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Repression of NHE1 Expression by PPARγ Activation Is a Potential New Approach for Specific Inhibition of the Growth of Tumor Cells *In vitro* and *In vivo*

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Abstract

Ligand-induced activation of peroxisome proliferator-activated receptor γ (PPARγ) inhibits proliferation in cancer cells *in vitro* and *in vivo*; however, the downstream targets remain undefined. We report the identification of a peroxisome proliferator response element in the promoter region of the Na^+/H^+ transporter gene *NHE1*, the overexpression of which has been associated with carcinogenesis. Exposure of breast cancer cells expressing high levels of PPARγ to its natural and synthetic agonists resulted in downregulation of *NHE1* transcription as well as protein expression. Furthermore, the inhibitory effect of activated PPARγ on tumor colony-forming ability was abrogated on overexpression of NHE1, whereas small interfering RNA–mediated gene silencing of NHE1 significantly increased the sensitivity of cancer cells to growth-inhibitory stimuli. Finally, histopathologic analysis of breast cancer biopsies obtained from patients with type II diabetes treated with the synthetic agonist rosiglitazone showed significant repression of NHE1 in the tumor tissue. These data provide evidence for tumor-selective downregulation of NHE1 by activated PPARγ *in vitro* and in pathologic specimens from breast cancer patients and could have potential implications for the judicious use of low doses of PPARγ ligands in combination chemotherapy regimens for an effective therapeutic response. [Cancer Res 2009;69(22):8636–44]

Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) belongs to the nuclear receptor superfamily (1). Putative endogenous ligands for the PPARγ receptor include polyunsaturated fatty acids (2, 3) and the eicosanoids 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2; refs. 2, 3), 13-hydroxyoctadecadienoic acid (4), and 15-hydroxyeicosatetraenoic acid (4). In addition to their role in lipid and glucose metabolism, PPARs play a role in cancer development and represent promising targets for cancer prevention and treatment strategies (5, 6). Activation of PPARγ has been identified as an approach for inducing differentiation and inhibiting proliferation in a variety of cancers. Breast tissue, in particular, was found to express PPARγ in amounts greater than those found in normal breast epithelium (7). Moreover, activation of PPARγ on exposure to specific ligands was shown to exert antitumor activity through growth inhibition and cellular differentiation (8–11). Despite these promising results, the target genes involved in the anticancer activity of PPARγ ligands are still not well understood.

Interestingly, a recent report implicated the pH regulator, NHE1, in the mechanism(s) underlying tumor cell growth arrest by activated PPARγ (11), a truly intriguing association considering that the activation of NHE1 is an oncogenic signal necessary for the development and maintenance of the transformed phenotype (12, 13). Supporting this, cancer cells invariably exhibit a strong tendency toward an alkaline intracellular milieu (14, 15). In addition, tumor cells deficient in NHE1 activity either fail to grow or show severely retarded growth when implanted in immunodeficient mice (16, 17). Along similar lines, we recently reported that decrease in NHE1 expression led to tumor cell growth arrest, intracellular acidification, and sensitization to death stimuli (18, 19). These data support that downregulation of NHE1 could be an avenue in the search for new strategies to induce growth arrest in cancer cells as well as increase sensitivity to anticancer treatment.

In light of the increased expression of PPARγ in breast cancer cell lines and its association with acidic intracellular pH, we hypothesized that, in addition to inhibiting NHE1 activity, ligand-induced activation of PPARγ could regulate NHE1 gene expression. Indeed, our results show that exposure of breast cancer cell lines expressing high levels of PPARγ to natural (2, 20) or synthetic (20) ligands of PPARγ significantly inhibited NHE1 gene expression compared with noncancerous cells or cancer cell lines expressing low levels of PPARγ. Furthermore, histopathologic analysis of breast cancer biopsies from patients treated for type 2 diabetes with the PPARγ agonist, rosiglitazone, showed significantly lower NHE1 protein expression in the tumor tissues.

Materials and Methods

Reagents. RPMI 1640, DMEM, PBS, fetal bovine serum, charcoal-stripped fetal bovine serum, L-glutamine, and trypsin were purchased from Hyclone. Pepstatin A, phenylmethylsulfonyl fluoride, leupeptin, sodium chloride, potassium chloride, calcium chloride, magnesium sulfate, D-glucose, HEPES, crystal violet, ammonium chloride, epidermal growth factor, paraformaldehyde, hygromycin, SDS, bovine serum albumin, and mouse anti–β-actin monoclonal antibody were supplied by Sigma-Aldrich.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Aprotinin was purchased from Applichem. Mouse anti-human NHE1 monoclonal antibody was purchased from Chemicon International, and mouse anti-human PPARγ E8 monoclonal and H100 polyclonal antibodies were purchased from Santa Cruz Biotechnology. Stabilized goat anti-mouse horseradish peroxidase was obtained from Pierce. Polyclonal goat anti-rabbit horseradish peroxidase was obtained from DAKO. Rosiglitazone, troglitazone, ciglitazone, and GW9662 were purchased from Cayman Chemical. 15d-PGJ_2 was purchased from Alexis Biochemical. Methanol, xylene, ethanol, and SDS were purchased from Merck. Cell lysis buffer (1×) was from BD Pharmingen. Biotinylated oligonucleotides were synthesized by Proligo (Singapore).

Cell lines and culture conditions. Human breast carcinoma cells MCF-7, MDA-MB-231, and T47D and human normal mammary cell line HTB125 were obtained from the American Type Culture Collection. MCF-7, MDA-MB-231, and T47D cells were maintained in RPMI 1640 containing 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1 mmol/L gentamicin sulfate (BioWhittaker). Normal mammary epithelial cell line HTB125 was maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1 mmol/L gentamicin sulfate and 30 ng/mL EGF.

Microscopic analysis of cell morphology. MDA-MB-231 cells were seeded on each coverslip placed in a 12-well plate culture plate. Cells were then transfected with 7.5 μg of either NHE1 or empty plasmids together with 0.5 μg plasmid encoding green fluorescent protein. Forty-eight hours following transfection, cells were treated with 3 μmol/L 15d-PGJ_2 for 16 h. After treatment, medium was aspirated and the coverslips were washed with 1× cold PBS. Cells were then fixed with 4% paraformaldehyde for 30 min at room temperature. Fluorescence images were analyzed with an Olympus system Fluoview 500 using the Fluoview imaging system (Olympus) using an excitation filer and emission filters at 488 and 506 to 538 nm, respectively.

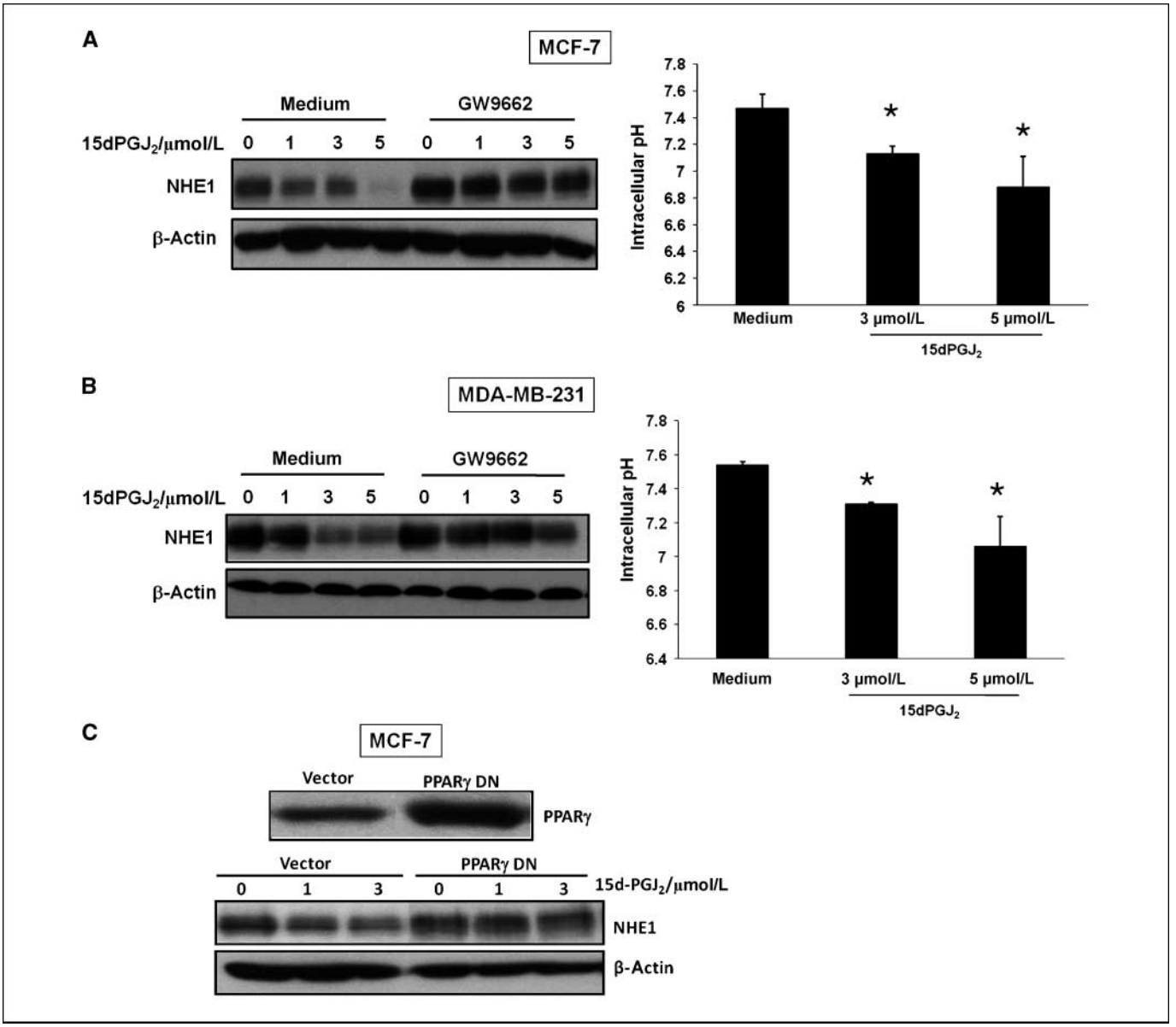

Figure 1. Active PPARγ represses NHE1 expression in a PPARγ-dependent manner. MCF-7 (*A*) and MDA-MB-231 (*B*) breast cancer cells were exposed for 24 h to 15d-PGJ_2 with and without 2 h preincubation with 15 μmol/L GW9662 before NHE1 protein levels were assessed by Western blot (*left*). Intracellular pH in MCF-7 and MDA-MB-231 cells was measured following 24 h exposure to 15d-PGJ_2. *C,* MCF-7 cells were transfected with either the empty vector pCDNA3 (*vector*) or a plasmid encoding a dominant-negative form of PPARγ ($PPAR\gamma^{C126A/E127A}$; *PPARγ DN*) and treated with increasing concentrations of 15d-PGJ_2 for 24 h. NHE1 protein expression was determined by Western blot.

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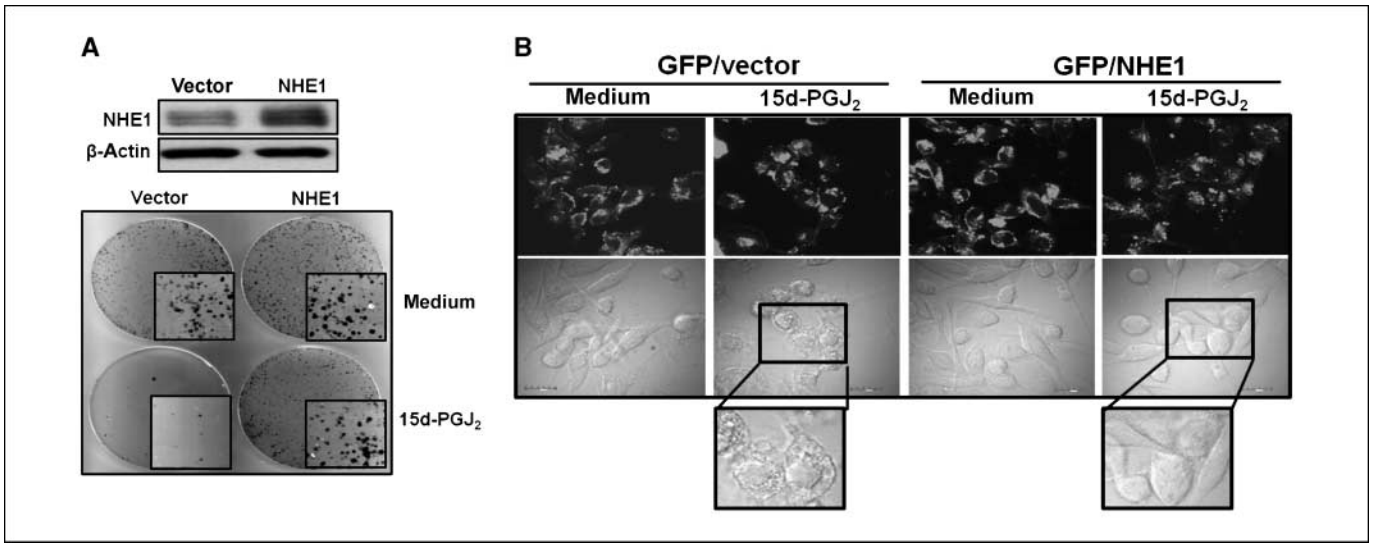

Figure 2. NHE1 gene expression regulates tumor cell growth. *A*, MDA-MB-231 cells were cotransfected with either the empty vector pCMV (vector)/pIRES-Hyg or pCMV-HA-NHE1/pIRES-Hyg. Cells were then treated with 3 μmol/L 15d-PGJ_2 for 16 h followed by seeding in 100 mm culture dishes for colony-forming assays in the hygromycin selection medium. Successful overexpression of NHE1 after 48 h of transfection was determined by Western blot (*top*). *B*, MDA-MB-231 cells seeded on coverslips placed in culture plates were cotransfected with either empty parent plasmid (*vector*) or pCMV-HA-NHE1 plasmid (*NHE1*) and green fluorescent protein (*GFP*) plasmid. Cells were treated with 3 μmol/L 15d-PGJ_2 for 24 h. Green fluorescent protein fluorescence and bright-field images were analyzed with an Olympus system Fluoview 500 using the Fluoview imaging system.

Determination of tumor clonogenic ability. MCF-7, MDA-MB-231, and T47D cells were treated with various concentrations of 15d-PGJ_2 for 16 h before they were left to grow for 10 to 15 days with complete medium and stained with crystal violet. Colony-forming assays with overexpression of NHE1 were done with MDA-MB-231 cells by cotransfecting 7.5 μg of either NHE1 or the control plasmid and 1 μg of the hygromycin encoding vector, pIRES-Hyg (Clontech). Further details on the tumor cells colony-forming capacity can be found in Supplementary Data.

DNA and small interfering RNA transfections. DNA was transfected using CalPhos Mammalian Transfection kit (Clontech) for 15 h before growth in fresh medium for another 24 h. Cotransfection with the *Renilla* plasmid (Clontech) was used to assess transfection efficiency in dual-luciferase reporter assay. For small interfering RNA–mediated gene silencing, 21-nucleotide RNAs were chemically synthesized by Qiagen. 5′-GAUAGGUUUCCAUGUGAUC sequence was used to silence NHE1 gene transcription (Si-NHE1) and 5′-AGCUUCAUAAGGCGCAUGCTT (luciferase gene sequence inverted) sequence was used as a control (Control Si). Cells were transfected with small interfering RNA using the CalPhos Mammalian Transfection kit using 200 nmol/L small interfering RNAs per well in a 6-well plate for 12 h. Gene silencing was assayed 48 h post-transfection by Western blot analysis of the total cell lysates.

RNA isolation and real-time PCR. Total RNA was extracted by Trizol reagent (Invitrogen) as described by the manufacturer's instructions with a DNase treatment step incorporated into the protocol. Primers and probes for human glyceraldehyde-3-phosphate dehydrogenase, human PPARγ, and human NHE1 were purchased as kits from Applied Biosystems (Assays-on-Demand). Details of the real-time PCR protocol can be found in Supplementary Data.

Chloramphenicol acetyltransferase ELISA. Quantification of chloramphenicol acetyltransferase (CAT) protein was done using a CAT antigen capture ELISA (Roche Molecular Biochemicals). All CAT quantitations were normalized to the protein concentration of the cell extract as determined using the Coomasie Plus Protein Assay Reagent kit (Pierce).

Luciferase reporter assay. The 3× peroxisome proliferator response element (PPRE) promoter activities were assessed on cells transfected with the pPPRE-tk-Luc plasmid with a dual-luciferase assay kit (Promega).

Western blot analysis. Western blot analysis was done as described previously (18).

LightShift Chemiluminescence electrophoretic mobility shift assay (nonradioactive gel shift assay). Electrophoretic mobility shift assay was applied to untreated and 15d-PGJ_2–treated MCF-7 nuclear extracts (5 μg) prepared using the nuclear and cytoplasmic extraction reagents N-PER kit (Pierce). For the detailed protocol, refer to Supplementary Data.

Clinical material from breast cancer patients. The study material comprised 15 cases of mammary carcinoma diagnosed at or referred to National University Hospital, Singapore, between 2004 and 2006. Tumors were previously classified according to histology and graded according to the Bloom-Richardson system. All 15 cases were invasive ductal carcinomas, 2 of which were micropapillary type. There were 6 grade 2 tumors and 9 grade 3 tumors. The 15 cases were then categorized into three groups. Patients in group I had diabetes and had been on rosiglitazone, patients in group II had diabetes and were on antidiabetic medication but not the thiazolidinedione class, and patients in group III did not have diabetes and were not on any antidiabetic medication.

Immunohistochemistry for NHE1. Immunohistochemical detection of NHE1 antigen was done on formalin-fixed, paraffin-embedded tumor breast tissues. Stained sections were viewed on an Arcturus PixCell II LCM System. Two pathologists blindly observed each sample and evaluated positively stained cells. Pictures of stained sections were taken using an Olympus camera (Model C5050).

Measurement of intracellular pH. Intracellular pH was measured using the pH-sensitive fluorogenic probe 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester as described previously (18).

***Renilla* assay for cell viability.** Cells were cotransfected *Renilla* expression plasmid together with either NHE1 or PPARγ expression plasmids in a 1:10 ratio. Control cells received *Renilla* plasmid together with appropriate empty vector in the same 1:10 ratio. Forty-eight hours post-transfection, cells were treated and death sensitivity was assessed by measuring the *Renilla* activity using a dual-luciferase assay kit (Promega). Percent survival was calculated as the *Renilla* activity of transfected cells incubated with drug treatment divided by the *Renilla* activity of transfected cell incubated without drug treatment multiplied by 100.

Statistical analysis. Statistical significance was evaluated using Student's *t* test. Significance was accepted at $P = 0.05$. Asterisk indicates $P < 0.05$.

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Results

Repression of NHE1 protein expression is involved in the inhibition of the growth of tumor cells on PPARγ activation. Recently, we showed that expression of the NHE1 was critical for the growth of tumor cells (18). In agreement with these previous findings, inhibition of NHE1 expression in the breast cancer cell line MDA-MB-231 efficiently blocked the ability of tumor cells to form colonies (Supplementary Fig. S1*A*). Similarly, exposure of MCF-7 or MDA-MB-231 breast cancer cell lines to 3 μmol/L 15d-PGJ_2 resulted in significant inhibition of long-term colony formation (Supplementary Fig. S1*B*). Intrigued by the similar effects of PPARγ activation and repression of NHE1 protein expression on tumor cells growth, we assessed the effect of 15d-PGJ_2 on the expression of NHE1 in both cell lines. Results show that exposure of MCF-7 and MDA-MB-231 cells to increasing concentrations of 15d-PGJ_2 inhibited NHE1 expression in a concentration-dependent manner that was accompanied with a decrease in intracellular pH (Fig. 1*A* and *B*). To confirm the involvement of PPARγ in the response of cells to 15d-PGJ_2, MCF-7 and MDA-MB-231 cells were preincubated with the PPARγ antagonist, GW9662 (21), before exposure to 15d-PGJ_2 and NHE1 protein level was assessed. GW9662 (15 μmol/L) was sufficient to block PPARγ activation, assessed by transfection with a 3× PPRE-luciferase reporter gene construct, in both cell lines (Supplementary Fig. S2). Moreover, the presence of GW9662 blocked the repression of NHE1 protein brought about by ligand-induced PPARγ activation (Fig. 1*A* and *B*). Similarly, overexpression of a dominant-negative form of PPARγ, $PPAR\gamma^{C126A/E127A}$, prevented the downregulation of NHE1 protein expression in MCF-7 cells in response to 15d-PGJ_2 (Fig. 1*C*). Taken together, these data indicate that the decrease in NHE1 protein expression could be a critical mediator of 15d-PGJ_2–induced inhibition of tumor colony formation. To test this hypothesis further, MDA-MB-231 cells were transfected with a plasmid containing the full-length NHE1 gene (22) before being exposed to 15d-PGJ_2 and the colony-forming ability and morphology of cells were assessed. It is to be noted that transient transfection with NHE1 did not affect the activation of PPARγ by 15d-PGJ_2 (data not shown). To assess the effect of 15d-PGJ_2 on cell morphology MDA-MB-231 cells were cotransfected with a construct encoding for the green fluorescent protein in addition to NHE1 or the empty

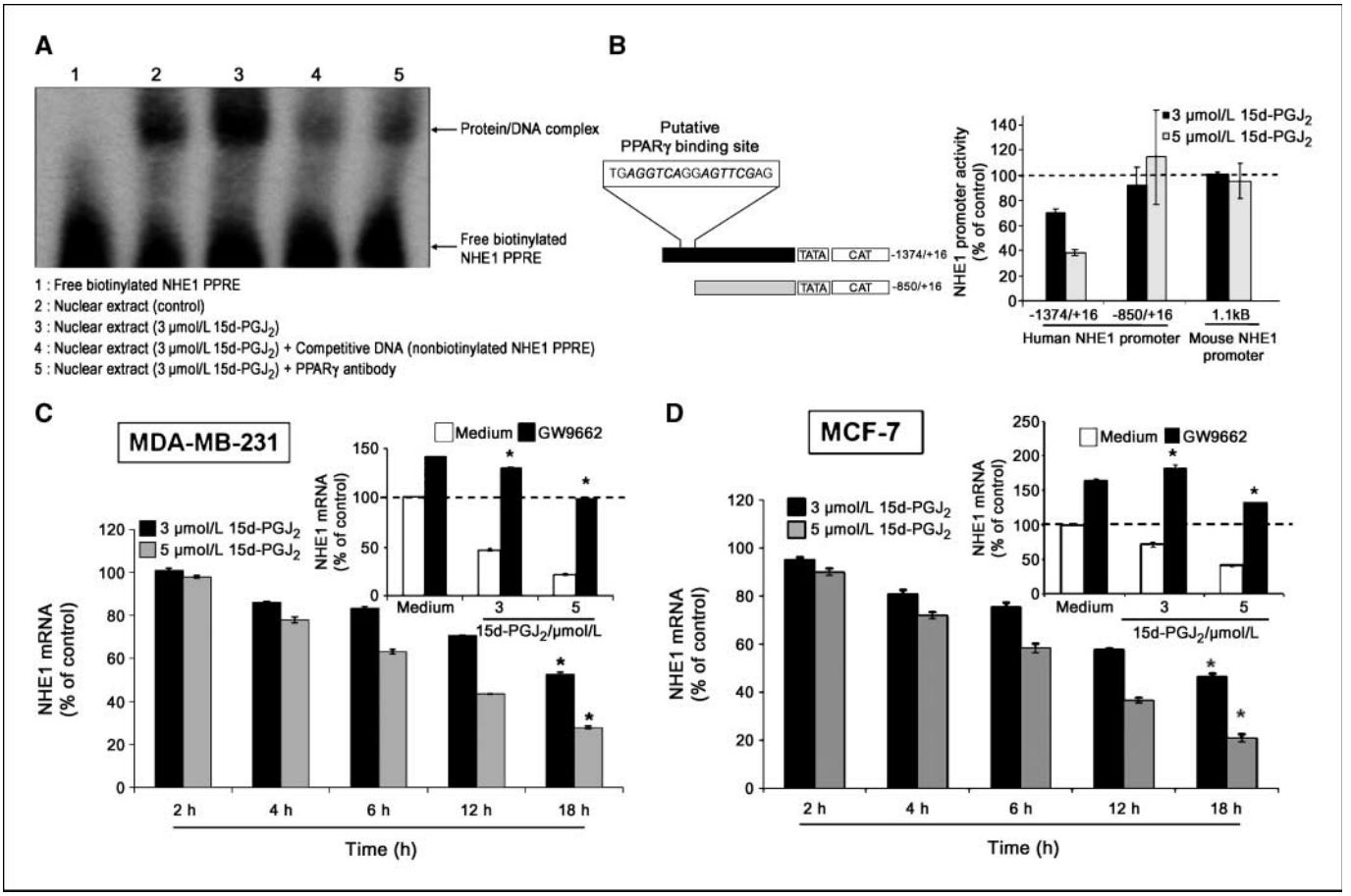

Figure 3. PPARγ binds to PPRE within NHE1 promoter in a ligand-dependent manner. *A,* LightShift Chemiluminescence electrophoretic mobility shift assay using nuclear extracts from MCF-7 with or without 6 h exposure to 15d-PGJ_2. *B,* MCF-7 cells were transiently transfected with a full-length human NHE1 promoter construct (–1374/+16) and a 5′-deletion derivative of the full-length lacking the PPRE site (–850/+16; $NHE1^{\Delta PPRE}$). Schematic representation of these two constructs with the PPRE site indicated. NHE1 promoter activities were calculated as CAT activity (A_{405nm})/μg total protein and expressed as percent decrease from untreated (*dotted line*). MCF-7 cells were cotransfected with 1.1-kb full-length mouse NHE1 luciferase and *Renilla* plasmids and treated with 15d-PGJ_2 for 24 h. NHE1 promoter activity was calculated as luciferase RLU/*Renilla*/μg total protein expressed as percent decrease from untreated cells (*dotted line*). Average ± SD of two experiments done in duplicate. MDA-MB-231 (*C*) and MCF-7 (*D*) cells were treated with 15d-PGJ_2 for various time points. Relative NHE1 mRNA expression is expressed as percent of untreated control (100%). *Inset,* cells were exposed for 24 h to 15d-PGJ_2 with and without 2 h preincubation with 15 μmol/L GW9662 before NHE1 mRNA levels were assessed. *, $P < 0.05$.

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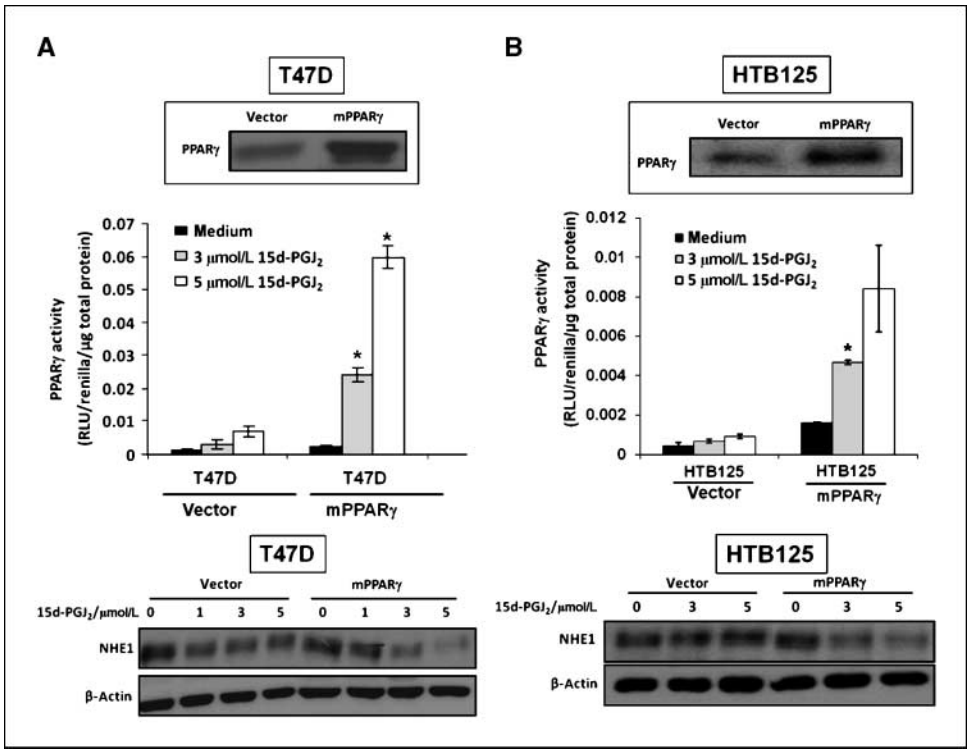

Figure 4. PPARγ protein level and activity correlates with the capacity of 15d-PGJ_2 to inhibit NHE1 expression. *Top,* T47D breast cancer (*A*) and HTB125 normal mammary (*B*) cells were transfected with plasmid encoding murine PPARγ (*mPPARγ*). Control cells were transfected with empty parent vector, pCMX (*vector*). *Middle,* T47D (*A*) and HTB125 (*B*) cells were cotransfected with empty vector or murine PPARγ, reporter 3× PPRE-luciferase, and *Renilla* plasmids. Cells were treated with 15d-PGJ_2 for 6 h. PPARγ activity was calculated as luciferase RLU/*Renilla*/μg total protein. Average ± SD of two experiments done in duplicate. *, $P < 0.05$. *Bottom,* T47D (*A*) and HTB125 (*B*) cells were transfected with empty vector (*vector*) or murine PPARγ. Cells were then treated with increasing doses of 15d-PGJ_2 for 24 h and NHE1 expression was determined by Western blot.

vector for monitoring the effect on NHE1-transfected cells only (green fluorescent cells). For the colony-forming assay, cells were cotransfected with a vector encoding for hygromycin resistance and selection for NHE1-transfected cells was achieved by the addition of hygromycin in the culture medium. Results show that overexpression of NHE1 afforded by transfection with NHE1 encoding plasmid prevented the effect of 15d-PGJ_2 (compared with empty vector transfected cells) on cell morphology as well as long-term colony formation in MDA-MB-231 cells (Fig. 2*A* and *B*). Cells that overexpressed NHE1 maintained a viable morphology, whereas cells exposed to 15d-PGJ_2 showed a lot of vacuoles (Fig. 2*B*) that correlated with the absence of colony formation in the presence of the PPARγ agonist (Fig. 2*A*). Vacuole formation and inhibition of colony formation were both prevented on overexpression of NHE1 (Fig. 2*A* and *B*).

Activated PPARγ binds to the PPRE motif at the 5′-proximal promoter region of the human NHE1 promoter. Interestingly, a putative PPRE with an AGGTCAnnAGTTCG motif in the 5′-proximal region of the human NHE1 gene promoter (nucleotides –977 to –990 with respect to the TATA box; accession number L25272) corresponding to a nonconsensus DR2 element was found. This motif is within a primate-specific Alu element, in a cluster of hexamers half-sites recognized by various nuclear receptors, termed as Alu receptor response element (refs. 23–25; Supplementary Fig. S3). To verify that activated PPARγ could bind to this motif, nuclear extract was prepared from MCF-7 cells exposed to 3 μmol/L 15d-PGJ_2 (2, 20), and biotinylated double-stranded oligonucleotide containing the PPRE site within the NHE1 promoter was used as a probe. Some basal PPARγ binding (retarded complex) could be detected in nuclear extract from untreated MCF-7 cells (Fig. 3*A, lane 2*); however, the intensity of the retarded complex increased significantly in nuclear extract from cells incubated with 15d-PGJ_2 (Fig. 3*A, lane 3*,). The PPRE binding of activated PPARγ could be inhibited by either nonbiotinylated oligonucleotides (Fig. 3*A, lane 4*) or the presence of anti-PPARγ antibody that blocks the formation of the DNA/PPARγ complex (ref. 23; Fig. 3*A, lane 5*).

Activation of PPARγ decreases NHE1 promoter activity and mRNA expression. To assess the effect of PPARγ activation on NHE1 promoter activity, MCF-7 breast carcinoma cells were transfected with a CAT reporter plasmid containing either the full-length human NHE1 promoter construct (–1374/+16; $NHE1^{-1374/+16}$), a 5′-deletion construct of the human NHE1 promoter in which the PPRE is absent (–850/+16; $NHE1^{\Delta PPRE}$), or a full-length mouse NHE1 promoter. The mouse NHE1 promoter was used because the putative PPRE motif present in the human NHE1 promoter is not found within the 1.1-kb mouse NHE1 promoter sequence (data not shown). Data show that exposure of transfected MCF-7 cells to 15d-PGJ_2 resulted in a dose-dependent decrease in the promoter activity of the human $NHE1^{-1374/+16}$ construct, whereas no significant decrease was observed in the $NHE1^{\Delta PPRE}$ construct or the 1.1-kb mouse NHE1 promoter (Fig. 3*B*). These results indicate that the 15d-PGJ_2–induced downregulation of NHE1 promoter activity in MCF-7 cells requires the presence of the PPRE motif and therefore is linked to the activation of PPARγ. To corroborate this result with the induction of mRNA expression, MCF-7 and MDA-MB-231 cells were preincubated with the PPARγ antagonist, GW9662 (21), before exposure to 15d-PGJ_2, and NHE1 mRNA was assessed. The presence of GW9662 blocked the repression of NHE1 mRNA level brought about by ligand-induced PPARγ activation (Fig. 3*C* and *D*).

Ligand-induced inhibition of NHE1 expression is a function of PPARγ expression. To assess if the inhibition of NHE1 expression by 15d-PGJ_2 was a function of PPARγ expression, we assessed the mRNA level as well as the activity of PPARγ (induced by 3 μmol/L 15d-PGJ_2) in three breast cancer cell lines (MCF-7, MDA-MB-231, and T47D) and one human normal mammary epithelial cell line (HTB125). The level of PPARγ mRNA in the three

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tumor cell lines was significantly higher than the normal mammary cell line (Supplementary Fig. S4*A*). Furthermore, within the tumor cell lines, the level of PPARγ mRNA was in the order of MDA-MB-231 > MCF-7 > T47D, which mirror-imaged their respective ligand-induced PPARγ activity (Supplementary Fig. S4*A*). Similar to the level of PPARγ mRNA expression, 15d-PGJ_2–induced PPARγ activity was hardly detected in the normal mammary HTB125 cells (Supplementary Fig. S4*B*). It is noteworthy that PPARγ mRNA expression level and activity in MDA-MB-231 and MCF-7 cells correlated well with the level of NHE1 suppression obtained with 15d-PGJ_2 (as shown in Fig. 1). Corroborating these data, the inhibitory effect of 15d-PGJ_2–induced PPARγ activation on NHE1 expression was significantly less pronounced in T47D cells that express low levels of PPARγ activity (Fig. 4*A*), whereas HTB125 normal mammary cells were completely refractory to the repressive effect of 15d-PGJ_2 on NHE1 expression (Fig. 4*B*). However, T47D and HTB125 cells became responsive to 15d-PGJ_2 in terms of both an increase in PPARγ activity and an inhibition of NHE1 expression (Fig. 4*A* and *B*) on transfection with a plasmid encoding murine PPARγ.

Thiazolidinedione ligands of PPARγ downregulate NHE1 gene expression. In addition to the endogenous PPARγ agonist 15d-PGJ_2, several synthetic PPARγ ligands are already in clinical use for the treatment of type 2 diabetes (20). Different concentrations of each of these ligands were tested for their effect on PPARγ activity in MCF-7 cells transfected with the PPARγ reporter gene construct. All three synthetic ligands induced strong activation of PPARγ albeit at different concentrations (Supplementary Fig. S5*A*) that was inhibited by preincubation of the cells with GW9662. More importantly, all three agents induced a significant decrease in NHE1 promoter activity in cells transiently transfected with the full-length promoter construct ($NHE1^{-1374/+16}$) but not in those expressing the $NHE1^{\Delta PPRE}$ (Fig. 5*A*). Similar effect was observed at the protein level with all three synthetic ligands significantly downregulating NHE1 mRNA and protein expression that could be rescued in the presence of the PPARγ antagonist GW9662 (Fig. 5*B–D*). Interestingly, the morphology of MCF-7 cells exposed to ciglitazone, troglitazone, or rosiglitazone resemble the one described in Fig. 1*B* on exposure to 15d-PGJ_2 (Fig. 5*B–D*).

NHE1 expression is downregulated in breast cancer tissues from diabetic patients treated with rosiglitazone. To provide a physiologic relevance to the finding that activation of PPARγ by endogenous or synthetic ligands induced a decrease in NHE1 expression in breast cancer cell lines, we exploited the clinical use of synthetic PPARγ ligands for the management of type 2 diabetes

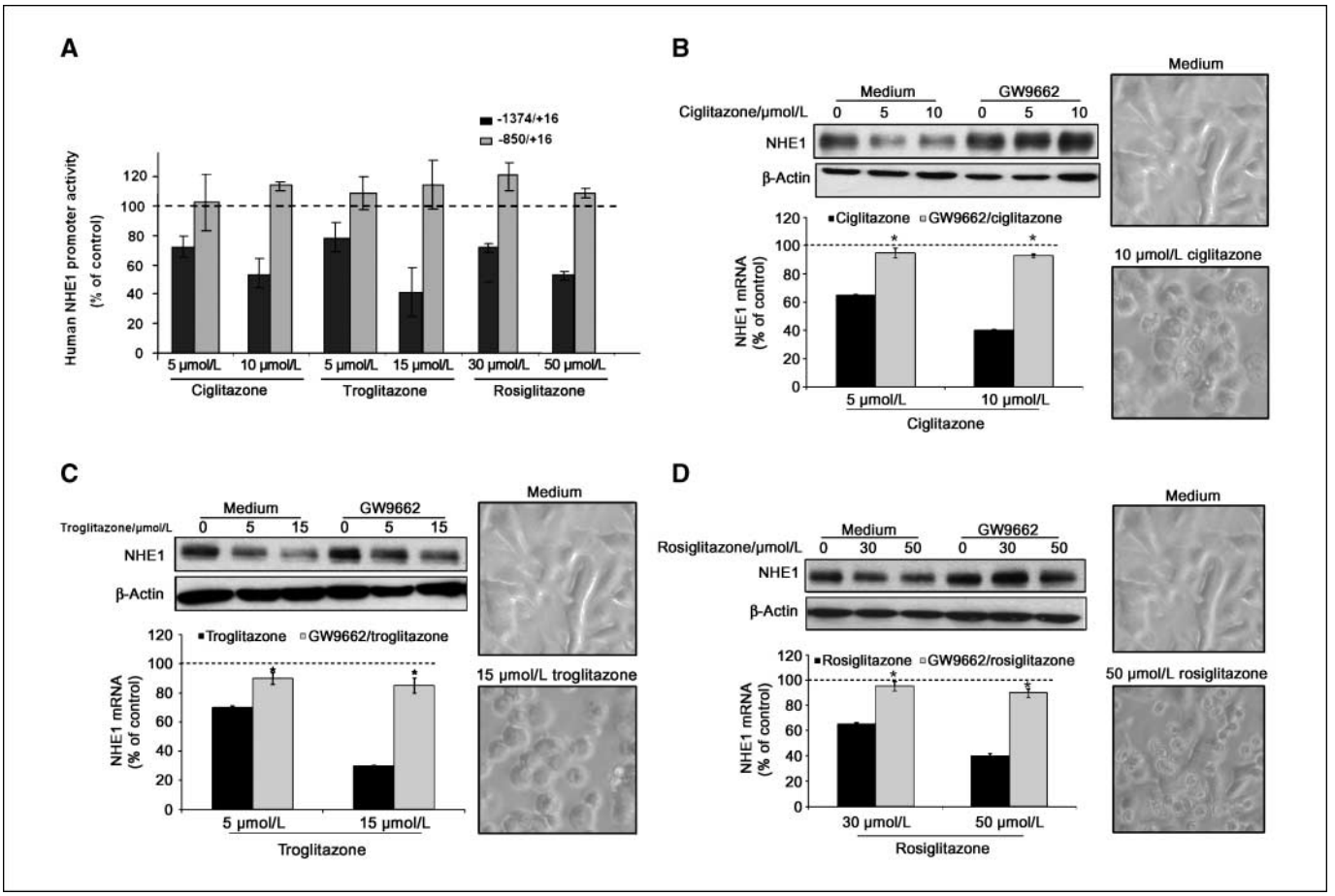

Figure 5. Glitazone treatment downregulates NHE1 gene expression via PPRE. *A,* MCF-7 cells were transiently transfected with the full-length human NHE1 promoter construct (–1374/+16) or a 5′-deletion derivative of the full-length lacking the PPRE site (–850/+16; $NHE1^{\Delta PPRE}$). Cells were treated for 24 h with ciglitazone, troglitazone, and rosiglitazone at the indicated concentrations. Human NHE1 promoter activity was calculated as CAT activity (A_{405nm})/μg total protein and expressed as percent decrease from untreated (*dotted line*). Average ± SD of two experiments done in duplicate. MCF-7 cells were exposed to (*B*) ciglitazone, (*C*) troglitazone, and (*D*) rosiglitazone at concentrations indicated for 24 h with and without 2 h preincubation with 15 μmol/L GW9662 before NHE1 protein levels and mRNA were assessed. NHE1 mRNA levels are expressed as percent decrease from non-15d-PGJ_2–treated cells (*dotted lines*). *, $P < 0.05$. Photographs of morphologic changes at ×100 magnification following respective glitazones treatment for 24 h are shown.

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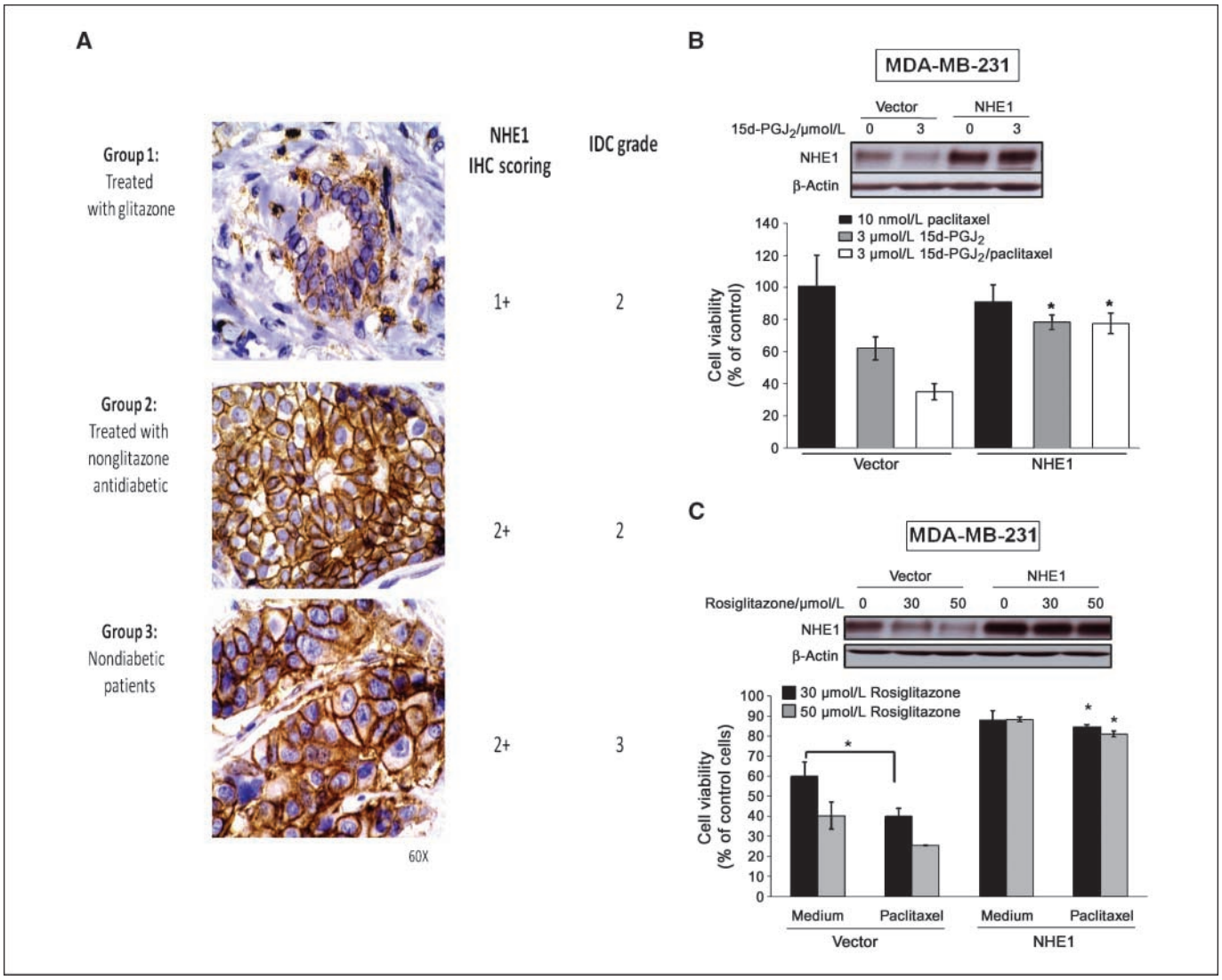

Figure 6. Reduced NHE1 gene expression observed in breast tissues from diabetic breast cancer patients treated with glitazones; repression of NHE1 by PPARγ ligands sensitizes tumor cells to paclitaxel. *A,* immunohistochemical (*IHC*) localization of NHE1 antigen done on formalin-fixed, paraffin-embedded tumor breast tissues. Tissue sections were incubated with mouse monoclonal NHE1 antibody. Positively stained cells were evaluated using the following intensity categories: 0 (no staining), 1+ (weak but detectable staining), 2+ (moderately intense staining), and 3+ (very intense staining). Tumor grade indicated as intraductal carcinoma (*IDC*) grade. *Top,* representative NHE1 immunostaining from patient case 1 in group I from Supplementary Table 1; *middle,* representative NHE1 immunostaining from patient case 5 in group II from Supplementary Table 1; *bottom,* representative NHE1 immunostaining from patient case 12 in group III from Supplementary Table 1. *B* and *C,* MDA-MB-231 cells cotransfected with either the empty parent vector or pCMV-HA-NHE1 and *Renilla* plasmids. Cells were first treated with either (*B*) 3 μmol/L 15d-PGJ_2 or (*C*) 30 and 50 μmol/L rosiglitazone for 16 h. After 16 h treatment, cells were then exposed to either 0 or 10 nmol/L paclitaxel for another 24 h. Cell viability was then assessed using a *Renilla* assay. *Renilla* readings were calculated as *Renilla*/μg total protein and expressed as percent decrease from untreated control. Average ± SD of two experiments done in duplicate. *, $P < 0.05$. NHE1 protein expression was determined by Western blot shown above the respective graphs.

mellitus. To that end, we compared the expression of NHE1 in breast cancer tissues of diabetic patients treated with PPARγ ligand (rosiglitazone) versus breast cancer patients who did not receive rosiglitazone (but other antidiabetic drugs) for their diabetic condition and/or nondiabetic patients with breast cancer. A total of 15 cases of carcinoma of the breast (intraductal carcinoma grades 2-3) with available paraffin-embedded tissue blocks were identified for the periods 2004 and 2006 from the pathology files of the Department of Pathology, National University of Singapore. These cases were divided into three groups: group I, diabetic breast cancer patients treated with rosiglitazone; group II, diabetic breast cancer patients treated with other antidiabetic drugs; and group III, nondiabetic breast cancer patients. Immunohistochemical analysis showed that the expression of NHE1 in clinical tissues corroborated our findings in breast cancer cell lines on the repressive effect of PPARγ activation on NHE1 expression; 3 of 4 patients in group I (diabetics on rosiglitazone) showed reduced levels of NHE1 expression compared with the tumors of all patients from the other two groups. Note that the intraductal carcinoma grade at diagnosis was similar within the different groups (Supplementary Table S1). A representative histopathology specimen stained for NHE1 expression from each group is shown in Fig. 6. In addition, we show that overexpression of NHE1 abrogated the chemosensitizing effect of PPARγ activation on MDA-MB-231 cells (Fig. 6*B* and *C*). Alternatively, small interfering RNA–mediated gene silencing of NHE1 significantly increased the sensitivity of MCF-7 and

MDA-MB-231 cells to paclitaxel and daunorubicine (Supplementary Fig. 6S*A* and *B*).

Discussion

The present study presents evidence supporting a relationship between two genes reported to be associated with carcinogenesis, PPARγ (5, 26–30) and NHE1 (11, 17, 31–36). Firstly, exposure of breast cancer cells to PPARγ agonists (endogenous as well as synthetic) specifically downregulated NHE1 expression in cells overexpressing PPARγ; secondly, a functional PPRE sequence was identified in the 5′-proximal promoter region of the human NHE1 gene. The repressive effect of ligand-induced PPARγ activation on NHE1 promoter activity was confirmed by the inhibition of this activity in the presence of the PPARγ antagonist GW9662.

As a first step to determining the pathway involved in the downregulation of NHE1 gene expression by activation of PPARγ, the 5′-proximal promoter region was examined for a potential PPRE. A putative PPRE in the human NHE1 promoter located at nucleotide −977 to −990 with respect to the TATA box (accession number L25272) was identified.

Interestingly, the PPRE found in the NHE1 promoter region is located within a primate-specific Alu element (37). An alignment AluRRE of the human NHE1 with that of human MPO gene showed considerable sequence identity (data not shown). Incidentally, it has been reported that the human MPO gene is regulated by PPARγ (23). The AGGTCAnnAGTTCG motif found in the NHE1 promoter corresponds to a nonconsensus DR2 element not only recognized by PPAR but also recognized by retinoic acid receptors functioning as retinoic acid response elements. This suggests that, in addition to respond to PPARγ the PPRE motif found in the NHE1 promoter may respond to retinoids.

The inhibition of target genes by PPARγ involves either the transrepression (negative cross-talk) of activating transcription factors (e.g., NF-κB and activator protein-1; refs. 38, 39) or the sequestration of limiting amounts of coactivator molecules such as CBP (40). Alternatively, repression of gene transcription by PPARγ activation could be due to the recruitment of corepressors, NCoR and SMRT, in a promoter-specific manner (41). In this regard, recruitment of sumolylated PPARγ was recently shown to downregulate inducible nitric oxide synthase gene transcription in response to PPARγ ligands (42). As for the mechanism underlying PPARγ-mediated repression of NHE1, our results show that PPARγ binds to a PPRE sequence on the NHE1 promoter. This supports that transrepression may not be the mechanism involved in the inhibition of gene transcription by PPARγ and a mechanism involving corepressors may be more likely to explain the inhibition of NHE1 expression by PPARγ ligands.

The decrease in NHE1 expression by PPARγ ligands was shown in breast cancer cell lines as well as in breast tissue of cancer patients incidentally treated with PPARγ agonists because of a type 2 diabetes mellitus. Although our data on clinical samples may not be statistically significant due to the low number of cases collected, we believe that these data support that downregulation of NHE1 expression is achievable *in vivo* on treatment with thiazolidinedione. Moreover, the inhibition of NHE1 expression on activation of PPARγ correlated with the level of PPARγ expression. Normal cells or tumor cells expressing low levels of PPARγ only became responsive following transfection with a plasmid encoding for a mouse PPARγ. These data support that decrease in NHE1 expression can be specifically induced in tumor cells overexpressing PPARγ, leaving normal cells unaffected. In light of the recent study showing that an antisense therapy targeting NHE1 gene in gastric cancer decreased invasive capacity, and loss of cloning efficiency, and tumorigenicity in nude mice (43), the repressive effect of PPARγ activation on NHE1 expression presents a window of opportunity for exploiting PPARγ agonists for selectively tailoring the expression of NHE1 to achieve a favorable therapeutic response. In addition, synergy between rosiglitazone and platinum-based drugs in several different cancers both *in vitro* and using transplantable and chemically induced "spontaneous" tumor models was recently reported (44, 45). Pertinent to this, we have shown previously that decreasing NHE1 expression sensitizes tumor cells to anticancer drugs (18) and Supplementary Fig. S6*A* and *B*.

In conclusion, our data provide evidence for a tumor-selective effect of PPARγ activation on downregulation of NHE1 expression *in vitro* and *in vivo*. In light of these data and our previously published experiments on the sensitization of tumor cells to anticancer drugs by a decrease in NHE1 expression, we surmise that, despite the current controversy surrounding the use of the rosiglitazone, intelligent use of PPARγ agonists singly or in combination chemotherapy for tumors such as breast cancer could be a promising novel therapeutic strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Repression of NHE1 Expression by PPARγ Activation Is a Potential New Approach for Specific Inhibition of the Growth of Tumor Cells *In vitro* and *In vivo*

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