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ORIGINAL ARTICLE

Modulation by phenylacetate of early estrogen-mediated events in MCF-7 breast cancer cells

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Abstract *Purpose*: Phenylacetate (PA) and its derivatives constitute a group of small aromatic fatty acids that have been of considerable interest due to their anticancer properties in a number of experimental systems. We previously showed that PA can inhibit the growth of estrogen receptor (ER)+ breast cancer cells and that this activity is, at least in part, mediated by the ability of the compound to inhibit transcriptional activation driven by estrogen response elements (EREs). We now shed additional light on the antiestrogenic action of PA by determining its effects on early events in the estrogen-signaling pathway. *Methods*: MCF-7 breast cancer cells were used in this study. ER–ERE binding activity, and subsequent effects on ER and progesterone receptors (PR), c-myc, and the cyclin-dependent kinase inhibitor $p21^{ASF1/CP1/MDA-6}$ (p21) were evaluated using electrophoretic mobility shift assays, real-time RT-PCR, and western blotting methodologies. Effects of PA on p21 promoter activity were assessed in transient transfection experiments utilizing p21 promoter–reporter gene constructs. *Results*: We demonstrate that PA treatment can block ER–ERE binding activity and that this effect is accompanied by downregulation of PR and c-myc, two genes which are transcriptionally regulated by estrogen through novel-ER-binding sites. Suppression of c-myc by PA is followed by increased mRNA levels of p21, an effect that is mediated by PA activation of the p21 promoter. Forced overexpression of c-myc through co-transfection of MCF-7 cells with a c-myc expression plasmid prevented PA upregulation of p21 promoter activity. *Conclusions*: These findings confirm the potent antiestrogenic properties of PA, indicate that its effects are mediated by inhibiting ER–ERE interactions, and suggest that downregulation of c-myc is an early event leading to increased p21 expression and cell growth inhibition.

Keywords Phenylacetate · Breast cancer · Antiestrogen · Estrogen receptor · C-myc · p21

Introduction

The group of small aromatic fatty acid exemplified by the compound phenylacetic acid [or its sodium salt phenylacetate (PA)] have demonstrated antiproliferative and/or differentiation inducing activity in a number of tumor systems [24, 25, 28], including hormone-driven tumors such as prostate and breast cancer [7, 27, 32]. Although there have been a number of published in vitro and in vivo studies with these compounds, their mechanisms of action remain unclear. Early work suggested that the ability of PA to deplete serum of glutamine due to the formation of phenylacetylglutamine might serve to selectively inhibit growth of certain types of tumor cells [19]. However, since many tumor cells shown to be affected by PA are not particularly sensitive to glutamine depletion, this property alone could not explain its antitumor activity. Subsequent

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studies have provided evidence for other mechanisms of PA action on tumor cells including inhibition of protein prenylation through its ability to block HMG-CoA reductase [15, 28], and its ability to function as a "low-affinity" ligand to peroxisome proliferator-activated receptors (PPARs). With respect to the latter activity, Samid et al. [26] demonstrated a possible link between activation of PPARγ by PA and induction of the cell cycle control cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1/MDA-6}$ (p21). These findings followed earlier work which showed that, in MCF-7 breast cancer cells, induction of p21 by PA plays a necessary role in the sequence of events leading to hypophosphorylation of pRB and growth arrest [12]. Genetic changes upstream of p21 induction by PA have not been elucidated.

We have demonstrated the ability of PA analogs to inhibit estrogen-dependent responses in human breast cancer cells [27]. These studies showed the preferential growth inhibitory effects of PA and its analog 4-chlorophenylacetate (4-CPA) on estrogen receptor (ER)+ vs. ER- breast cancer cells. The antiproliferative effects of the PA derivatives on ER+ cells were, at least partly, mediated at the level of gene transcription via direct effects on estrogen response elements (EREs). These finding supported reports showing the ability of PA to exert antiproliferative effects on ER+ breast cancer cells in vivo [1]. In the present work, we have further investigated mechanisms of the antiestrogenic activity of PA by determining its effects on early events in the estrogen-signaling pathway; ER–ERE binding activity and modulation of direct ER target genes.

Materials and methods

Reagents and cell culture

MCF-7 was obtained from the American Type Culture Collection for use in our study. Cells were propagated in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 1 μg/ml fungizone (complete medium). Some experiments were performed under estrogen-free conditions in which the cells were preincubated for at least 2 days in medium containing phenol red-free RPMI 1640 and 2% charcoal-stripped FCS. The sodium salt of phenylacetic acid (PA) was obtained from Sigma (Sigma Chemical Co., St Louis, MO, USA) and dissolved in sterile distilled water (pH 7.4). ICI 182,780, a selective ER modulator, was purchased from TOCRIS Cookson INC (Ballwin, MO, USA). Antibodies to c-myc, p21, estrogen receptor α (ERα) were from Neomarkers (Fermont, CA, USA). The Luc reporter plasmid containing the full-length (2.3-kb fragment) p21 promoter region was from Dr Bert Vogelstein (John Hopkins University School of Medicine, Baltimore, MD, USA). The synthetic Renilla luciferase reporter vector (phRL-SV40) was obtained from Promega (Madison, WI, USA). The c-myc expression plasmid was obtained through the generosity of Dr Andrei Gartel (University of Illinois, Chicago, IL, USA).

RT-PCR

Total RNA was prepared from MCF-7 cells with TRIzol Reagent (Life Technologies, Inc., Rockville, MD, USA) according to the manufacturer's instructions. To amplify 354 bp p21, 110 bp progesterone receptor (PR), 108 bp pS2, 71 bp c-myc, and 185 bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragments, the sequences of PCR primers (Sigma Genosys, Woodlands, TX, USA) were as follows: for p21 sense (5′-GCGATGGAACTTCGACTTTGT-3′), antisense (5′-GGGCTTCCTCTTGGAGAAGAT-3′); for PR sense (5′-CGCGCTACCCTGCACTC-3′), antisense (5′-TGAATCCGGCCTCAGGTAGTT-3′); for pS2 sense (5′-CATCGACGTCCCTCCAGAAGAG-3′), antisense (5′-CTCTGGGACTAATCACCGTGC TG-3′); for c-myc sense (5′-GCCACGTCTCCACAC ATCAG-3′), antisense (5′-TCTTGGCAGCA GGA TAGTCCTT-3′); and for GAPDH sense (5′-CC AT GGAGAAGGCTGGGG-3′), antisense (5′-CAAAG TTGTCATGGATGACC-3′) according to published data [34]. RT-PCR was carried out as described previously [14]. The samples were first denatured at 95°C for 30 s, followed by 35 cycles, each with temperature variations as follows: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The last cycle was followed by an additional extension incubation of 10 min at 72°C. Analysis of products was accomplished on 1% agarose gel containing 0.2 μg/ml ethidium bromide and visualized under UV transilluminator. The densitometric analysis of PCR products was performed by computer software (Bio-Rad Quantity One), GS-800 Imaging Densitometer (Bio-Rad, Hercules, CA, USA), and standardized to the GAPDH product.

Real-time RT-PCR was utilized as previously described [29] for quantifying p21, PR, pS2, and c-myc mRNA levels. All PCR reactions using LightCycler-FastStart DNA Master SYBR Green I kits were performed in the Cepheid Smart-Cycler real-time PCR cycler (Sunnyvale, CA, USA). Final results were expressed as relative mRNA levels normalized to GAPDH gene expression. Melt curve analysis of each sample was supplemented with agarose gel electropho-

resis of randomly selected samples to confirm the success of reactions.

Western blot analysis

Protein concentrations were determined by the Bio-Rad protein assay. Equal amounts of protein (50 μg) from whole cell lysates were solubilized in 4× SDS-sample buffer, separated on SDS polyacrylamide gels. The separated proteins were transferred onto nitrocellulose and blocked with Blotto {1× Tris-buffered saline [10 mM Tris–HCl (pH 8.0), 150 mM NaCl]} with 5% nonfat dry milk and 0.1% Tween 20 for overnight at 4°C. Blots were incubated with antibodies against mouse p21 (1:500), or c-myc (1:1,000 dilution) for 4°C overnight, then incubated with a secondary antibody raised against rabbit IgG conjugated to horseradish peroxidase (1:1,000 dilution; Sigma, St Louis, MO, USA) for 1 h at room temperature. The blots were washed, transferred to freshly made enhanced chemiluminescence solution (Amersham, Arlington, IL, USA), and exposed to X-ray film. Protein bands were quantified by densitometric scanning using a Bio-Rad GS-800-calibrated densitometer. In controls, the p21 or c-myc antibodies were omitted or replaced by serum IgG.

Transient transfection assays

MCF-7 cells were seeded at a density of 1×10^5 cells/well in six-well dishes and grown to 60% confluence. For each well, 2 μg of the plasmid DNA containing wild-type p21 promoter construct and/or c-myc expression plasmid [11], and 0.2 μg of the internal control plasmid phRL-SV40 were co-transfected into the cells using 10 μl of Qiagen Polyfect transfection reagent (Alameda, CA, USA). After 24 h of incubation, cells were treated with PA, ICI 182,780, or solvent for an additional 24 h. The preparation of cell extracts and measurement of luciferase activities were carried out using the dual-luciferase reporter kit according to recommendations by the manufacturer (Promega, Madison, WI, USA). Results were calculated as the ratio of the optical densities of luciferase activities (firefly/renilla)

Electrophoretic mobility shift assay

Nuclear protein extracts were prepared for electrophoretic mobility shift assay as described earlier [9]. The protein content of the nuclear extract was determined using the Bradford protein assay kit (Sigma, St Louis, MO, USA). Electrophoretic mobility shift assay experiments were performed as described previously [13]. The probe of double-stranded oligonucleotides for ERE was synthesized by Santa Cruz Biotechnology (Santa Cruz, CA, USA) based on a human ER consensus binding site (5′-GGATCTAGGTCACTGTG ACCCCGGATC-3′). The complimentary oligonucleotide was annealed and purified following the manufacturer's instructions. The ERE oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase as recommended by the manufacturer. Ten micrograms of nuclear proteins from control and treated cells were incubated with the ^{32}P-labeled oligonucleotide probe under binding conditions (Promega, Madison, WI, USA) for 20 min at room temperature in a final volume of 20 μl. For cold competition, a 100-fold excess of the respective unlabeled consensus oligonucleotide was added in reaction buffer containing nuclear protein for 10 min before adding probe. For assessing specificity, 2 μl anti-ERα antibody (Neomarkers, Fermont, CA, USA) was added to the appropriate sample before ^{32}P-ERE addition. After binding, protein–DNA complexes were electrophoresed on a native 4.5% polyacrylamide gel using 1× Tris–glycine buffer. Each gel was then dried and subjected to autoradiography at −80°C.

Statistical analysis

All experiments were repeated a minimum of three times. All data collected from electrophoresis gel mobility shift assays, luciferase activity assays, RT-PCR, or real-time RT-PCR, and western blot were expressed as means ± SE. The data presented in some figures are from a representative experiment, which was qualitatively similar in the replicate experiments. Statistical significance was determined with Student's *t*-test (two-tailed) comparison between two groups of data sets or one-way ANOVA. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition ($P < 0.05$, see figure legends).

Results

PA inhibits estrogen-mediated transactivation

Our previous work has shown that PA inhibits promoter activation mediated through a canonical ERE as assessed in transient transfection studies using the ERE_v-tk-Luc reporter construct (ERE_v is the vitellogenin A2 ERE sequence from −336 to −310, reference [5]). In those studies, we also demonstrated that PA at

5 and 10 mM significantly inhibited the growth of ER+ breast cancer cell lines, but was much less effective against ER- lines. To investigate whether PA inhibition of transcriptional activation may extend to other natural estrogen regulatory sites, we first determined whether the estrogenic responses of our MCF-7 cells were intact as reflected by induction of PR and pS2, two faithful genomic markers of estrogens action [17, 22, 23]. Figure 1 shows that mRNA levels of both of these genes were strongly upregulated by the addition of 10 nM estradiol to cells cultured under estrogen-free conditions. This induction was suppressed when PA, as well as ICI 182,780, was added to the cultures. Similar effects were observed when the experiments were performed in complete medium which contains FCS and phenol red pH indicator, known sources of estrogenic activity [3, 8]. In the absence of estrogen, neither PA nor ICI 182,780 had significant effects on the expression of these genes. To more precisely evaluate the ability of PA to inhibit the expression of genes containing noncanonical EREs, its dose-dependent effects on the expression of PR and c-myc were assessed. Although both of these genes are known to be transcriptionally regulated by estrogen in MCF-7 cells in an ERα-dependent manner [10, 22, 23], neither the PR nor c-myc promoters contain a "classical" ERE that binds ER homodimers. However, in both cases, half-ERE/Sp1 sites have been identified that bind ER as well as Sp1 proteins while half-site/AP-1 binding motifs are also present in the PR promoter [10, 22, 23]. These chimeric sites are thought to be responsible for mediating estrogen-dependent transcriptional activation of these genes. Figure 2 compares the ability of PA to inhibit endogenous levels of PR and c-myc mRNA expression. The effects of PA on the expression of these genes showed similar dose-dependent characteristics; concentrations of 2.5–10 mM caused inhibition of approximately 30–70% while little effects were seen at lower PA concentrations. Treatment of cells with 100 nM ICI 182,780 showed inhibition of PR and c-myc of 80 and 90%, respectively.

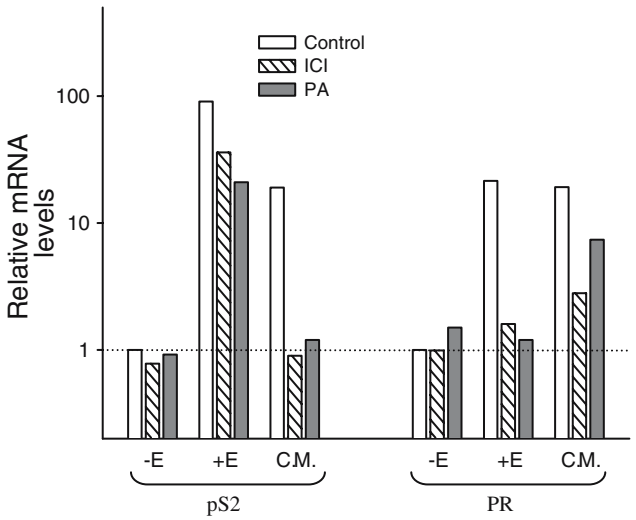

Fig. 1 Activation of estrogen-responsive genes in MCF-7 cells. Replicate cultures growing under estrogen-free conditions as described in Sect. "Materials and methods" were treated with β-estradiol (10 nM) or switched to complete medium (C.M.) and further treated with solvent control, ICI 182,780 (100 nM), or phenylacetate (10 mM) for 48 h. Total RNA was then isolated and subjected to real-time RT-PCR analysis using primers for the estrogen-responsive genes pS2 and progesterone receptor as indicated. *Results* shown are representative and qualitatively similar results were obtained in three experiments

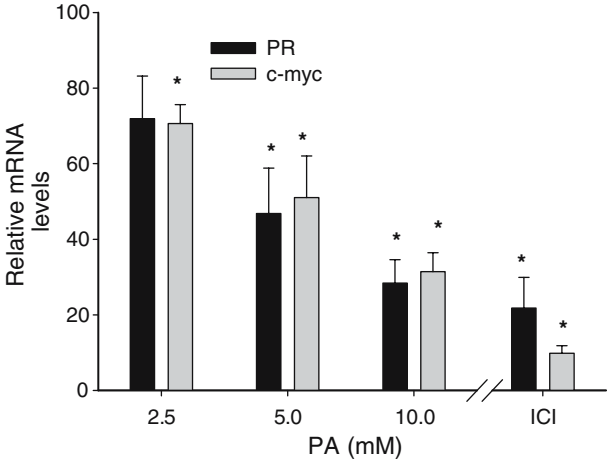

Fig. 2 Dose-dependent inhibition by phenylacetate (PA) of progesterone receptors and c-myc mRNA levels. MCF-7 cells were treated with solvent control, ICI 182,780 (100 nM), or PA at the indicated concentrations for 48 h, after which total RNA was isolated and subjected to real-time RT-PCR analysis. *Results* represent percent (±SEM) of control cultures for at least three determinations for each treatment condition. *, different from solvent-treated controls, $P < 0.05$

PA inhibition of ERα–ERE binding activity

Our studies showing the ability of PA to inhibit signaling through ERE_v in ER+ breast cancer cells [27] suggests that the compound may alter ERα–ERE binding levels. To address this possibility, we tested for the effects of PA on ERα expression and ER–ERE binding activity as assessed by EMSA. Figure 3a shows that treatment of MCF-7 cells with 5 and 10 mM PA resulted in a significant reduction in ERα protein at the higher, but not the lower, PA concentration. As seen in Fig. 3b (lane 1), nuclear extracts prepared from untreated MCF-7 cells produced a prominent band that corresponded in size to an ERα–ERE binding complex whereas a weaker band was detected using

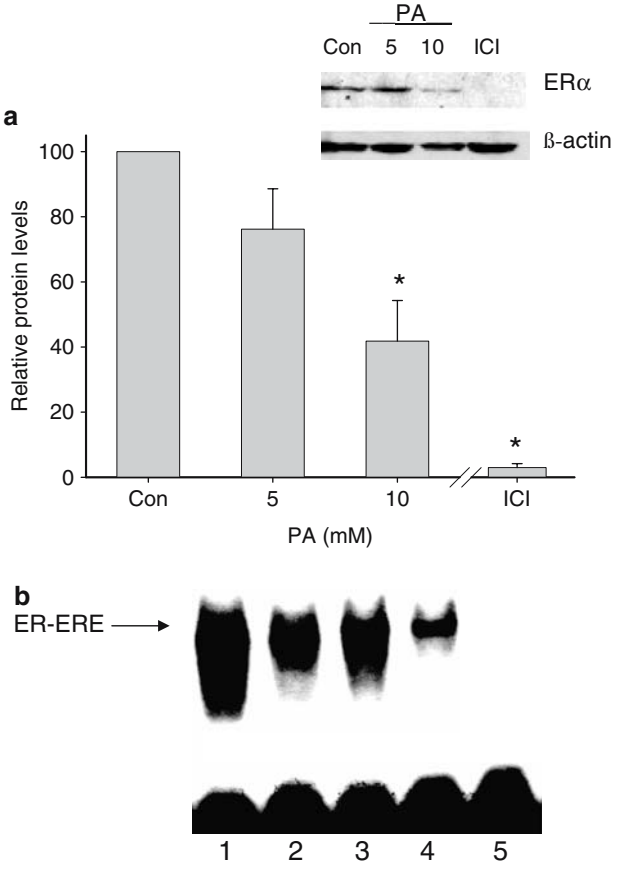

Fig. 3 Phenylacetate (PA) suppresses estrogen receptor α (ERα) expression and ERα–ERE (estrogen response element) binding activity. Nuclear protein was isolated from MCF-7 cells treated with ICI 182,780 (100 nM), PA, or solvent control as indicated for 48 h and analyzed for **a** ERα protein levels by western blotting. *Values* in the graphic represent the means (± SEM) of ERα/GAPDH band densities of three independent experiments. *, different from solvent-treated controls, $P < 0.05$. **b** ERα–ERE binding activity by EMSA as described under Sect. "Materials and methods" using nuclear protein from: *lane 1* control cultures, *lane 2* control cultures where the nuclear protein was preincubated with anti-Eα antibody, *lane 3* PA (10 mM)-treated cells, *lane 4* PA-treated cells where the nuclear protein was incubated with anti-ERα antibody, and *lane 5* control cultures where the ^{32}P-labeled ERE probe was competing with 100-fold molar excess of unlabeled ERE. Results shown are representative and although relative band density values varied from experiment to experiment, qualitatively similar results were obtained in three independent experiments

extract from PA-treated cells (lane 3). The presence of ERα within the complex was confirmed by the addition of anti-ERα antibody, which resulted in a reduction of the intensity of the ERα–ERE band (lanes 2 and 4). As shown, supershift bands were not always observed following incubation of lysate with anti-ERα antibody, a finding consistent with results frequently reported by other investigators [21, 23]. However, a reduction in ERα–ERE binding with anti-ERα antibody was reproducible, confirming the specificity of the reaction. The specificity of binding was further demonstrated by the disappearance of the band with excess unlabeled oligonucleotide corresponding to ERE (lane 5).

Induction of p21 by PA

Having demonstrated that PA treatment inhibited the expression of well-characterized estrogen-responsive target genes, we were interested in investigating the relationship of the inhibition of c-myc to alteration of p21 expression, an important cyclin-dependent kinase inhibitor. Induction of p21 by PA has been implicated in playing an important role in the growth inhibitory effects of this compound on human breast cancer cells [12]. Figure 4a shows that 48-h treatment with PA induced p21 mRNA levels as determined by RT-PCR. In accordance with the changes observed in p21 mRNA, western blot analysis revealed that PA enhanced the expression of p21 protein levels after 72 h (Fig. 4b). To determine whether these effects by PA may reflect increased transactivation of the p21 promoter, transient transfections were performed with human p21 promoter constructs. As shown in Fig. 4c, PA as well as ICI 182,780 was found to significantly activate the 2.3-kb fragment of the p21 promoter.

PA regulation of p21 is dependent on its effects on c-myc

Studies have demonstrated that c-myc can negatively regulate p21 in breast cancer and other cell lines [4, 5, 11, 18]. To assess whether upregulation of p21 by PA may be downstream of its effect on c-myc, we initially evaluated the temporal sequence of drug effect on c-myc protein and its relation to p21 protein and mRNA levels. MCF-7 cultures were treated with PA for various durations of time and examined for protein expression of c-myc and p21. As shown in Fig. 5a, c-myc protein showed a decrease within 12 h of treatment followed by an increase in p21 12–36 h later. The time-dependent upregulation by PA of p21 mRNA levels are shown in Fig. 5b and indicate that significant enhancement required 48 h of treatment. These results are consistent with the possibility that PA induction of p21 may be downstream of c-myc modulation. To directly address this question, we assessed the ability of PA to alter p21-promoter-Luc reporter gene activity in MCF-7 cells when the cells were co-transfected with a c-myc expression plasmid. Transfection of this expression vector resulted in levels of c-myc protein greater than fivefold higher than the endogenous level (Fig. 6 inset). In the presence of 10 mM PA, there was approx-

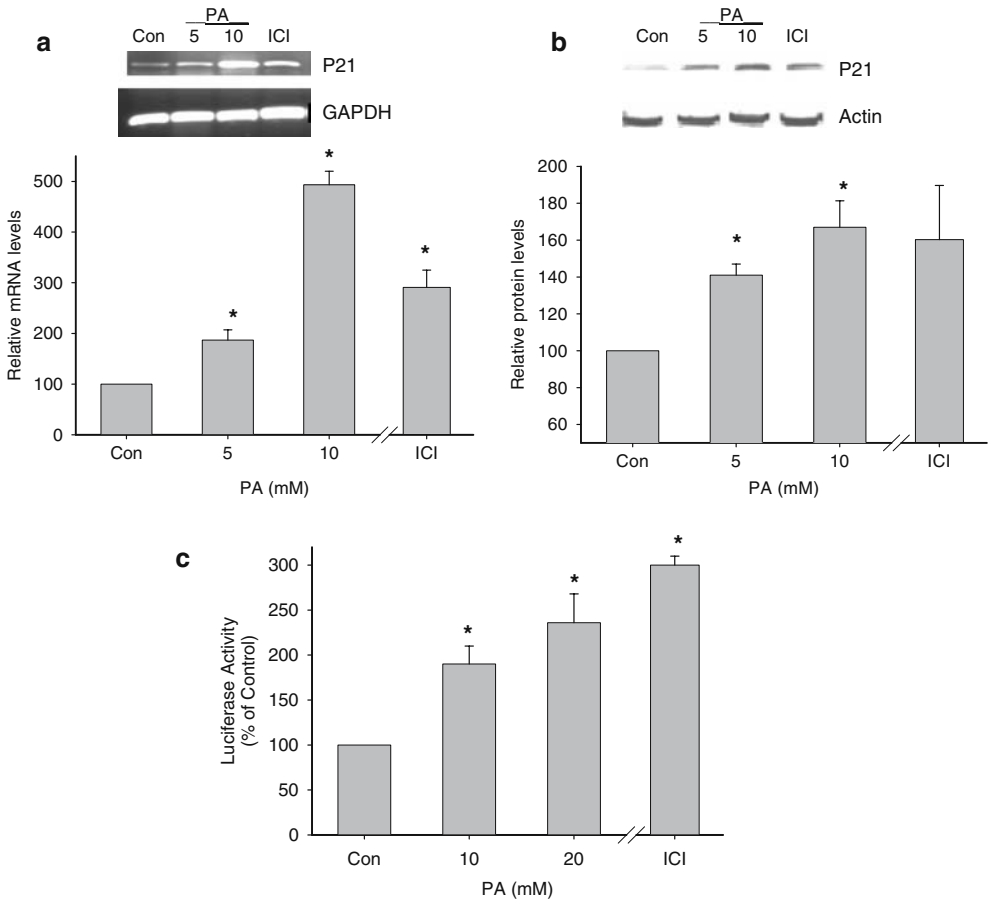

Fig. 4 Induction of p21$^{WAF1/CIP1/MDA-6}$ (p21) by phenylacetate (PA). Total RNA and cellular protein were isolated from MCF-7 cells treated with the indicated concentrations of PA for 48 and 72 h, respectively. RT-PCR (**a**) and western blot analysis (**b**) were then performed as described under Sect. "Materials and methods." The lower graphics in *A* and *B* were obtained by real-time RT-PCR and densitometry, respectively. **c** MCF-7 cells were transiently transfected with a 2.3-kb wild-type p21 promoter construct along with an internal control plasmid and the degree of promoter activation (firefly/renilla luciferase activity) determined after 48-h exposure to the indicated concentration of PA. *Values* are expressed relative to control cultures and represent the mean (± SEM) of three determinations. *, different from control values, $P < 0.05$

imately a 50% reduction in the level of endogenous c-myc protein. In the c-myc-transfected cells, there was only slight reduction of the protein which could basically be accounted for by the reduction in the endogenous component of the total c-myc expressed. Figure 6 shows that overexpression of c-myc downregulated p21 promoter activity and prevented its upregulation by PA treatment.

Discussion

The results of the present study support the contention that PA possesses potent antiestrogenic properties. Previous work demonstrated the ability of this compound to inhibit estrogen signaling through "classical" ERE regulatory motifs [27]. We have now shown that this activity corresponds to lower ER–ERE binding activity in PA-treated cells. Although PA was shown to reduce ERα protein levels at the highest concentration tested (10 mM), significant reduction was not observed at lower PA concentrations that still showed effects on the various parameters measured (e.g., reduction of c-myc and upregulation of p21), including inhibition of ER–ERE binding (not shown). In support of an argument that PA downregulation of ERα cannot completely account for reduced ER–ERE binding activity, our previous study showed that forced overexpression of ERα in MCF-7 cells did not prevent PA from inhibiting ERE reporter gene activation [27]. Johnson et al. [16] demonstrated that PA and its derivatives were not competitive ligands to the ER although the antiproliferative effects of the compounds were found to be as potent as tamoxifen on MCF-7 cells. Thus, unlike most known antiestrogens, PA does not seem to bind to ER and compete with estrogen for interaction with its

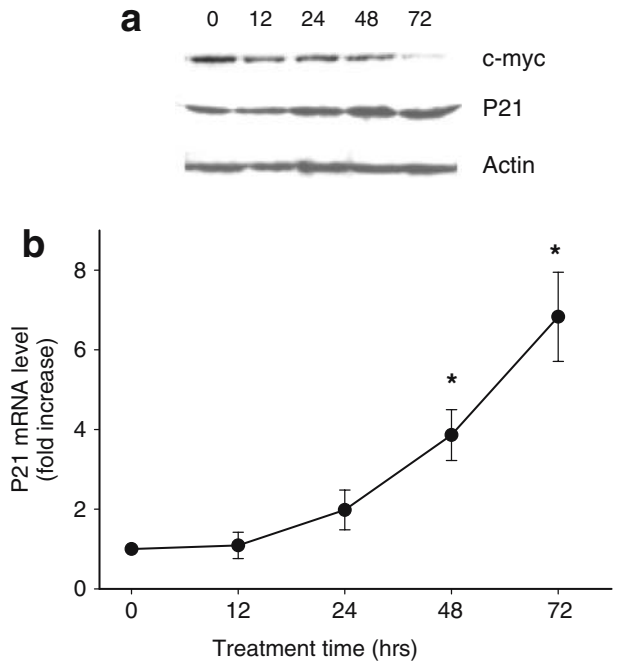

Fig. 5 Time-dependent effects of phenylacetate (PA) on c-myc and p21$^{WAF1/CIP1/MDA-6}$ (p21) expression. Cellular protein and total RNA were isolated from MCF-7 cells treated with 10 mM PA for the time periods as indicated. Western blot analysis (**a**) and real-time RT-PCR (**b**) were then performed to assess levels of c-myc, p21 protein, and p21 mRNA, respectively. Results in **a** are representative with qualitatively similar results obtained in three independent experiments. *Values* in **b** represent the fold-increased p21 mRNA compared to solvent-treated controls and are the mean (± SEM) of three determinations. *, different form control values, $P < 0.05$

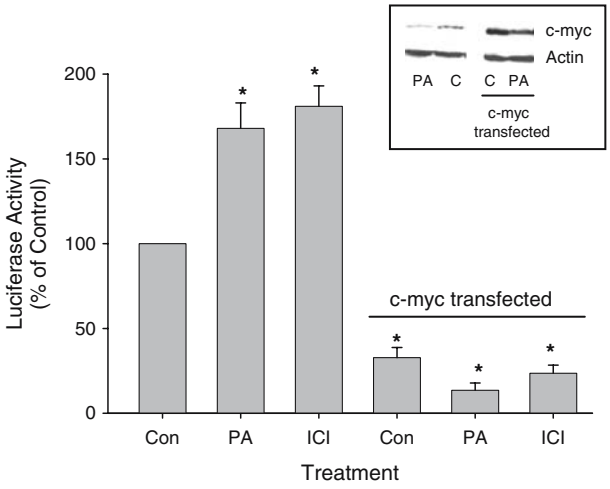

Fig. 6 Overexpression of c-myc inhibited p21$^{WAF1/CIP1/MDA-6}$ (p21) promoter activity and prevented its upregulation by phenylacetate (PA). MCF-7 cells were co-transfected with the p21 promoter–reporter construct, an internal control plasmid, and either a c-myc expression vector or a nonexpressing control vector as indicated. The degree of promoter activation (firefly/renilla luciferase activity) was determined after 48-h exposure to PA (10 mM), ICI 182,780 (100 nM), or solvent control. Values are expressed relative to control cultures and represent the mean (± SEM) of three determinations. *, different from control cultures co-transfected with the nonexpressing control vector, $P < 0.05$. The *inset* confirms overexpression of c-myc in cells transfected with the c-myc expression plasmid. C-myc protein levels were assessed by western blotting after 48 h of treatment with 10 mM PA or solvent control in cell transfected with either the nonexpressing control (*first two lanes*) or c-myc expression (*last two lanes*) vectors

receptor. Our recent studies have illustrated that PA and other piperidinedione derivatives can directly bind to canonical ERE motifs through intercalation into 5′-dTdG-3′:5′-dCdA-3′ sites and that the energetic fit of the compounds is correlated with their potency to inhibit ERE reporter gene activity and proliferation of MCF-7 cells [30]. Thus, although the mechanism(s) of antiestrogenic activity of PA is unknown, it may be due to its direct binding to DNA at certain critical sites within the ERE, thereby interfering with ER–ERE interactions. Such a mechanism of action has recently been demonstrated in the case of the potent anticancer agent XR5944 which has been shown to bis-intercalate into sequence specific DNA motifs such as TG sequences found in AP-1 and NF-1 regulatory sites [6]. This intercalation was shown to directly interfere with AP-1 protein (i.e., fos, jun dimers)–DNA binding which resulted in inhibition of AP-1-mediated transcriptional activation.

The present work suggests that the antiestrogenic properties of PA extents to nonclassical estrogen regulatory elements such as that demonstrated in the promoters of the PR and c-myc genes. Although both promoters contain half-ERE sites that are thought to bind ER in conjunction with other protein cofactors (e.g., Sp1) [10, 22], recent studies have implicated the "tethering" of ER with the cofactors as a mechanism of its transcriptional regulation [33]. The role of the half-ERE motifs in this mechanism of action is unknown but is thought to be involved in the dimeric binding of liganded ER as part of in its initial interactions with the relevant cofactor proteins. Previous studies have shown that PA also inhibits cyclin D1 promoter activity in MCF-7 cells and other breast cancer cell lines [16, 27]. This gene is overexpressed in approximately 40% of all breast cancer and has been shown to be positively correlated with ER expression in primary tumors [20, 31]. Gene transcription of cyclin D1 is rapidly induced via an ERE regulatory region that is also different from the canonical ERE [2]. Thus, our demonstration that PA can interfere with ER–ERE binding appears to extent to a variety of "nonclassical" ER–ERE interactions that play fundamental roles in cellular growth and function.

As a means of investigation the role(s) that suppression of ER–ERE binding by PA may play in its antiproliferative activity, the effects of the compound on p21 expression was determined. Results showed that PA induced p21 mRNA and protein levels, and activated its promoter in MCF-7 cells in a dose-dependent fashion. To characterize the temporal sequence of drug effect on c-myc as an estrogen-responsive gene and its relationship to upregulation of p21, MCF-7 cells were treated with PA for various lengths of time and examined for expression of these two genes. Results showed that inhibition of c-myc following PA treatment preceded upregulation of p21 expression. Recent work has shown that c-myc protein can affect p21 transcription through sequestration of Sp1 [11]. Thus, our finding that inhibition of c-myc protein preceded an increase in the levels of p21 mRNA is consistent with the contention that upregulation of p21 is downstream of PA's effect on c-myc. This hypothesis was confirmed by transient transfection experiments showing that overexpression of c-myc protein prevented PA-induced activation of the p21 promoter.

Taken together, the results presented herein provide significant new information concerning the sequence of events leading to growth arrest of breast cancer cells by PA and its derivatives. In previous work, we have shown that PA can intercalate with specific base-pair configurations located within the ERE, and proposed that this activity can interfere with ER–ERE interactions [30]. In those studies, the predicted higher binding energy of halide derivatives of PA with ERE was found to correlate with the compounds' antiestrogenic effects. Thus, the PA derivative 4-CPA was shown to be effective in inhibiting estrogen signaling at threefold lower concentrations that the parent compound and at plasma levels which are pharmacologically achievable in mice [29]. The present findings confirmed the ability of PA to inhibit ER–ERE binding which results in suppression of certain estrogen target genes such as c-myc. Our data indicate that PA inhibition of c-myc is upstream of its ability to induce p21 expression which has been shown to play a necessary role in events leading to hypophosphorylation of pRB and growth arrest [12]. The new information obtained in this study will encourage the future development and testing of other PA compounds with predicted high energy of binding within the ERE and correspondingly high antiestrogenic activity. The ability to block estrogen signaling through compound interactions with EREs rather than with ERs should have major implications for the treatment of breast cancer and other estrogen-dependent diseases.

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