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A Novel Antiestrogenic Mechanism in Progesterone Receptor-transfected Breast Cancer Cells*

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The expression of progesterone receptor (PR) is normally estrogen-dependent, and progesterone is only active in target cells following estrogen exposure. This study revealed that the effect of estrogen was markedly disrupted by estrogen-independent expression of PR. Transfection of PR in estrogen receptor (ER)-positive MCF-7 cells abolished the estradiol-17β growth stimulatory effect that was observed in the parental cells and the vector-transfected controls in a ligand-independent manner. The antiestrogenic effect was also observed at the level of gene transcription. Estradiol-17β (E2)-induced gene expression of pS2 and GREB1 was impaired by 50–75% after 24–72 h of E2 treatment in PR-transfected cells. Promoter interference assay revealed that PR transfection drastically inhibited E2-mediated ER binding to estrogen response elements (ERE). The antiestrogenic effects of transfected PR are associated with enhanced metabolism of E2. HPLC analysis of [^{3}H]E2 in the samples indicated that the percentage of [^{3}H]E2 metabolized by PR-transfected cells in 6 h is similar to that by vector-transfected control cells in 24 h (77 and 80%, respectively). The increased metabolism of E2 may, in turn, be caused by increased cellular uptake of E2, as demonstrated by whole cell binding of [^{3}H]E2. The findings open up a new window for a hitherto unknown functional relationship between the PR and ER. The antiestrogenic effect of transfected PR also provides a potential therapeutic strategy for estrogen-dependent breast cancer.

Ovarian steroid hormones estrogen and progesterone are essential for the normal growth and development of the breast. The two hormones also play critical roles in the regulation of breast cancer development. It has been established that estrogen stimulates the growth of breast cancer cells both *in vivo* and *in vitro* (1–3). Antiestrogens have been the front-line therapy for hormone-dependent breast cancers (4), which are estrogen receptor (ER)[1]- and progesterone receptor (PR)-positive. Nonetheless, antiestrogen-induced remissions are often followed by acquisition of antiestrogen resistance and ultimately disease relapse (5). The acquired resistance is mostly manifested by insensitivity to antiestrogens such as tamoxifen and increased ER activity (6). It has been shown that signaling via the epidermal growth factor receptor and HER-2/*neu* can activate both ER and the important ER coactivator AIB1 (7). Breast tumors with high levels of AIB1 and HER-2 may be resistant to tamoxifen because of an increase in its estrogen agonist activity. The development of strategies for the effective treatment of tamoxifen-resistant breast cancer is one of the main challenges for breast cancer research.

Whereas it is established that estrogen stimulates the growth of breast cancer cells, the function of progesterone in breast cancer remains controversial. Progestins were found to stimulate growth, have no effect, or inhibit growth depending on the experimental conditions and the status of hormone receptors (8–11). This controversy reflects our insufficient understanding of progesterone biology and has hampered effective applications of progestins or antiprogestins in breast cancer treatment.

The controversies over the effect of progesterone in breast cancer are due to several complexities in the PR system. One of the complexities is that PR is an estrogen receptor-dependent gene product (12, 13), and the action of progesterone requires priming treatment of estrogen to induce PR. It is conceivable that the prior presence of estrogen may significantly confound assessment of the role of progesterone on growth and other cellular processes in breast cancer cells. On the other hand, the effects of progesterone also depend on a complex interaction between estrogen, progesterone, and their receptors. Studies have demonstrated the suppression of estrogen-stimulated ER activity by agonist- and antagonist-occupied PR (14–15). However, ER can transmit signals received from the agonist-activated PR to the Src/p21 (ras)/Erk pathway (16), suggesting a synergistic interaction between the ER and PR.

Our previous work has shown that estrogen-independent expression of PR by transfection in the ER- and PR-negative breast cancer cells MDA-MB-231, facilitated a striking inhibition of cell growth by progesterone *in vivo* and *in vitro* (11, 17). Progesterone also induced remarkable focal adhesions in the PR-transfected MDA-MB-231 cells (18). This present study reveals that estrogen-independent expression of PR in MCF-7 cells exhibited strong antiestrogenic and antiproliferative effect that is independent of PR ligands. The antiestrogenic effect is associated with a marked decrease of estradiol-17β (E2) in the culture medium of PR-transfected cells. This suggests that the transfected PR exerted the antiestrogenic effect by modulating cellular uptake and metabolism of E2. The study pro-

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[1] The abbreviations used are: ER, estrogen receptor; ERE, estrogen response element; E2, estradiol-17β; PR, progesterone receptor; PR-A, PR isoform A; PR-B, PR isoform B; eiPR, estrogen-independent PR; RT, reverse transcription; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; Ab, antibody.

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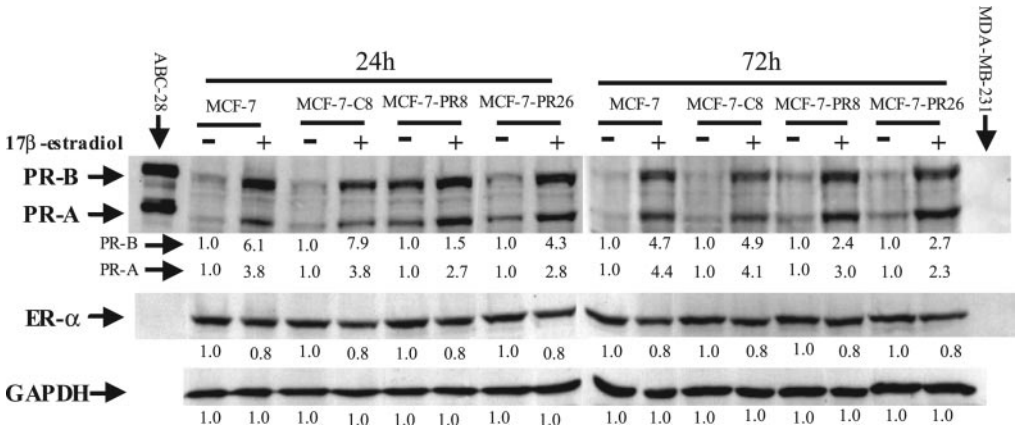

FIG. 1. **Levels of PR and ERα in MCF-7 cells, vector-transfected MCF-7 cells (MCF-7-C8), and PR-transfected MCF-7 cells (MCF-7-PR8 and MCF-7-PR26).** Cell lysates were collected from cells treated with 0.1% ethanol (−) or 10^{-9} M E2 (+) for 24 or 72 h, and analyzed for PR and ERα by Western blot. GAPDH was used as loading control. ER- and PR-negative breast cancer cell line MDA-MB-231 and ABC28 cells (MDA-MB-231 cells transfected with PR-A and PR-B) were used as negative and positive controls, respectively.

vides the basis for a novel antiestrogenic mechanism that may be used for breast cancer treatment.

MATERIALS AND METHODS

Chemicals—All tissue culture reagents were from Invitrogen. Propidium iodide (PI), progesterone, 17β-estradiol, estrone, estriol, 6α-hydroxyestradiol, and 16α-hydroxyestrone were purchased from Sigma. Trichloroacetic acid was purchased from Fisher Scientific. Nonidet P-40 was purchased from USB Corp. (Cleveland, OH). [2,4,6,7,16,17-^{3}H]Estradiol-17β (~149 Ci/mmol) was from Amersham Biosciences.

Cell Culture—MCF-7 cells were obtained from the American Type Culture Collection in 1995 at passage 147. Cells were routinely maintained in phenol red-containing Dulbecco's modified Eagle's medium supplemented with 7.5% fetal calf serum (Hyclone, Logan, UT), 2 mM glutamine, and 40 mg/liter gentamicin. For all experiments involving cell culture, phenol red-free medium containing 5% dextran-coated charcoal-fetal calf serum (DCC-FCS), and 2 mM glutamine was used, and this medium will be referred as Test Medium in the subsequent description.

Transfection—PR expression vectors hPR1 and hPR2 contain human PR cDNA coding for PR isoform B and A, respectively, in pSG5 plasmid (18). Vector pBK-CMV (Stratagene) containing the neomycin-resistant gene was cotransfected with hPR1 and hPR2 into MCF-7 cells using Lipofectin reagent (Invitrogen). Neomycin-resistant clones selected in medium containing G418 (500 μg/ml) were further screened for vector pSG5 sequence by PCR using primers specific for pSG5. Cells stably transfected with both pBK-CMV and pSG5 (Stratagene) plasmids were used as transfection controls.

Western Blotting Analysis—Total proteins were extracted from the cells by three cycles of freezing (liquid nitrogen) and thawing (37 °C water bath) in buffer containing 0.25 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 2% β-mercapthoethanol. Lysates containing 50 μg of protein were separated on 8.5% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Human PR isoforms A and B were probed with anti-PR antibody Ab-8 from Neomarker (Fremont, CA); human ERα was probed with anti-ER antibody Ab-15 from Neomarker; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probed by anti-GAPDH antibody (Ambion) was used as loading control.

Cell Growth Assay—1×10^4 cells were seeded onto 6-well plates in Test Medium. Two days later, the cells were treated with 10^{-9} M E2 in fresh medium from 1000-fold stock in ethanol, which gave a final concentration of ethanol of 0.1%. Treatment controls received 0.1% ethanol only. The medium with the test compounds was changed every 2 days, and cells were counted with a hemocytometer.

Cell Cycle Analysis—Cells plated onto 6-well plates were grown in Test Medium for 48 h before they were treated with E2 or progesterone for the indicated periods of time. Trypsinized cells were stained with PI in Vindelov's mixture (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 50 mg PI/liter, 10 mg/liter RNaseA, and 0.1% Nonidet P-40) for 1 h in the dark. The stained cells were analyzed in FACS Caliber flow cytometer (BD Biosciences) with excitation wavelength of 488 nm. The resulting histograms were analyzed by program MODFIT for cell cycle distribution.

Real Time RT-PCR—cDNAs were synthesized from 5 μg of total RNA. Real time PCR was performed with SYBR Green Master Mix on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers for pS2 are 5′-ATGGCCACCATGGAGA-ACAAGG-3′ (forward) and 5′-CTAAAATTCACACTCCTCTTCTGG-3′ (reverse). The primers for GREB1 are 5′-CCCCGTGGCCCCGCAGA-G-3′ (forward) 5′-AGGATGAGCCCGAGGAGGAGGACA-3′ (reverse). 36B4 (forward: 5′-GATTGGCTACCCAACTGTTGCA-3′ and reverse: 5′-CAGGGGCAGCAGCCACAAAGGC-3′), which codes for human acidic ribosomal phosphoprotein, was used as internal control for normalizing the quantity of cDNA analyzed. PCR for each gene fragment were performed in triplicates, and each experiment was repeated twice. The relative amount of PCR products generated from each primer set was determined on the basis of threshold cycle (Ct).

Promoter Interference Assay—Cells were grown in Test Medium for 48 h before they were transfected with 2 μg of CMV-TATA-ERE$_2$-CAT (cytomegalovirus-estrogen response element-chloramphenicol acetyltransferase) or 2 μg of control vector CMV-TATA-CAT. 16 h later, cells were given fresh medium and treated with 10^{-9} M E2 or 0.1% ethanol as control. Cells were harvested at 16, 24, and 48 h after E2 treatment. CAT activity was measured using the CAT-ELISA kit (Roche Applied Sciences, Indianapolis, IN).

Cellular Uptake of [^{3}H]E2—Cells (1×10^5) were grown on 12-well plates in Test Medium for 48 h before they were incubated with 10^{-9} M [^{3}H]E2 in phenol red-free Dulbecco's modified Eagle's medium for 1 h. Nonspecific uptake was determined by incubating 10^{-9} M [^{3}H]E2 in the presence of 200-fold excess of unlabeled E2. Following incubation, the cells were washed with cold Mg^{2+}- and Ca^{2+}-free DBPS three times, and cellular [^{3}H]E2 was extracted with 500 μl of absolute ethanol and measured by a Microbeta liquid scintillation counter (PerkinElmer Life Sciences).

Analysis of Estradiol Metabolism by HPLC—Cells were seeded in 6-well plates for 48 h before they were treated with 10^{-9} M [^{3}H]E2. The conditioned media were harvested following 3, 6, and 24 h treatment. Cell-free media incubated with 10^{-9} M [^{3}H]E2 were used as controls for each time point.

The proteins in the media were precipitated with 10% trichloroacetic acid followed by washing with acetone twice. The pH of the deproteinated medium was adjusted to ~5.0 with 10 N NaOH. HPLC was performed on a Supercosil LC-18-DB column (5 μm, 25 cm × 4.6 mm, Supelco Bellefonte, PA) at room temperature. The mobile phase A contains 21% acetonitrile, 22% methanol, 57% water, 0.1% acetic acid; B contains 40% acetonitrile, 60% water, 0.33% acetic acid. The gradient was as follows: 0–15 min, 100% solvent A; 15–25 min, a linear increase to 19% solvent B; 25–30 min, a linear increase to 20% solvent B; 30–53 min, a linear increase to 100% solvent B; 53–60 min, 100% solvent B. The flow rate was 1.5 ml/min. 0.5 min flow fractions were collected and the radioactivity was measured on a Microbeta liquid scintillation counter.

Statistical Analysis—Differences among treatment groups with regard to cell proliferation and growth were analyzed by analysis of variance followed by the least significant difference (LSD) test. Differences of gene expression, reporter gene activity, and E2 concentration between control and E2-treated cells were tested by Student's *t* test.

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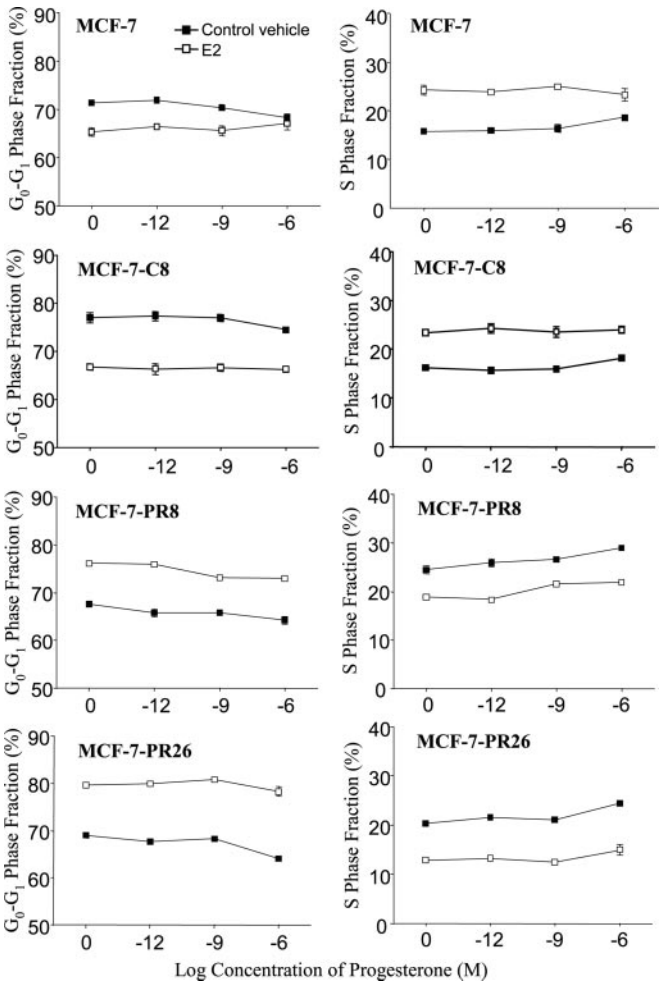

FIG. 2. **E2 inhibited cell cycle progression in PR-transfected MCF-7 cells.** Cells were treated with 0.05% ethanol (■) or 10^{-9} M E2 (□) for 24 h before they were treated with 0, 10^{-12} M, 10^{-9} M, or 10^{-6} M progesterone for 48 h. They were then harvested, stained, and analyzed for cell cycle distribution using flow cytometry. Results are expressed as mean ± S.D., $n = 3$.

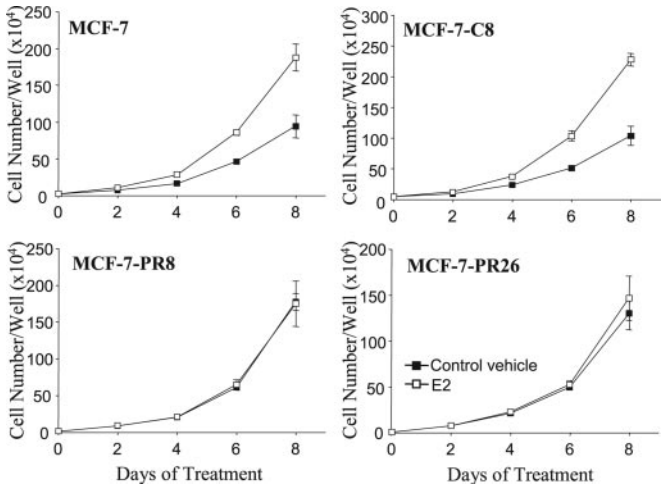

FIG. 3. **The growth stimulatory effect of E2 was abolished in PR-transfected MCF-7 cells.** Cells were treated with 10^{-9} M E2 or 0.1% ethanol for various periods of time. The cell numbers were determined by counting on a hemocytometer at 0, 2, 4, 6, and 8 days after the initial treatment. Results are expressed as mean ± S.D., $n = 4$.

RESULTS

Characterization of PR Transfectants—Fig. 1 shows the levels of PR and ER proteins in the parental cells MCF-7, the vector-transfected control cells MCF-7-C8 and in PR-transfected cells MCF-7-PR8 and MCF-7-PR26. MCF-7-PR8 and MCF-7-PR26 cells express ~3–5 times more PR than MCF-7 and MCF-7-C8 cells in the absence of E2. MCF-7-PR8 cells express ~three times as much PR-B as PR-A, whereas MCF-7-PR26 express twice as much PR-A as PR-B. Despite the expression of estrogen-independent PR (eiPR), E2 was able to induce endogenous PR in PR-transfected cells. Fig. 1 also revealed that the ER protein was reduced by 20% following E2 treatment in all the cell lines. On the other hand, PR transfection did not affect the levels of ER in the presence or absence of E2.

PR-transfected MCF-7 cell clones MCF-7-PR8 and MCF-7-PR26 were studied for their responses to progesterone and estrogen. The MCF-7 parental cells and the vector-transfected MCF-7 clones 8 (MCF-7-C8) served as control cells.

E2 Inhibited Cell Cycle Progression in PR-transfected MCF-7 Cells after 72 h of Treatment—The experiment depicted in Fig. 2 was designed to determine the effect of progesterone on cell cycle progression in MCF-7 cells transfected with PR. The cells had been treated with either 0.05% ethanol or 10^{-9} M E2 for 24 h before they were treated with various concentrations (0, 10^{-12} M, 10^{-9} M, and 10^{-6} M) of progesterone for 48 h. The duration of E2 treatment is thus 72 h. The estradiol treatment was to induce PR so that the effect of progesterone can be compared between PR-transfected cells and the control cells. The results revealed that increase of PR protein by transfection in MCF-7 cells did not affect the action of progesterone on cell proliferation. Whereas having no affect at lower concentrations, progesterone at 10^{-6} M elicited about an 8–10% increase of the S-phase fraction in both PR-transfected cells (MCF-7-PR8 and MCF-7-PR26 cells) and control cells (MCF-7 and MCF-7-C8) ($p < 0.01$) (Fig. 2). However, the affect of E2 on PR-transfected MCF-7 cells is the opposite of that on control cells. In accordance with the known effect, E2 increased the S-phase fraction of MCF-7 and vector-transfected control MCF-7-C8 cells by an average of 35% regardless of the presence or absence of progesterone. In contrast, E2 decreased S-phase cells by an average of 30–40% in PR-transfected cells MCF-7-PR8 and MCF-7-PR26. This effect of transfected PR is ligand-independent as progesterone at various concentrations did not modify this antiproliferative effect of E2 in PR-transfected MCF-7 cells.

PR Transfection in MCF-7 Cells Abolished the Growth Stimulatory Effect of E2—Fig. 3 shows that the numbers of E2-treated MCF-7 and MCF-7-C8 cells were double that of vehicle-treated controls after 8 days. In contrast, the cell numbers of E2-treated MCF-7-PR8 and MCF-7-PR26 cells were the same as that of vehicle-treated cells. Although the cell numbers of PR-transfected cell clones appears to be higher than that of control cells after 8 days in culture, this does not necessarily indicate an increased rate of cell growth in PR-transfected cells. As shown by Fig. 6, the S-phase fraction of PR-transfected cells receiving control vehicle is similar to that of parental and vector-transfected cells (19 *versus* 22%).

Antiestrogenic Effect of PR Transfection Is Also Exerted at the Level of Gene Transcription—ER is a transcription factor, and the effect of E2 is mediated by ER through its gene regulation activities. To determine if transfected PR inhibits the effect of E2 by modifying the transcriptional activity of ER, the expression of two well known estrogen target genes pS2 and GREB1 were studied by real-time RT-PCR analysis (Fig. 4). E2 induced the increase of pS2 expression by 6–10-fold after 24 h of treatment in all cell lines tested. This induction of pS2 gene expression was sustained in MCF-7 and MCF-7-C8 cells after 48 and 72 h of treatment. In MCF-7-PR8 and MCF-7-PR26 cells; how-

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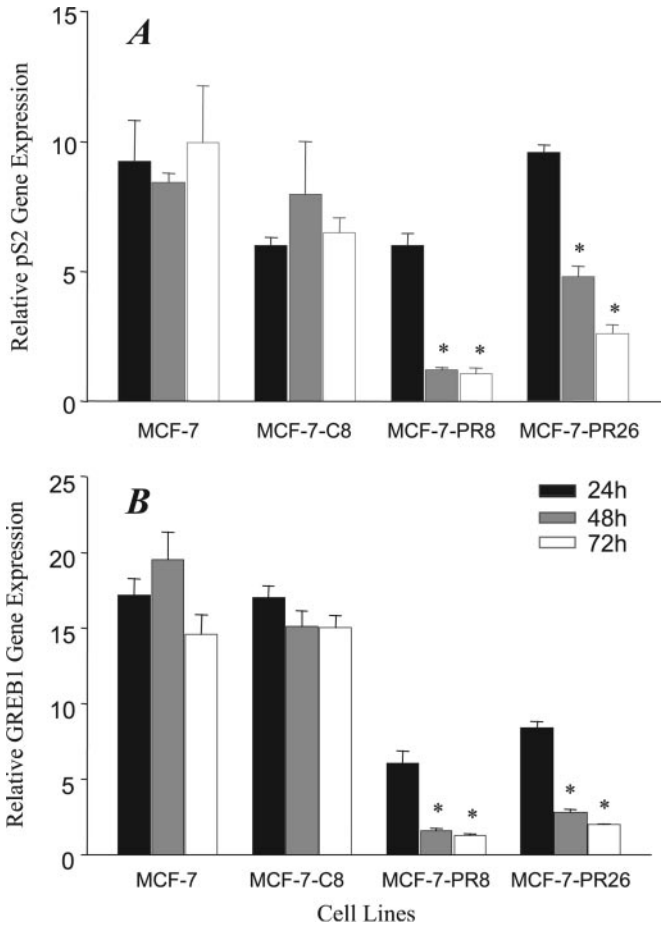

FIG. 4. **Induction of the gene expression of pS2 and GREB1 by E2 was repressed in PR-transfected MCF-7 cell.** Cells were treated with 0.1% ethanol or 10^{-9} M E2 in 0.1% ethanol, and total RNA was extracted from the cells at 24, 48, and 72 h after treatments. The expression of pS2 (*A*) and GREB1 (*B*) was analyzed by real time RT-PCR. The expression of each gene is expressed relative to vehicle-treated controls, which are given the value of 1. The results are the means of three replicates. *Asterisk* indicates that the gene expression at 48 and 72 h time points are significantly ($p < 0.01$) less than the expression at the 24-h time point.

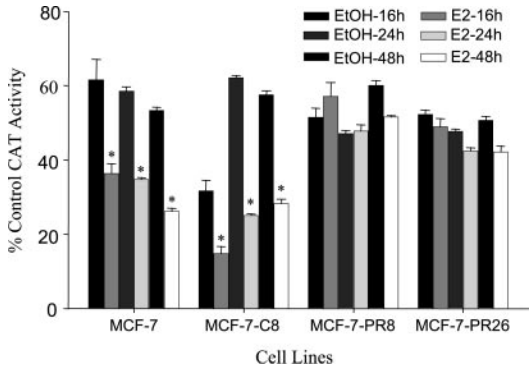

FIG. 5. **Effect of PR transfection in MCF-7 cells on ER-ERE binding in the promoter interference assay.** Cells were transfected with CMV-TATA-ERE_2-CAT or CMV-TATA-CAT for 16 h before they were treated with 0.1% ethanol or 10^{-9} M E2. CAT activity was measured by ELISA assay at 16, 24, and 48 h after E2 treatment. The CAT activity in CMV-TATA-ERE_2-CAT-transfected cells was calculated as the percentages of that in cells transfected with CMV-TATA-CAT. Results are means ± S.E., $n = 3$. *Asterisk* indicates that CMV-driven CAT activity in E2-treated cells is significantly ($p < 0.01$) less than that in vehicle-treated control cells.

ever, the induction of *pS2* gene expression by E2 was impaired by 50–75% after 48 and 72 h of treatment ($p < 0.001$). Similarly, estradiol-induced GREB1 expression following 48 and 72 h of treatment was impaired by 70% compared with that at the 24 h time point in PR-transfected cells. In contrast, the induction of gene expression in MCF-7 and MCF-7-C8 cells for both pS2 and GREB1 remained statistically similar at all time points studied. It is interesting to note that the inhibition of transcription activity of ER by PR transfection at 24 h was not as prominent as that at 48 and 72 h.

Transfected PR Interferes with ER Binding to Estrogen Response Elements (ERE)—To determine if the transfected PR modify the effect of E2 by interfering with ER binding to specific ERE, we conducted promoter interference assay by transient transfection. The reporter plasmid (CMV-ERE_2-CAT) contains two ERE sequences positioned between the TATA box and the start of transcription. The ER binding to ERE will disrupt the assembly of the general transcription complex, resulting in the reduction of the CMV promoter-driven CAT reporter gene expression. It has been validated that insertion of up to three ERE into CMV-TATA-CAT plasmids did not disrupt the expression of CAT activity in the absence of ER (20, 21). Cells transfected with CMV-TATA-CAT reporter construct were used as transfection controls for each treatment. The result of each treatment in Fig. 5 was expressed as the percentage of CAT expression by CMV-TATA-ERE_2-CAT relative to that by CMV-TATA-CAT. CAT expression by CMV-TATA-ERE_2-CAT in vehicle-treated cells is on the average 50% of that by CMV-TATA-CAT, suggesting some intrinsic interference of CMV promoter activity by cellular proteins. In MCF-7 and vector-treated MCF-7-C8 cells, E2 treatment for 16, 24, and 48 h caused a 40–60% reduction in CAT activity compared with vehicle-treated controls. In contrast, estradiol-17β-mediated promoter interference activity was largely abolished in PR-transfected cells at these time points. Hence transfected PR inhibited ER binding to ERE. This effect appeared as early as 16 h after treatment.

The Antiestrogenic Effect of Transfected PR on Cell Proliferation Exhibited a Delay of ~60 h—We have shown earlier that the inhibitory effect of E2 on cell cycle was demonstrated after 72 h of treatment (Fig. 2). Gene expression studies showed that the antiestrogenic effect of transfected PR appeared following a delay of ~24 h. Further studies were carried out to determine if this delay also occurs in cell cycle regulation. Surprisingly, the effect of E2 on cell cycle progression was stimulatory in these PR-transfected cells up to the time point of 60 h after treatment. The S-phase fraction of these cells plunged to about 60% of the vehicle-treated controls only after 72 h of treatment (Fig. 6*A*). In contrast, S-phase fractions of E2-treated MCF-7 and MCF-7-C8 cells were consistently higher than that in vehicle-treated control cells throughout the 72-h period examined. These results suggest E2 treatment turned growth inhibitory after 72 h.

Studies of both gene expression and cell cycle suggest that E2 functions normally during the first 24–48 h of treatment depending on the parameter tested. We asked the question whether PR transfection affected the metabolism of E2 such that E2 is short-lived. In this scenario, a second dose of E2 should still be growth stimulatory initially. This hypothesis was tested by studying cells in culture over a period of 7 days, and the culture medium was replaced by fresh medium with E2 after 72 and 120 h. Fig. 6*B* shows that freshly added E2 was able to induce an increase of S-phase fraction after 24 h in culture in PR-transfected cells. But it again turned antimitogenic after 48 h in culture. The same stimulatory and inhibitory pattern occurred following the third addition of E2. This is in sharp contrast to the effect of E2 on vector-transfected cells MCF-7-C8 cells in which the number of S-phase cells was

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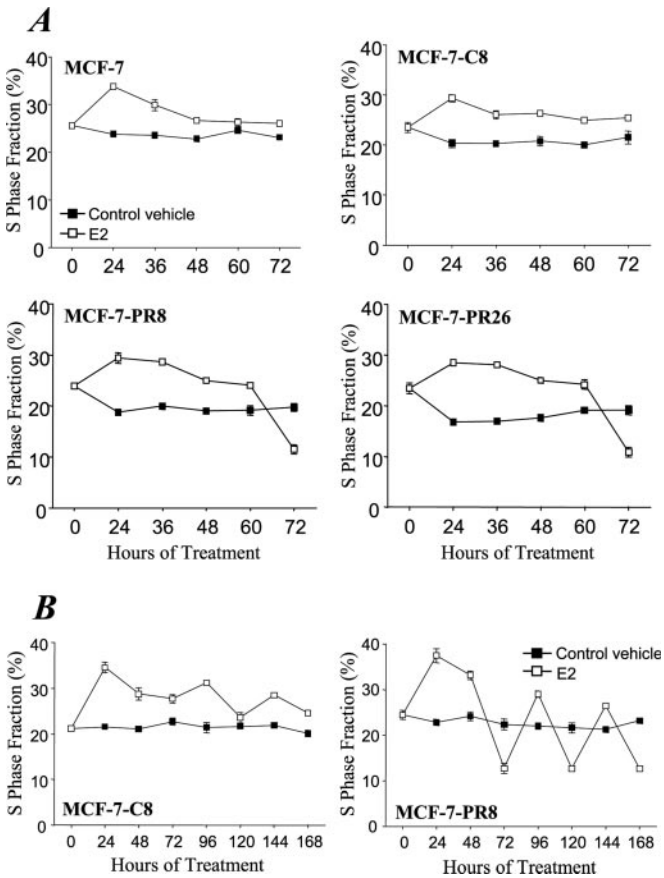

FIG. 6. *A*, the effect of E2 on cell cycle progression as a function of time. Cells were treated with 0.1% ethanol as vehicle control (■) or 10^{-9} M E2 (□) for various time before were harvested and analyzed for cell cycle distribution by flow cytometer. Results are expressed as mean ± S.D., $n = 3$. *B*, effect of replacement of fresh E2 on cell cycle progression. Cells were treated with 0.1% ethanol as vehicle control (■) or 10^{-9} M E2 (□) for various periods of time and analyzed for cell cycle distribution. Medium was changed with fresh E2 at 72 and 120 h after the initial treatment. Results are expressed as mean ± S.D., $n = 3$.

higher in E2-treated cells than that of vehicle-treated control cells throughout the 7-day period. These data support the hypothesis that E2 in PR-transfected cells may be metabolized at a faster rate than that in control cells, and E2 may be converted to a growth inhibitory compound in PR-transfected MCF-7 cells.

Heightened Metabolism of E2 in PR-transfected MCF-7 Cells—The metabolic changes of [^{3}H]E2 in the media of MCF-7-PR8 and MCF-7-C8 cells were monitored by scintillation counting of radioactivity in the fractions of eluate following HPLC separation (Fig. 7). The whole media were analyzed without solvent extraction so that metabolites of both a hydrophobic and hydrophilic nature were present in the sample. The protein components were removed by trichloroacetic acid precipitation to prevent interference in separation. [^{3}H]E2 was eluted as peak *y* since it was the only peak in cell-free medium, and its retention time also matched that of E2 standard (23.9 ± 1.5 min). Peak *x* is a mixture of [^{3}H]E2 metabolites, and this peak was not present in cell-free medium. There was a progressive decrease of the [^{3}H]E2 peak and increase of the metabolites peak *x* with time in both cell lines. However, these changes occurred more rapidly in culture medium of MCF-7-PR8 cells compared with that of MCF-7-C8 cells. After 3 h of treatment, 58% of [^{3}H]E2 was metabolized by MCF-7-PR8 cells compared with 26% (Table I) by MCF-7-C8 cells. Following 6 h of treatment, 77% of [^{3}H]E2 was metabolized by MCF-7-PR8 cells compared with 40% by MCF-7-C8 cells. It is remarkable that the percentage of [^{3}H]E2 metabolized by MCF-7-PR8 cells in 6 h (77%) is similar to that by MCF-7-C8 cells in 24 h (80%), suggesting a much faster metabolic conversion of E2 in PR-transfected cells than the vector-transfected controls.

The retention time of [^{3}H]E2 metabolites in peak *x* is between 4 and 9 min. This overlaps the retention time for 6α-hydroxyestradiol (4.3 min), estriol (5.1 min), and 16α-hydroxyestrone (7.9 min) (Table II). Although these three compounds are some of the major metabolites of E2, there are insufficient data for us to confirm the identities of the metabolites in peak *x*.

We also determined whether the heightened E2 metabolism is caused by enhanced cellular uptake. Fig. 8 shows that after just 1 h of incubation with [^{3}H]E2, cellular [^{3}H]E2 in MCF-7-PR8 and MCF-7-PR26 cells was 32% higher ($p < 0.05$) than that in control cells MCF-7 and MCF-7-C8. It appears that enhanced cellular uptake of E2 in PR-transfected cells is possibly one of the mechanisms for increased metabolism of E2.

DISCUSSION

Phylogenetic analysis and genome mapping of steroid receptors revealed that the first steroid receptor to evolve is the ER, followed by the progesterone receptor (22, 23). Estrogen-dependent expression of PR in target tissues may carry selective advantages from an evolutionary point of view. The emergence of PR before estrogen may inflict certain threats to homeostasis of cells. This study demonstrates that the expression of PR prior to estrogenic signal severely hampered the function of estrogen. eiPR inhibited E2-mediated gene expression and abolished its growth stimulatory effect. While the antiestrogenic effects of progestin have been documented (14, 15, 24), the antiestrogenic effects of these eiPR are ligand-independent. Neither progesterone nor antiprogestins such as RU486 or ZK98299 were able to modify the antiestrogenic property of these PR-transfected MCF-7 cells (data not shown).

Several lines of evidence suggest that the antiestrogenic effect of eiPR may be mediated through enhancing the metabolism of E2. The first indication was that the affect of E2 on gene expression and cell proliferation was only impaired following 48 or 72 h of treatment. Because Western blotting analysis revealed no difference in the ER levels between PR-transfected cells and control cells, we speculate that this antiestrogenic effect may be caused by the depletion of E2. Indeed, when the S-phase fraction of E2-treated PR-transfected cells fell to 40% below vehicle-treated controls at the 72-h point, freshly added E2 was able to resume the growth stimulatory effect in these cells. Results of HPLC analysis of [^{3}H]E2 in the conditioned media strongly supported the notion of enhanced metabolism of [^{3}H]E2 in PR-transfected cells; MCF-7-PR8 cells metabolized 77% [^{3}H]E2 in 6 h compared with a similar percentage metabolized (80%) by MCF-7-C8 cells in 24 h. The study also suggests that increased cellular uptake of E2 is one of the mechanisms for the increased E2 metabolism in PR-transfected cells. This is conceivable as the metabolism of E2 in culture occurs mainly inside the cells (25).

Though the retention time of 6α-hydroxyestradiol, estriol, and 16α-hydroxyestrone match that of the metabolite peak, the true identities of the metabolites in the mixture remain to be determined. It has been suggested that estriol and 6α-hydroxyestradiol are among the main end products of follicular metabolism (26). On the other hand, studies in MCF-7 cells suggested estrone, 2-hydroxyestrone, and estradiol 3-sulfate as the main metabolites of E2 (27–30). All three compounds were not detectable in our study based on their retention time (27, 31). The discrepancies may be because of many differences in experimental conditions such as the position and the amount of tritium labeling in E2, the concentration of E2 used, and the

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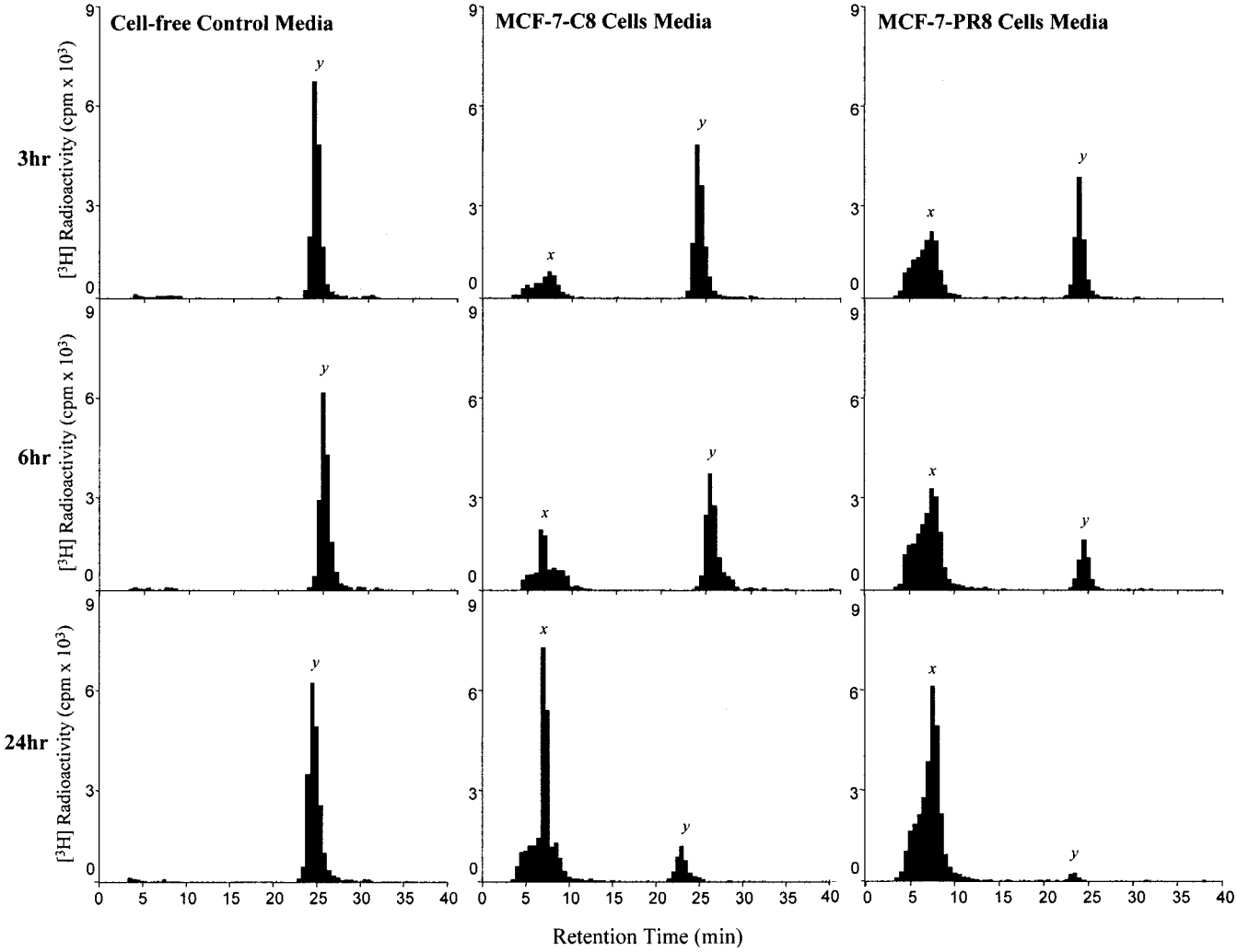

FIG. 7. **Estradiol metabolic profiles in vector-transfected and PR-transfected MCF-7 cells.** The vector-transfected and PR-transfected MCF-7 cells were incubated with 10^{-9} M [^{3}H]E2 for 3, 6, or 24 h. The conditioned media were harvested and analyzed by reverse-phase HPLC as described under "Materials and Methods." The radioactivity in 0.5-min flow fractions were counted on a liquid scintillation counter. The data are presented as counts per minute (cpm) in each fraction *versus* the retention time. Peak *x* is a mixture of [^{3}H]E2 metabolites, and [^{3}H]E2 was eluted in peak *y*.

TABLE I
The percentage of radioactivity in peak x and peak y for the histogram presented in Fig. 7

The percent radioactivity is calculated as the sum of the counts of all the fractions in each peak as a percentage of the total counts detected.

E2 and E2 metabolite(s)	Radioactivity		
	3 h	6 h	24 h
		%	
Cell-free control media			
Peak *x*	–	–	–
Estradiol (Peak *y*)	92.0	92.8	93.9
MCF-7-C8 cell media			
Peak *x*	25.8	40.4	80.4
Estradiol (Peak *y*)	67.6	55.3	13.2
MCF-7-PR8 cell media			
Peak *x*	57.9	77.0	93.4
Estradiol (Peak *y*)	38.8	17.5	2.0

TABLE II
The retention time of unlabeled E2 and E2 metabolite standards

Results are expressed as mean ± S.D. $n = 3$.

Steroid	Retention time
	min
6α-Hydroxyestradiol	4.3 ± 0.5
Estriol	5.1 ± 0.8
16α-Hydroxyesterone	7.9 ± 0.8
17β-Estradiol	23.9 ± 1.5
Estrone	30.2 ± 1.3

method of extraction.

The time lag between the antiestrogenic effects on gene expression and on cell cycle progression also supports the theory of heightened metabolism of E2 in PR-transfected cells. The effect of E2 during the first few hours has probably initiated the genomic events that were sufficient to commit cells to 2 rounds of accelerated cell cycles. That explains why the antiestrogenic effect on cell cycle distribution was only observed following 72 h of treatment. In contrast, E2 induction of gene expression is a relatively early event, and the effect can be observed at 6–12 h following E2 depletion. Because 77–94% E2 was metabolized in PR-transfected cells between 6 and 24 h following E2 treatment, a decline in E2-activated gene transcription of cell cycle genes would be seen after 24–48 of E2 treatment, as is shown in Fig. 4.

It is intriguing that the S-phase fraction of E2-treated PR-transfected cells at 72 h is 40% lower than vehicle-treated controls. It seems to suggest that E2 has been converted to growth inhibitory metabolites that can counteract the effect of E2. Compounds such as estriol and 2-methoxyestradiol have

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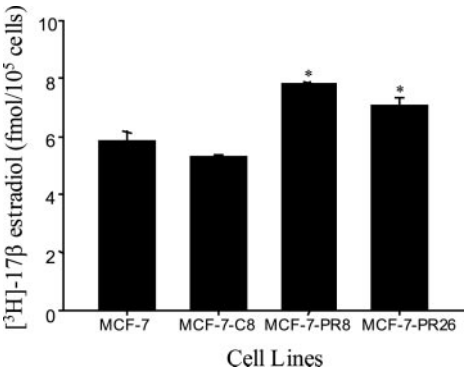

FIG. 8. **Transfection of PR increases the uptake of E2.** Cells were incubated with 10^{-9} M [^{3}H]E2 in the presence or absence of 200-fold molar excess of unlabeled E2 for 1 h. [^{3}H]E2 in the cells was extracted, and its radioactivity was determined by counting in a liquid scintillation counter. Specific uptake of [^{3}H]E2 was determined by subtracting the [^{3}H]E2 bound to the cells in the presence of unlabeled E2 from the total [^{3}H]E2 bound (in the absence of unlabeled E2). Results are expressed as mean ± S.E., $n = 3$. *Asterisk* indicates that the [^{3}H]E2 uptake in PR-transfected cells are significantly greater than that in control cells ($p < 0.05$).

been shown to inhibit the growth of breast cancer cells, but these effects were only exhibited at high concentrations ($>10^{-6}$ M) (32, 33). The E2 concentration of 10^{-9} M used in this study is unlikely to generate higher concentrations of metabolites.

E2-mediated promoter interference was abolished as early as 16 h after treatment in eiPR-expressing cells. This antiestrogenic event seems to be too early to be attributable to the depletion of E2. Therefore, there may be additional mechanisms leading to the impaired ER-ERE interaction. In this regard, the mechanism of competition for cofactors between eiPR and ER is more plausible. It has been reported (34) that transcriptional activity of ER was inhibited in HeLa cells by co-expressing ER and PR in a ligand-dependent manner. This inhibition could be relieved by increasing the expression level of ER. The above study appears to suggest that PR may compete with ER for transcription cofactors or co-regulators so as to impair the ER transcriptional activity. It is also possible that eiPR compete for binding with chaperone proteins such as heat shock protein 90 and various immunophilins that are required to maintain the receptor in a favorable conformation for ligand binding (35).

It is presently unclear how transfected PR mediates increased cellular uptake of E2. Early studies showed that the relative binding affinity of E2 for PR is less than 0.3% of progesterone (36). With a dissociation constant of 10^{-9} M for progesterone binding, the estimated dissociation constant for E2 would be in the range of 10^{-6} M. It is thus theoretically not possible for E2 at 10^{-9} M to bind to PR significantly. However, we observed that E2 treatments facilitate an inhibition of the PR progesterone response element binding in the promoter interference assay,[2] suggesting an inhibitory effect of E2 on PR-DNA interaction. Whether this is mediated through E2-PR binding remains to be investigated.

Evidence supporting the antiestrogenic potential of eiPR has been recently reported in an *in vivo* study. Breast cancer cells T47D expressing eiPR grew into smaller estrogen-dependent tumors in nude mice than PR-negative cells, and this phenomenon is independent of the PR ligand (37). The effect was PR isoform-selective as tumors expressing PR-A were only half the size of PR-B-expressing tumors. However, there was no indication of one PR isoform being more potent than the other in our study. Although MCF-7-PR8 expresses more PR-B than PR-A and MCF-7-PR26 expresses more PR-A than PR-B, the two cell lines have similar potential to abolish the growth stimulatory effect of E2. Studies of gene expression of pS2 and GREB1 indicate that the antiestrogenic potential of MCF-7-PR8 is greater than that in MCF-7-PR26. This may be related to the higher level of PR in MCF-7-PR8 cells instead of the relative expression of PR isoforms.

In summary, the study demonstrated that estrogen-independent expression of PR in MCF-7 cells antagonized the stimulatory effect of E2 on cell proliferation and on gene expression in a PR ligand-independent manner, and interfered with ER binding to ERE. This antiestrogenic effect of eiPR is associated with an accelerated metabolism of E2 in PR-transfected MCF-7 cells, which is likely mediated through increased cellular uptake of E2. Squelching of cofactors by transfected PR may explain the reduced ER-ERE interaction in a promoter interference assay. The findings open up a new window for a hitherto unknown functional relationship between the PR and ER. This antiestrogenic mechanism is also of therapeutic relevance to the group of ER-positive breast tumors, which do not respond to antiestrogen therapy, and to the antiestrogen-resistant tumors that remain ERα-positive and estrogen-responsive.

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