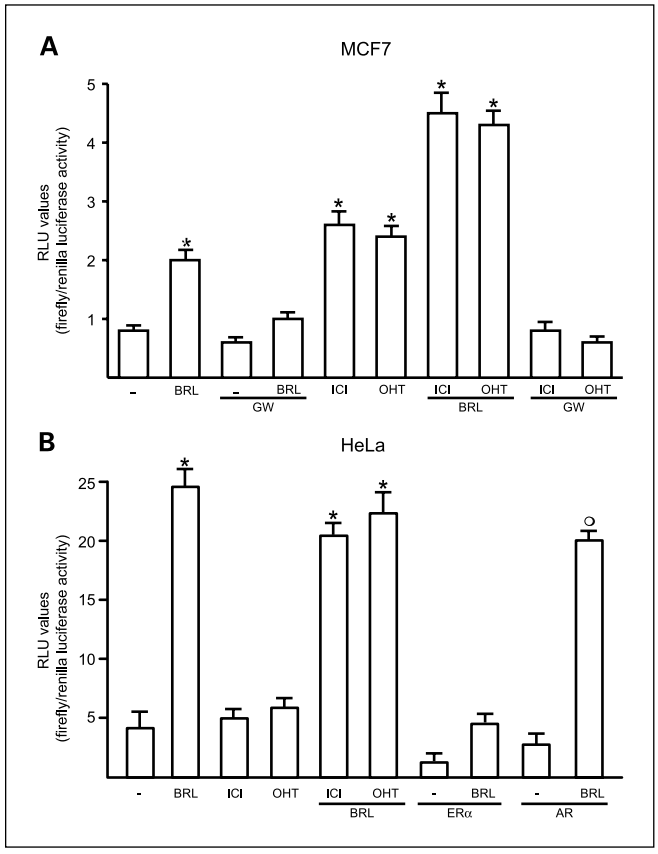

Fig. 1. ERα negatively regulates the PPRE-mediated transcriptional activity. *A*, MCF7 cells were transfected with a PPRE reporter gene and treated with 10 μmol/L BRL49653 (*BRL*), 10 μmol/L PPARγ antagonist GW9662 (*GW*), 1 μmol/L ICI182780 (*ICI*), and 1 μmol/L hydroxytamoxifen (*OHT*), as indicated. *B*, HeLa cells were cotransfected with a PPRE reporter gene and expression vectors of ERα and androgen receptor (*AR*), treated with 10 μmol/L BRL49653, 1 μmol/L ICI182780, and 1 μmol/L hydroxytamoxifen, as indicated. *Columns,* mean of three independent experiments done in triplicate; bars, SD. *, $P < 0.05$, treated versus untreated cells; ○, $P < 0.05$, BRL49653-treated versus untreated cells transfected with androgen receptor. RLU, relative light units.

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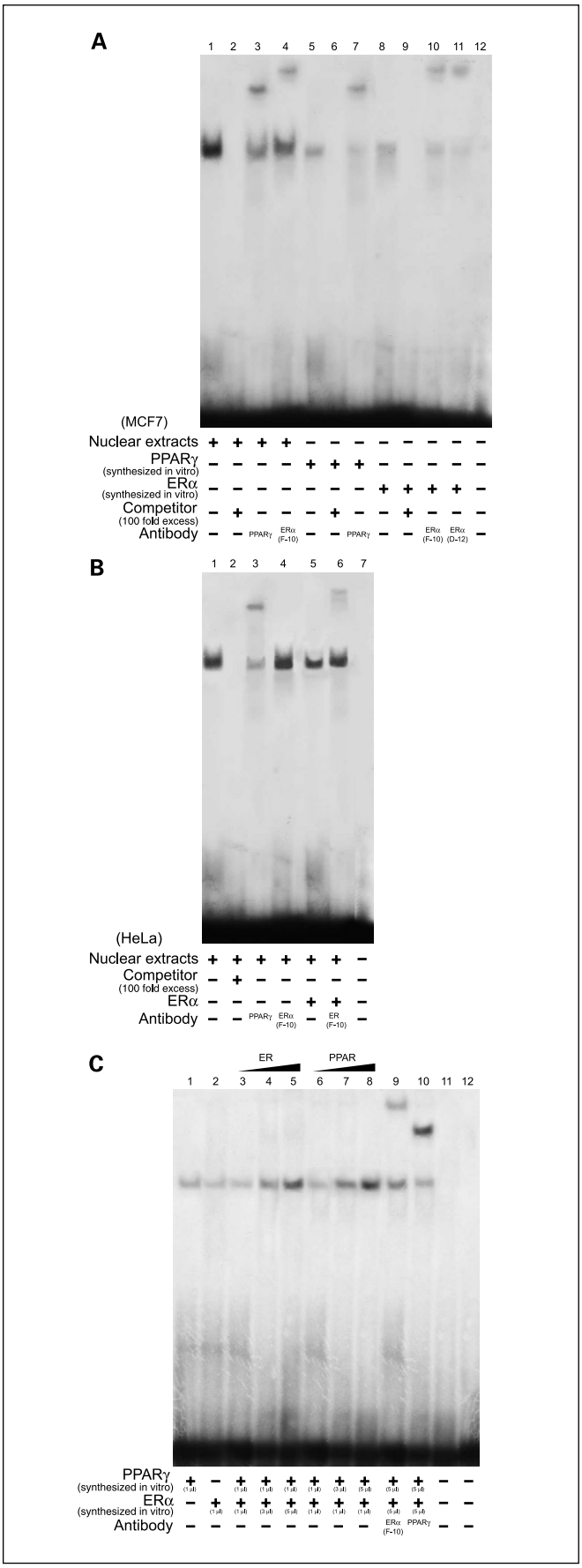

10 mmol/L HEPES (pH 7.4), 10 mmol/L $MgCl_2$, 50 μmol/L ATP, 20 μCi [γ-^{32}P]ATP, and 10 μg of phosphatidylinositol for 20 minutes at 37°C. The reactions were stopped by adding 100 μL of 1 mol/L HCl. Phospholipids were extracted with 200 μL of $CHCl_3$/methanol. Phase separation was facilitated by centrifugation at 5,000 rpm for 2 minutes in a tabletop centrifuge. The upper phase was removed, and the lower chloroform phase was washed once more with clear upper phase. The washed chloroform phase was dried under a stream of nitrogen gas and redissolved in 30 μL of chloroform. The labeled products of the kinase reaction, the phosphatidylinositol phosphates, were then spotted onto *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid – treated silica gel 60 TLC plates. Radioactive spots were visualized by autoradiography.

[^{3}H]Thymidine incorporation. MCF7 and HeLa cells were seeded in six-well plates in regular growth medium. On the second day, cells were incubated in DMEM-F12 or DMEM supplemented with 1% charcoal-stripped FCS for the indicated times in the presence of increasing BRL49653 concentrations or GW9662, 17β-estradiol, and ICI182780.

The medium was renewed every 2 days together with the appropriate treatments. [^{3}H]Thymidine (1 μCi/mL; New England Nuclear, Newton, MA) was added to the medium for the last 6 hours. After rinsing with PBS, the cells were washed once with 10% and thrice with 5% trichloroacetic acid. Cells were lysed by adding 0.1 N NaOH and then incubated 30 minutes at 37°C. Thymidine incorporation was determined by scintillation counting.

Statistical analysis. Statistical analysis was done using ANOVA followed by Newman-Keuls testing to determine differences in means. $P < 0.05$ was considered as statistically significant.

Results

Estrogen receptor α negatively regulates the peroxisome proliferator–activated receptor response element–mediated transcriptional activity. We first aimed to evaluate in MCF7 cells the response of a PPRE reporter gene to BRL49653, a synthetic ligand of PPARγ. As reported in Fig. 1A, BRL49653 activated PPARγ directly because the transcriptional activity was abrogated by the specific PPARγ antagonist GW9662. Interestingly, the ER antagonists ICI182780 and hydroxytamoxifen were able to stimulate the PPRE transactivation, which was reversed by GW9662 treatment (Fig. 1A). Furthermore, the response to BRL49653 treatment was potentiated by both antiestrogens (Fig. 1A).

To ascertain whether ERα is involved in the PPRE-mediated transcriptional activity induced by ICI182780 and hydroxytamoxifen in MCF7 cells, we turned to the ER-negative HeLa cells. Basal and BRL49653-stimulated reporter activity was higher

Fig. 2. ERα binds to PPRE in EMSA. *A*, nuclear extracts from MCF7 cells (*lane 1*) or 2 μL of PPARγ and ERα (*lanes 5* and *8*, respectively) translated proteins were incubated with a double-stranded PPRE consensus sequence probe labeled with [γ-^{32}P] and subjected to electrophoresis in a 6% polyacrylamide gel. Competition experiments were done, adding as competitor a 100-fold molar excess of unlabeled PPRE probe (*lanes 2, 6*, and *9*). Anti-PPARγ or anti-ERα antibodies were incubated with nuclear extracts from MCF7 cells (*lanes 3* and *4*, respectively) or added to PPARγ (*lane 7*) and ERα translated proteins (*lanes 10* and *11*). Lane 12 contains probe alone. *B*, nuclear extracts from HeLa cells were subjected to similar experimental conditions of MCF7 cells (*lane 1*). Competition experiment was done, adding as competitor a 100-fold molar excess of unlabeled PPRE probe (*lane 2*). Anti-PPARγ or anti-ERα antibodies were incubated with nuclear extracts from HeLa cells (*lanes 3* and *4*, respectively). HeLa cells were transfected with 5 μg of an ERα expression vector (*lane 5*) and incubated with an anti-ERα antibody (*lane 6*). Lane 7 contains probe alone. *C*, PPARγ translated proteins were incubated in the absence (*lane 1*) or in the presence of increasing amounts of ERα translated proteins (*lanes 3-5*). ERα translated proteins were incubated alone (*lane 2*) or together with increasing amounts of PPARγ translated proteins (*lanes 6-8*). Anti-ERα or anti-PPARγ antibodies were added to the reaction (*lanes 9* and *10*, respectively). Lane 11 contains probe alone; lane 12 contains 2 μL of unprogrammed rabbit reticulocyte lysate incubated with PPRE.

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in HeLa than in MCF7 cells, whereas both antiestrogens had no effects alone or in combination with BRL49653 treatment (Fig. 1B). According to a previous study (29), the PPARγ transactivation by BRL49653 was no longer noticeable transfecting ERα (Fig. 1B) and ERβ (data not shown) in HeLa cells, whereas an expression vector encoding the androgen receptor did not alter the PPRE-mediated transcriptional activity on BRL49653 (Fig. 1B).

Estrogen receptor α binds to peroxisome proliferator–activated receptor response element in electrophoretic mobility shift assay. To provide further insight into the mechanisms by which ERα negatively interferes with PPRE-mediated transcriptional activity, we did EMSA using a [γ-^{32}P]-labeled consensus sequence of PPRE as probe. Nuclear extracts from MCF7 cells showed strong DNA binding activity for PPRE (Fig. 2A, *lane 1*) in a specific manner because a 100-fold molar excess of unlabeled probe abrogated the formation of this complex (Fig. 2A, *lane 2*). The inclusion in the reaction mix of an anti-PPARγ antibody attenuated the specific band and induced the formation of a supershifted complex (Fig. 2A, *lane 3*). Surprisingly, an anti-ERα antibody also caused a supershift of the band together with a reduced intensity of the specific signal (Fig. 2A, *lane 4*). On the basis of these observations, we aimed to determine whether ERα is capable of binding directly to the PPRE sequence. Thus, we did EMSA using the same radiolabeled PPRE probe with PPARγ and ERα proteins transcribed and translated *in vitro* in a cell-free system. As expected, the synthesized PPARγ protein bound specifically to PPRE because the complex was absent in the presence of a 100-fold molar excess of unlabeled probe (Fig. 2A, *lanes 5* and *6*). Adding an anti-PPARγ antibody to the reaction, the signal was drastically reduced due to the formation of a supershifted complex (Fig. 2A, *lane 7*). Of note, using the synthesized ERα protein, we obtained a single band migrating at the same level as that of PPARγ (Fig. 2A, *lane 8*). The incubation of a 100-fold excess of unlabeled probe abrogated this signal, indicating its specificity (Fig. 2A, *lane 9*). In addition, two distinct monoclonal antibodies against ERα (see Materials and Methods) were both able to form supershifts and showed a reduced intensity of the ERα-PPRE band (Fig. 2A, *lanes 10* and *11*). To confirm the above-mentioned results, we turned to HeLa cells which were transfected with an ERα expression vector. Results obtained were similar to those observed in MCF7 cells (Fig. 2B). Next, using equal amounts of PPARγ and increasing concentrations of ERα and *viceversa*, a progressive enhancement of bands was observed in both cases (Fig. 2C, *lanes 3-8*). Incubating the highest amount of both ERα and PPARγ in the presence of anti-ERα or anti-PPARγ antibodies, supershifted complexes were formed together with an attenuation of the specific signals (Fig. 2C, *lanes 9* and *10*).

Physical and functional interactions of peroxisome proliferator–activated receptor γ and estrogen receptor α. To determine whether crosstalk between ERα and PPARγ transduction pathways may also occur at the protein-protein level, we did coimmunoprecipitation assays in MCF7 cells. It is worth noting that PPARγ was constitutively associated with ERα (Fig. 3A). Treatment with BRL49653 or 17β-estradiol slightly decreased this association, whereas ICI182780 strongly inhibited the interaction (Fig. 3A). Given that ERα binds to the p85 regulatory subunit of PI3K (22), we investigated a possible association of ERα, PPARγ, and p85. We observed the formation of this ternary complex in an ERα-dependent manner because ICI182780 was able to abrogate the coprecipitation (Fig. 3A).

Considering the above-mentioned observations, we investigated whether ERα, alone or together with PPARγ, binds to an endogenous PPRE sequence like that contained in the promoter region of the *PTEN* gene (36). Thus, we did a ChIP

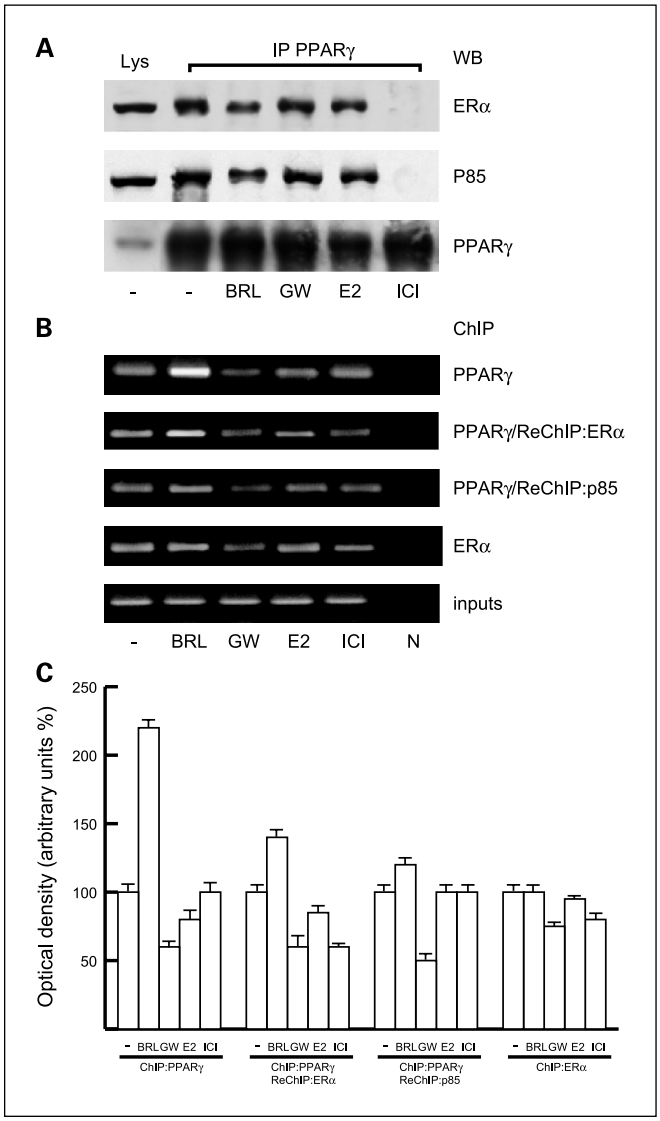

Fig. 3. Physical and functional interactions of PPARγ and ERα. *A*, MCF7 cells were treated for 24 hours with 10 μmol/L BRL49653, 10 μmol/L GW9662, 100 nmol/L 17β-estradiol (*E2*), and 1 μmol/L ICI182780. Cell lysates were immunoprecipitated with an antiserum against PPARγ (IP: anti-PPARγ) and then the immunocomplexes were resolved in SDS-PAGE. The membrane was probed with ERα and p85 antibodies. To verify equal loading, the membrane was probed with an antibody against PPARγ. *B*, MCF7 cells were treated for 1 hour with 10 μmol/L BRL49653, 10 μmol/L GW9662, 100 nmol/L 17β-estradiol, and 1 μmol/L ICI182780, then cross-linked with formaldehyde and lysed. The soluble chromatin was immunoprecipitated with either anti-PPARγ (reverse ChIP with anti-ERα or anti-p85) or anti-ERα. The immunocomplexes were reverse cross-linked, and DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The PTEN promoter sequences containing PPRE were detected by PCR with specific primers, as described in Materials and Methods. To control input DNA, PTEN promoter was amplified from 30 μL of initial preparations of soluble chromatin (before immunoprecipitations). N, negative control provided by PCR amplification without DNA sample. *C*, quantitative representation of data of three independent experiments including that of *B*.

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assay in MCF7 cells using agonists and antagonists of both receptors. The results indicated that PPARγ as well as ERα bound to the *PTEN* promoter along with p85 in untreated cells (Fig. 3B). Interestingly, on treatment with BRL49653 and GW9662, we observed an enhanced and a decreased recruitment of PPARγ to the *PTEN* promoter sequence, respectively. 17β-Estradiol did not induce substantial changes whereas ICI182780 reduced the recruitment of ERα (Fig. 3B). To evaluate the specificity of the reactions, we also used normal mouse and rabbit immunoglobulin G that did not reveal DNA amplifications (data not shown).

BRL49653 down-regulates the phosphatidylinositol 3-kinase/AKT pathway in MCF7 cells. To assess the influence of the complex formed by PPARγ/ERα/p85 on the PI3K/AKT transduction cascade, we evaluated the short (30 minutes) and late (24 hours) effects of BRL49653 on PI3K activity and AKT phosphorylation. Interestingly, BRL49653 showed a dose-dependent negative interference with this pathway at both times (Fig. 4A-D). In agreement with previous reports (22, 37), an opposite regulation was induced by 17β-estradiol (data not shown). Besides, the overexpression of ERα in MCF7 cells enhanced the PI3K activity, which was further potentiated in the presence of 17β-estradiol (Fig. 4E, *lanes 3* and *4*). On the contrary, the overexpression of PPARγ in MCF7 cells reduced the PI3K activity, which resulted to further repression with BRL49653 (Fig. 4E, *lanes 5* and *6*). Of note, the latter inhibitory effects were no longer noticeable with a combination of ERα overexpression and 17β-estradiol treatment (Fig. 4E, *lane 7*).

17β-Estradiol reverses the up-regulation of PTEN by BRL49653 in MCF7 cells. It has been reported that PPARγ up-regulates PTEN expression through two cognate response elements located upstream to the promoter region (36). It is worth noting that PTEN controls several cell functions, including survival and proliferation, by antagonizing the PI3K signaling cascade (38). These observations and our findings prompted us to evaluate the potential effects of both BRL49653 and 17β-estradiol on PTEN expression. Both compounds had no rapid effects (up to 3 hours; data not shown), whereas a 24-hour exposure to 10 μmol/L BRL49653 induced a significant enhancement of PTEN mRNA and protein levels, which was

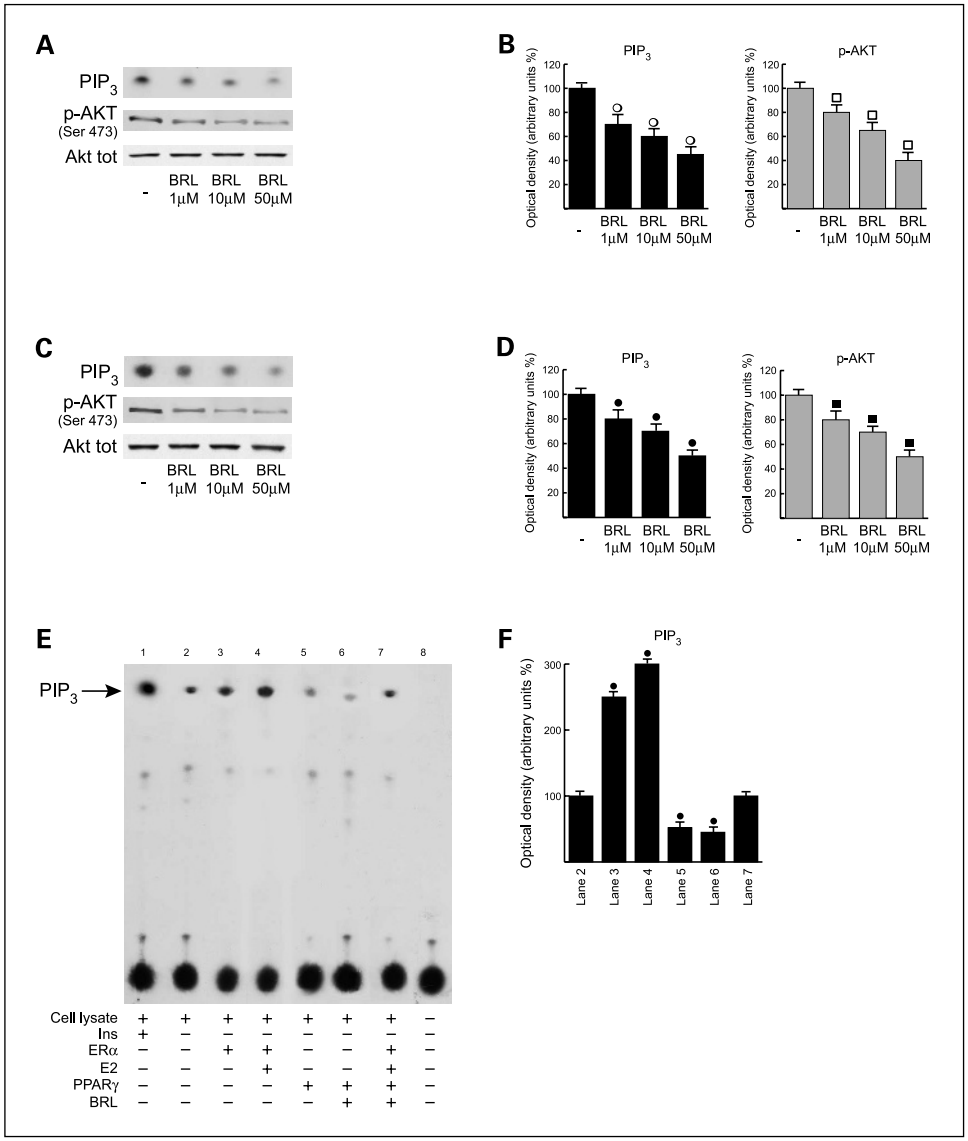

Fig. 4. BRL49653 negatively interferes with PI3K/AKT pathway in MCF7 cells. MCF7 cells were treated for 30 minutes (*A*) and 24 hours (*C*) with increasing concentrations of BRL49653. p85-associated PI3K activity was measured in MCF7 lysates immunoprecipitated with the anti-p85 antibody and incubated in the presence of 200 μmol/L phosphatidylinositol and 10 μCi of [γ-^{32}P]ATP for 20 minutes as described in Materials and Methods. Protein lysates were immunoblotted for phospho-AKT (Ser473) and total AKT. Autoradiographs are representative of six independent experiments, which are cumulatively represented in *B* and *D*, respectively (*columns,* mean; *bars,* SD). *E*, MCF7 cells were transfected with an empty vector (*lane 2*), with 5 μg of an ERα expression vector (*lane 3*) and in the presence of 100 nmol/L 17β-estradiol (*lane 4*), with 5 μg of PPARγ expression vector (*lane 5*) and in the presence of 10 μmol/L BRL49653 (*lane 6*), with 5 μg of both ERα and PPARγ plasmids in the presence of 100 nmol/L 17β-estradiol and 10 μmol/L BRL49653 (*lane 7*). Cell lysates were immunoprecipitated using the anti-p85 antibody, incubated in the presence of 200 μmol/L phosphatidylinositol and 10 μCi of [γ-^{32}P]ATP for 20 minutes. As a positive control, we used MCF-7 cells treated with 100 nmol/L insulin for 30 minutes before lysis and immunoprecipitated with anti – IRS-1 from 500 μg of cell lysates (*lane 1*). As a negative control, we used MCF7 cell lysates in which p85 was previously removed by preincubation with specific antibody (1 hour at room temperature) and subsequent immunoprecipitation with protein A/G-agarose (*lane 8*). *F*, quantitative representations of data of three independent experiments including that of *E*. ○, ●, □, ■: $P < 0.05$, treated versus untreated cells. PIP_3, phosphatidylinositol 3,4,5-triphosphate.

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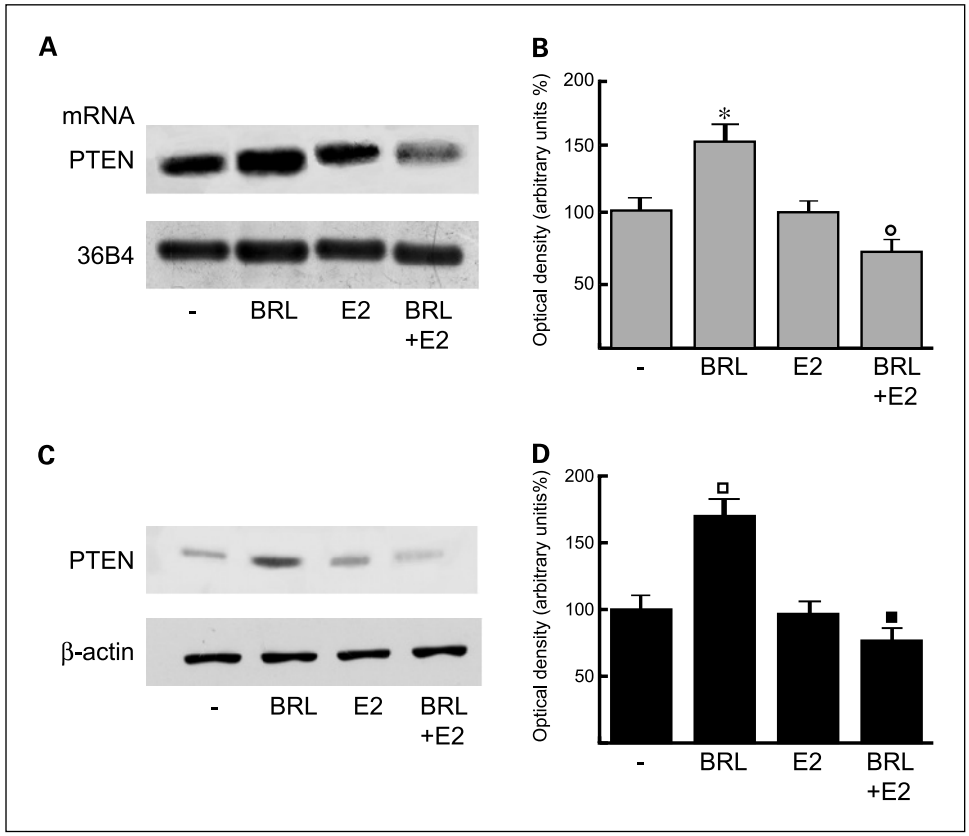

Fig. 5. 17β-Estradiol reverses the up-regulation of PTEN induced by BRL49653 in MCF7 cells. *A*, semiquantitative reverse transcription-PCR evaluation of PTEN mRNA. MCF7 cells were treated for 24 hours with 10 μmol/L BRL49653 and/or 100 nmol/L 17β-estradiol as indicated. 36B4 mRNA levels were determined as a control. *B*, quantitative representation of data of three independent experiments including that of *A* after densitometry and correction for 36B4. *C*, immunoblots of PTEN from MCF7 cells treated as in *A*. β-Actin was used as loading control. *D*, quantitative representations of data of three independent experiments including that of *C*. *, □: $P < 0.05$, BRL49653-treated versus untreated cells; ○, ■: $P < 0.05$, cells treated with BRL49653 and 17β-estradiol versus cells treated with BRL49653 alone.

no longer noticeable in the presence of 17β-estradiol (Fig. 5A-C). Collectively, our results argue that the rapid inhibition of the PI3K/AKT pathway induced by BRL49653 does not directly involve PTEN, which may only contribute to long-term repression.

Growth inhibitory effects of BRL49653 in MCF7 cells. Having shown a functional interaction between ERα and PPARγ and their ability to modulate the PI3K transduction pathway, we evaluated the effects on cell proliferation as a biological counterpart. BRL49653 treatments elicited a time- and dose-dependent growth inhibition in MCF7 cells and, to a higher extent, in HeLa cells (Fig. 6A). The PPARγ antagonist GW9662 reversed the growth inhibitory effects induced by BRL49653, indicating that the repressive action was directly PPARγ-mediated (Fig. 6B). Considering the ability of PPARγ to down-regulate the PI3K/AKT pathway, we did a growth assay using as a model system MCF7 cells transfected with a constitutively active myr-AKT. Notably, under these conditions, the growth inhibition observed on BRL49653 was no longer noticeable (Fig. 6B), suggesting the involvement of the PI3K/AKT pathway in the biological effects triggered by PPARγ activation. Next, the antiproliferative activity exerted by ICI182780 in MCF7 cells was potentiated in the presence of BRL49653 irrespective of 17β-estradiol treatment (Fig. 6B).

Discussion

The present study shows for the first time, to our knowledge, that ERα binds to PPRE sequences and forms a ternary complex with PPARγ and the p85 regulatory subunit of PI3K. As a biological counterpart, the crosstalk between ERα and PPARγ signaling pathways modulates the growth response to cognate ligands in hormone-dependent breast cancer cells.

A large body of evidence has been accumulated about the mechanisms by which nuclear receptors interact at different levels of the transduction cascades, including (*a*) utilization of common response elements such as androgen receptors, glucocorticoid receptors, progesterone receptors, and mineralocorticoid receptors (39); (*b*) heterodimerization of RXR with other receptors (40); and (*c*) receptor associations with several transcription factors and/or other components of the signaling systems located at the level of the cell membrane (41–44).

For PPARs, the heterodimers formed with RXR are able to bind to diverse hormone responsive elements such as ERE (26, 28, 45–47), which can occur independently of the ERs (47). However, natural ERE-containing promoters including those for PS2, the very-low-density apolipoprotein II, and the vitellogenin A2 genes exhibited considerable differences in the binding to PPAR/RXR heterodimers because the ERE flanking sequences influence the binding affinity (26). On the other hand, functional analysis of the vitellogenin A2 promoter showed that the PPAR/RXR complex binds to ERE but cannot transactivate due to a nonpermissive promoter structure (26). Hence, cross-talk between the PPAR/RXR complex and ERE-mediated signals requires further studies to be completely understood.

In the current study, we have provided new evidence on the molecular mechanisms by which ERα negatively interferes with PPRE-mediated transcriptional activity. Of note, in MCF7 cells the ER antagonists ICI182780 and hydroxytamoxifen were both able to stimulate PPRE transcription, which was potentiated by the cognate ligand BRL49653 and reversed by the PPARγ-antagonist GW9662. To better define the inhibitory

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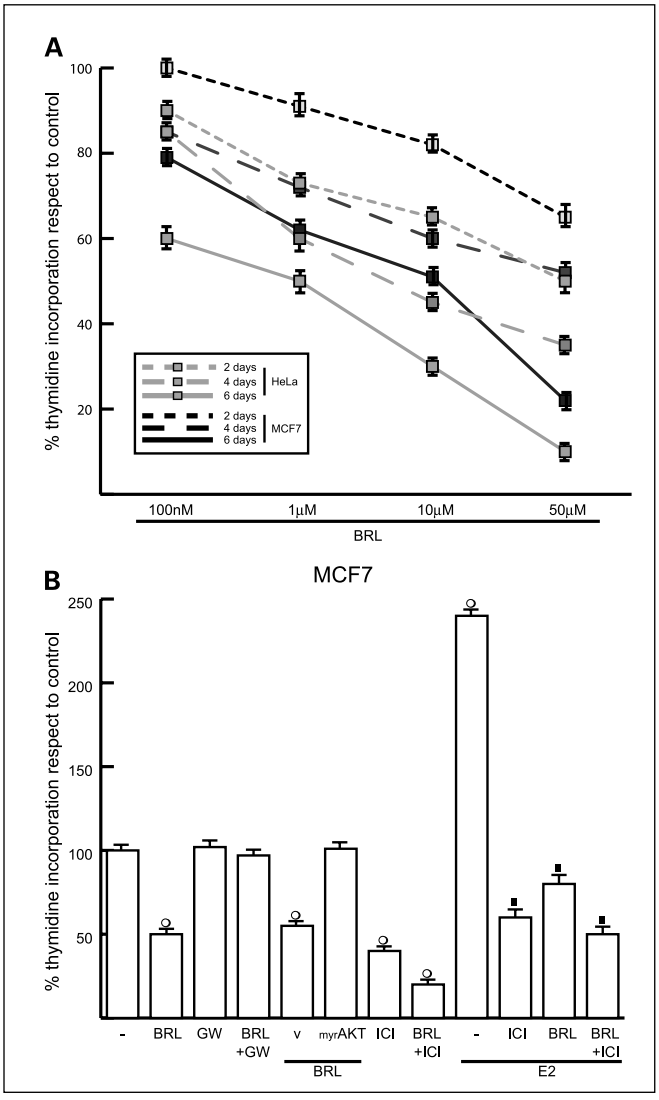

Fig. 6. Antiproliferative effects exerted by BRL49653 in MCF7 and HeLa cells. *A*, MCF7 and HeLa cells were cultured in the presence of increasing concentrations of BRL49653. Six hours before lysis, [^{3}H]thymidine was added and cells were counted. *Columns,* mean of three independent experiments; *bars,* SD. *B*, MCF7 cells were treated with 10 μmol/L BRL49653, 10 μmol/L GW9662, 1 μmol/L ICI182780, and 100 nmol/L 17β-estradiol as indicated, or transfected with an empty vector (*v*) or with 0.5 μg of myr-AKT where applicable (see Materials and Methods for other details). On day 6, 6 hours before lysis, [^{3}H]thymidine was added and cells were counted. Columns, mean of three independent experiments done in triplicate; bars, SD. ○, $P < 0.05$, treated versus untreated cells; ○, $P < 0.05$, BRL49653-treated cells transfected with an empty vector versus cells transfected with a constitutive active myr-AKT; ■, $P < 0.05$, cells treated with 17β-estradiol + ICI182780, 17β-estradiol + BRL49653, or 17β-estradiol + BRL49653 + ICI182780 versus cells treated with 17β-estradiol alone.

action of ERα on the PPARγ transduction pathway, we did EMSA experiments using a [γ-^{32}P]-labeled consensus sequence of PPRE. Nuclear extracts of MCF7 and HeLa cells transfected with an ERα expression vector showed a single band that, in the presence of an anti-ERα antibody, supershifted and reduced the signal intensity. These intriguing observations prompted us to evaluate the binding of PPARγ and ERα translated proteins to the [γ-^{32}P]-labeled PPRE sequence. The band generated by ERα was similar in size to that of PPARγ and, using two distinct ERα antibodies, seemed immunodepleted and supershifted. Taken together, our data show that ERα binds to the PPRE sequence mimicking the ability of the PPAR/RXR complex to interact with ERE (26, 29). Hence, ERα and PPARγ share the ability to bind to the AGGTCA half-sites contained as a palindrome and as a direct repeat in the ERE and PPRE sequences, respectively (26). Consequently, both receptors can potentially influence ERE- and PPRE-mediated responses, likely depending on the cell and promoter context. In this respect, our findings documented a functional interaction between ERα and PPRE contained in an endogenous *PTEN* promoter sequence. Besides, the crosstalk of ERα and PPARγ involves their physical association at the protein level, which is even extended to p85, as we have shown. Such phenomenon may provide an explanation for the opposite functional interplay on PI3K/AKT signaling exerted by ERα and PPARγ transduction pathways. Previous studies have reported the ability of PPARγ to up-regulate the expression of the *PTEN* tumor suppressor gene, which in turn antagonizes the PI3K/AKT cascade (48, 49). Of note, the binding of PPARγ to a pair of PPRE sequences located upstream to the transcription starting site of *PTEN* is responsible for the modulation of its expression (37). Our data confirmed that PPARγ mediates the up-regulation of PTEN because it was enhanced by a 24-hour exposure to BRL49653. Interestingly, this effect was no longer noticeable in the presence of 17β-estradiol, demonstrating the opposite action of ERα in respect to PPARγ on the PI3K pathway. On the basis of our findings, the rapid inhibition of PI3K/AKT signaling with BRL49653 is not mediated by PTEN; however, it may act to prolong the PI3K/AKT repression.

The possible cellular localization of the complex formed by ERα with PPARγ and p85 remains an interesting open question. In resting cells, inactive PI3K and AKT are located in the cytoplasm and activator signals recruit p85 to cell membrane through phosphatidylinositide phosphorylation (50). This process induces the activation of AKT, which in turn moves to the nucleus and other subcellular compartments. As for ERα, its localization mainly at the nuclear level has been clearly established; however, numerous studies have shown the involvement of membrane-associated ER in several cellular responses (ref. 22 and references therein). Our findings provide further evidence on the intriguing interplay between the rapid effects triggered at the membrane level and genomic events requiring different mechanisms which control cell survival and proliferation.

Indeed, the opposite functional role elicited by ERα and PPARγ was recapitulated in the biological responses provided by the growth assay. BRL49653 repressed the PI3K/AKT pathway and induced antiproliferative effects in MCF7 cells. The constitutively active myr-AKT reversed the inhibitory action of BRL49653, indicating that the PI3K/AKT pathway is involved in the negative growth regulation mediated by PPARγ. The ER antagonist ICI182780 potentiated the antiproliferative activity exerted by BRL49653 in MCF7 cells, suggesting that the combination of such compounds could be considered as an adjuvant pharmacologic tool in ERα-positive breast tumors.

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