

RESVERATROL ACTS AS AN ESTROGEN RECEPTOR (ER) AGONIST IN BREAST CANCER CELLS STABLY TRANSFECTED WITH ER α

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Resveratrol (Res) is a phytoestrogen found in grapes and present in red wine. Res has been shown to function as an estrogen receptor (ER) agonist, but it remains unclear whether it may also exert antagonist activity. Our aim was to study the effects of Res at both the molecular (TGF α gene activation) and the cellular (cell growth) levels in breast cancer cells stably transfected with wild-type (wt) ER(D351) and mutant (mut) ER (D351Y). TGF α mRNA induction was used as a specific marker of estradiol (E₂) responsiveness. Res caused a concentration-dependent (10⁻⁸–10⁻⁴ M) stimulation of TGF α mRNA, indicating that it acts as an estrogen agonist in these cell lines. The pure antiestrogen ICI 182,780 (ICI) blocked Res-induced activation of TGF α , consistent with action through an ER-mediated pathway. Further studies that combined treatments with E₂ and Res showed that Res does not act as an antagonist in the presence of various (10⁻¹¹–10⁻⁸ M) concentrations of E₂. To determine whether Res can be classified as a type I or type II estrogen (Jordan et al., Cancer Res 2001;61:6619–23), we examined Res with the D351G ER in the TGF α assay and found that Res belongs to the type I estrogens. Both Res and E₂ had concentration-dependent growth inhibitory effects in cells expressing wtER and D351Y ER. Although the pure antiestrogen ICI blocked the growth inhibitory effects of E₂, it did not block the inhibitory effects of Res, suggesting that the antiproliferative effects of Res also involve ER-independent pathways. Interestingly, Res differentially affected the levels of ER protein in these 2 cell lines: Res down-regulated wtER levels while significantly up-regulating the amount of mutD351Y ER. Co-treatment with ICI resulted in strongly reduced ER levels in both cell lines. Gene array studies revealed Res-induced up-regulation of more than 80 genes, among them a profound activation of p21^{CIP1}/WAF1, a gene associated with growth arrest. The p21^{CIP1}/WAF1 protein levels measured by Western blotting confirmed Res-induced significant up-regulation of this protein in both cell lines. In summary, Res acts as an ER agonist at low doses but also activates ER-independent pathways, some of which inhibit cell growth.

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Key words: Resveratrol; ER α ; agonistic activity; gene arrays; p21^{CIP1}/WAF1; breast cancer cells

Resveratrol (Res) (3,5,4'-trihydroxy-*trans*-stilbene) is a phytochemical found in grapes and present in red wine. Its structure is related to known estrogens (Fig. 1). Res exhibits a variety of pharmacological effects, some of which may be associated with cardioprotective activities.^{1–3} There is also increasing interest in Res because of its activity as a cancer chemopreventive.^{4–6} The chemopreventive effect of flavonoid phytoestrogens has been suggested as a possible explanation for the low incidence of breast cancer among vegetarians and Orientals who have higher blood levels of these compounds.⁷ Although not a flavonoid, Res has been categorized as phytoestrogen based on its ability to bind and activate the estrogen receptor α (ER).⁸ More recent data have shown that Res also binds to a second subtype of ER, ER β .^{9,10} However, in contrast to some other phytoestrogens, e.g., genistein and coumestrol, which bind with greater affinity to ER β than ER α ,^{11,12} Res binds both ER α and ER β with comparable affinity⁹ or with only a small preference to ER β .¹⁰

Res inhibits growth proliferation of cultured cancer cells of different origin (leukemia cell lines, colon cancer cells, breast carcinoma cell lines, hepatoblastoma cell lines and prostate cancer cell lines) *via* induction of apoptotic cell death and/or through other mechanisms.^{13–19} However, the effect of Res on breast cancer cell growth and ER-mediated activation of gene transcription are controversial. Gehm *et al.*⁸ found that Res stimulated growth of the estrogen-dependent breast cancer cell line T47D, and that the stimulation was blocked by the estrogen antagonist ICI 182,780 (ICI). Other researchers have reported that Res decreases cell proliferation of both ER-positive (MCF-7 and T47D) and ER-negative (MDA-MB-231) breast cancer cells,^{19–21} which suggests that the interaction of Res with the ER may not fully explain its inhibitory effect on proliferation. Moreover, Res exerted a greater inhibitory effect on the ER-negative highly invasive MDA-MB-435 than on MCF-7 cells.²²

Res has both estrogenic and antiestrogenic properties when bound to the ER and therefore has characteristics similar to the selective ER modulators (SERMs),^{10,23–25} although data examining tissue selectivity *in vivo* is limited. Whether the Res-bound ER has agonist or antagonist activity in breast cancer is not clear. Res has shown “superagonist” activity in MCF-7 cells,^{8,26} whereas other reports found agonism but not superagonism in cells transiently transfected with ER and luciferase reporter plasmids.^{9,27} Agonist activity of Res was also demonstrated in MCF-7 cells by activating the expression of 2 estrogen-responsive genes: PgR and pS2.⁸ In contrast, Lu and Serrero²⁰ demonstrated antiestrogenic activity of Res on the expression of mRNA for TGF α , IGF-IR and

Abbreviations: 4OHT, 4-hydroxytamoxifen; AF-1, activation function 1; AF-2, activation function 2; E₂, 17 β -estradiol; ER, estrogen receptor α ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GW, GW 7604; IC₅₀, the concentration producing 50% maximal inhibition; ICI, ICI 182,780; IGF-IR, insulin-like growth factor I receptor; LBD, ligand-binding domain; mut, mutant; PgR, progesterone receptor; Ral, raloxifene; Res, resveratrol; SERM, selective estrogen receptor modulator; TGF α , transforming growth factor alpha; TGF β 2, transforming growth factor beta 2; wt, wild type.

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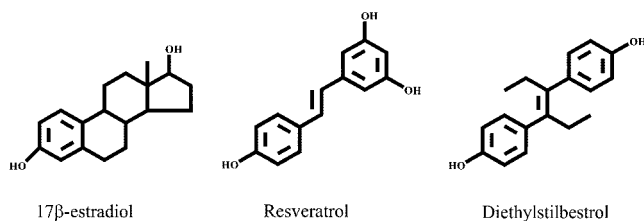


FIGURE 1 – The chemical structures of 17 β -estradiol, resveratrol and diethylstilbestrol

TGF β 2 in MCF-7 cells. Furthermore, it was shown that Res acted as a mixed agonist/antagonist in different mammary cancer cell lines in the absence of E $_2$, but in the presence of E $_2$, Res functioned as an antiestrogen.²⁸ In accord with these data, it was stated that the ER agonist activities of Res are dependent on cell context.²⁹

In order to evaluate the involvement of the ER in the growth effects of Res and its agonistic/antagonistic properties, we used well-characterized breast cancer cell lines stably transfected with wtER (D351) and mutER (D351Y). We have previously shown that the surface amino acid D351 of the ER is important for the estrogen-like actions of SERMs.^{30–35} Furthermore, we have developed an assay system that can discriminate between tamoxifen-like estrogens and true estrogens and have proposed the existence of 2 classes of estrogens, based on the structures of the ER complexes.³⁶

We examined the effects of Res at both the cellular (cell growth) and the molecular (TGF α gene expression) levels in breast cancer cells stably transfected with wtER and mutER (D351Y). To evaluate whether Res belongs to type I or II estrogens, we used another mutER (D351G),³⁷ which causes tamoxifen-like compounds to act as a complete antiestrogens at the TGF α gene but shows agonist activity with true estrogens.³⁶

MATERIAL AND METHODS

Cell culture

The stable ER-transfectants used in this study were constructed from the ER-negative MDA-MB-231 human breast cancer cells as described previously.^{37–39} These cells stably express either wtER (D351) (S30 cells),³⁸ mutER (D351Y) (BC-2 cells)³⁹ or mutER (D351G) (JM-6 cells).³⁷ Cells were maintained in phenol red-free MEM with 5% charcoal-stripped calf serum, supplemented with 100 μ g/ml streptomycin, 100 U/ml penicillin, 6 ng/ml bovine insulin, 2 mM L-glutamine, 100 mM nonessential amino acids and 500 μ g/ml G418. All materials were obtained from Gibco BRL (Gaithersburg, MD).

Res and 17 β -estradiol were purchased from Sigma Chemical Co. (St. Louis, MO); 4OHT was obtained from ICI Pharmaceuticals (Macclesfield, UK). ICI 182,780 and EM 652 were gifts from Dr. A. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Raloxifene was a gift from Lilly Research Laboratories (Indianapolis, IN). Compound GW 7604 was a gift from Dr. T. Willson (Glaxo Wellcome Research and Development, Research Triangle Park, NC). All compounds used in the experiments were dissolved in 100% ethanol.

Growth assays

Cells were seeded at a density of 4–5 \times 10 4 cells/well into 24-well plates using the media described above. The following day, media containing the appropriate compound or different concentrations of 1 compound were added. The compound-containing media was changed every other day for 6 days. The cells were then lysed by sonication (Kontes, Micro Ultrasonic cell Disrupter). DNA content was measured as described previously^{30,40} using a VersaFluorTM Fluorometer (Bio-Rad Laboratories, Hercules, CA). Each data point represents a mean of triplicate wells.

Northern blot analysis

RNA extraction and Northern blot were performed essentially as described previously.^{30–34} Briefly, total RNA was isolated using Trizol reagent (Gibco, BRL) from cells treated for 24 hr with different concentrations of Res or other compounds and EtOH (vehicle). RNA samples (15–20 μ g) were fractionated in a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane (Hybond-N $^+$, Amersham, Arlington Heights, IL). The membranes were hybridized at 42°C with 32 P-labeled TGF α probe (plasmid was provided by Dr. Rik Derynck, Genentech, Inc., CA). The membranes were then washed and exposed to Kodak film with intensifying screens at –80°C for 2–3 days. Subsequently, the membranes were stripped and reprobated with 32 P-labeled β -Actin cDNA. The signals were quantitated using densitometric analyses of the autoradiographs (Molecular Dynamics, Image Quant software).

Western blot analysis

Whole-cell extracts were prepared from cells treated for 48 hr with compounds by lysis of cold PBS-washed cells in lysis buffer [50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.5% NP40 and 10 mM β -glycerophosphate, pH 8, containing a 1:100 dilution of a protease inhibitor cocktail (Sigma Chemical Co., St Louis, MO)]. The protein concentration was measured using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (30–50 μ g) were resolved over 12% polyacrylamide-SDS gels and transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with blocking solution (0.5 \times PBS, 0.05% Tween and 2.5% dry milk) overnight. The membrane was cut into 3 strips, with each strip corresponding to the molecular weight of ER α , p21 or β -Actin. The appropriate strip was incubated with primary antibody in blocking solution for 2 hr at RT. We used the ER α G-20 antibody (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and p21/WAF1 (Ab-11; Lab Vision, Fremont, CA) or anti-p21 antibody (1:100, CalBiochem, San Diego, CA) for p21^{CIP1}/WAF1. The blots were also probed with β -Actin antibodies (1:20,000, Sigma Chemical Co., St Louis, MO) as a loading control. After 3 \times 5 min washes in PBS-Tween, HRP-conjugated secondary antibodies (1:3,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the membrane for 1 hr at RT. Protein visualization was achieved by using an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL) and autoradiography with Hyperfilm-ECL film (Kodak Co., Rochester, NY). The signals on the X-ray films were quantified using Scion Image 4.0.2., and statistics were performed using SPSS 9.0 software (SPSS, Inc., Chicago, IL).

Array-based expression profiling and data processing

We used AtlasTM Human cDNA Expression Arrays (Clontech, Palo Alto, CA), positively charged nylon membranes that are spotted in duplicate with 200–600 base-pair cDNA fragments representing 588 known cancer-related genes and 9 housekeeping genes. A complete list of genes on the AtlasTM Human Expression Array used can be accessed through <http://www.clontech.com>.

Preparation of radiolabeled cDNA probes and hybridization with cDNA arrays was performed according to the manufacturer's protocol (Clontech, Palo Alto, CA) and described previously.^{41,42} Atlas Array expression data for each condition was obtained from 3 independent Atlas experiments using at least 2 different populations of RNA and 2 different membranes.

Signals were detected by phosphorimage analysis using a Molecular Dynamic Storm phosphorimager (Molecular Dynamics, Sunnyvale, CA). Initial analysis of the data, including background calculation and correction, normalization to the house-keeping gene GAPDH for each array, production of Composite Arrays (the average of 3 independent Atlas Array experiments for each compound) and generation of the customized report of up- and down-

regulated genes, was performed using AtlasImage™ 1.5 software (Clontech, Palo Alto, CA).^{41,42} Comparisons were done between Composite Array of cells treated with Res or E₂ vs. Composite Array of untreated Control cells. Ratios of 2 and higher (for up-regulation) and of 0.5 and lower (for down-regulation) between background-corrected normalized gene expression levels were considered to be significant. Prior to statistical analysis and graphing, data were normalized (GeneSpring, the default function) to account for systematic differences across data sets. Complete data sets for expression profiles are available at http://www.math.mtu.edu/~igor/Gene_index.html.

RESULTS

Concentration-dependent induction of TGF α mRNA by resveratrol in cells expressing wtER and mut D351Y ER

To determine whether Res acts as an agonist or antagonist in breast cancer cells stably transfected with ER, we evaluated the effect of Res on an estrogen-inducible gene. We performed Northern blot analysis using TGF α as a marker of estrogen responsiveness. We have previously shown a concentration-dependent expression of TGF α mRNA by E₂ in cells expressing wtER and D351YER.³⁰ The effects of various concentrations of Res on the expression of TGF α mRNA in this system were determined following a 24 hr treatment (Fig. 2a). Experiments were performed in the presence of 1% stripped serum as well as 5%; serum concen-

tration did not affect the ability of Res to induce TGF α expression (data not shown). As expected, higher concentrations of Res were required for the induction of TGF α in cells expressing mut D351Y ER, which has a lower binding affinity for ligands than the wtER.⁴³

Next, we addressed the issue of ER-mediated activation of TGF α and the interactions between E₂ and Res. The pure antiestrogen ICI was able to block both E₂- and Res-induced activation of TGF α in both cell lines, implicating ER-mediated activation of this estrogen-responsive gene with known putative EREs in the promoter region (Fig. 2b and data not shown). Res maximally stimulated the expression of TGF α mRNA at a concentration of 5×10^{-5} M in both cell lines, to the same extent as 10^{-9} M E₂ for the wtER (Fig. 2b) and as 10^{-8} M E₂ for mutD351YER (data not shown). However, when Res and E₂ were both present at their optimal concentrations, there was little or no further stimulation of TGF α mRNA. More detailed studies with both cell lines demonstrated that Res acted as an agonist in a dose-dependent manner in the presence of low concentrations of E₂ (10^{-11} M for wtER and 10^{-10} M for mutD351Y ER) (Fig. 3a) and did not have any detectable antagonistic effects in the presence of higher concentrations (10^{-9} – 10^{-8} M) of E₂ (Fig. 3b).

Resveratrol belongs to type I estrogens

Recently we proposed 2 classes of estrogens (type I and type II) with different ER conformations that may incorporate various coactivators in order to function.³⁶ We also suggested that phy-

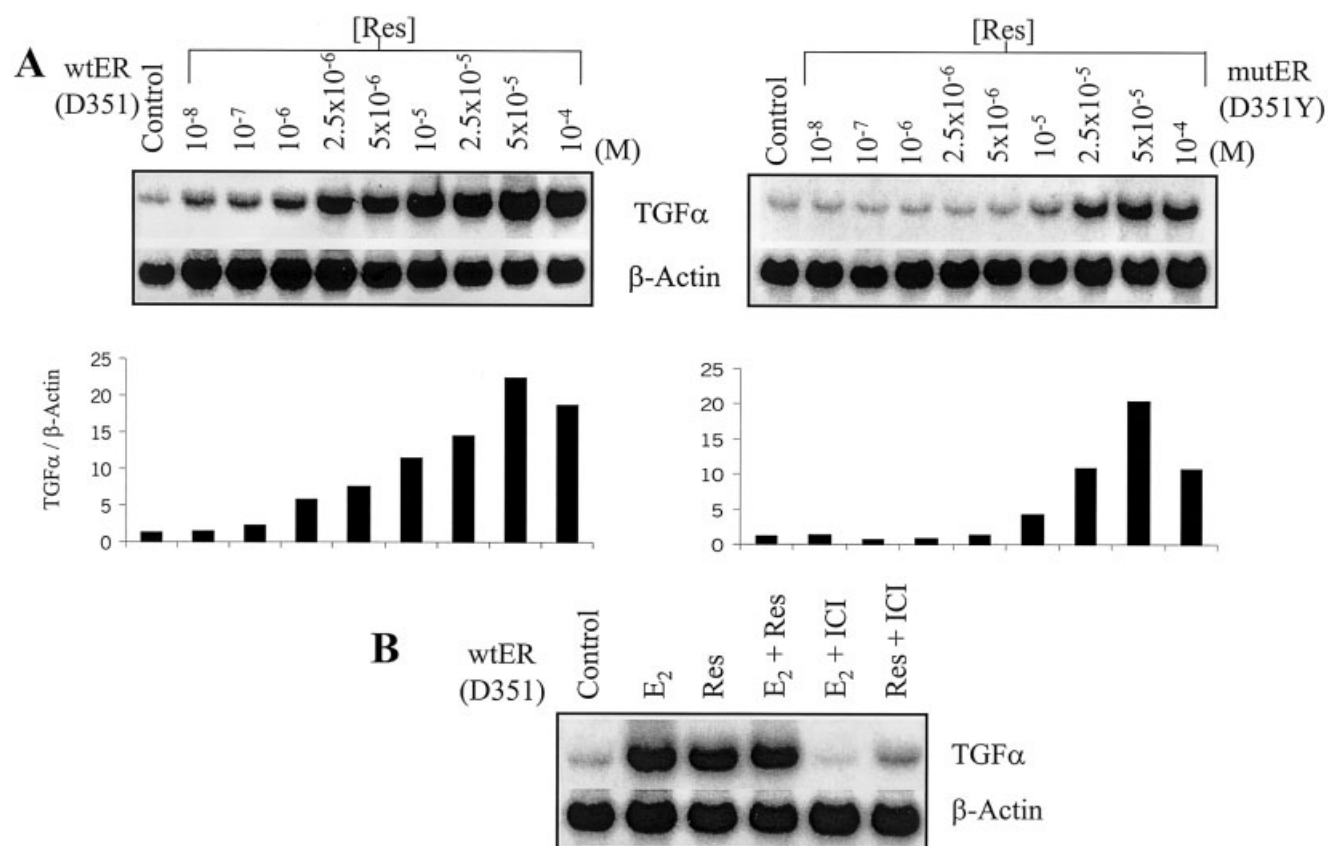


FIGURE 2 – (a) Concentration-dependent effect of Res on the TGF α mRNA expression in cells expressing wtER (D351) and mutER (D351Y) analyzed by Northern blotting. Total RNA was isolated from cells following treatment with EtOH (control) and different doses of Res for 24 hr as described in Materials and Methods. β -Actin was used as a loading control. (b) Effect of Res in combination with E₂ and ICI on TGF α mRNA expression in cells expressing wtER (D351) analyzed by Northern blotting. The sources for RNAs were the following: control, cells treated with EtOH; E₂, cells treated with 10^{-9} M estradiol; Res, cells treated with 5×10^{-5} M resveratrol; E₂ + Res, cells treated with 10^{-9} M estradiol and 5×10^{-5} M resveratrol; E₂ + ICI, cells treated with 10^{-9} M estradiol and 10^{-6} M ICI 182,780; Res + ICI, cells treated with 5×10^{-5} M resveratrol and 10^{-6} M ICI 182,780. β -Actin was used as a loading control.

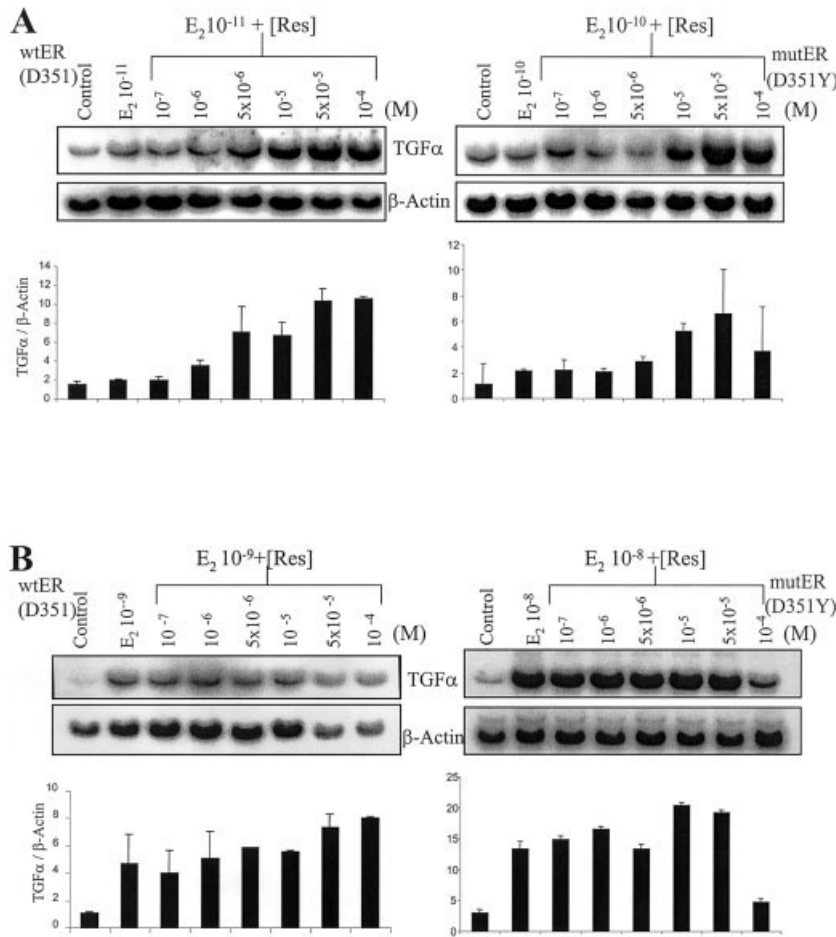


FIGURE 3 – The effect of Res on TGFα mRNA expression in the presence of low and high concentrations of E₂. (a) Res exhibits concentration-dependent agonistic activity with both wtER and D351Y ER in the presence of low concentrations of E₂ (10⁻¹¹ M and 10⁻¹⁰ M). (b) Res is not acting as an antiestrogen with high concentrations of E₂ (10⁻⁹ M and 10⁻⁸ M). Total RNA was isolated from cells following treatments for 24 hr as described in Material and Methods. β-Actin was used as a loading control. Data are presented as the mean ± SD of 2 independent experiments.

toestrogens and environmental xenoestrogens will fall into different classes based on structure and may exhibit different carcinogenic potential based on different ER conformations. One goal was to investigate whether Res belongs to type I or type II estrogens. We examined Res with D351G ER³⁷ in the TGFα assay. The effects of various concentrations of Res on the expression of TGFα mRNA in cells expressing D351G ER were determined following 24 hr treatment. We found concentration-dependent activation of TGFα mRNA by Res starting from 10⁻⁵ M, with the maximum effect at 2.5 × 10⁻⁵ M (data not shown). Moreover, this agonistic action of Res was blocked by various SERMs (4OHT, Ral, EM and GW) and ICI (Fig. 4). We conclude that Res belongs to the type I estrogens based on the results with mutD351G ER at the TGFα gene and by reference to its planar structure (Fig. 1).

Resveratrol has growth inhibitory effects on cells stably transfected with wtER and mutD351Y ER

We examined the effect of Res and E₂ either alone or in combination with ICI 182,780 on the growth of cells expressing either wtER or mutER (D351Y). Cells were plated in appropriate media and cultured in the presence of various concentrations of E₂, Res or in combination with the pure antiestrogen ICI (10⁻⁶ M) for 6 days. Seven different concentrations were used for each compound ranging from 10⁻¹⁴ to 10⁻⁸ M for E₂ and from 10⁻⁸ to 10⁻⁴ M for Res. Control cells were treated with the same volume of vehicle (EtOH). As shown in Figure 5a, (wtER) and 5b (D351Y ER), Res, like E₂, inhibited the growth of both cell lines in a concentration-dependent manner. The growth-inhibitory effects of E₂ on these cells has been shown previously.^{30,40} IC₅₀ values, the compound concentration causing 50% maximal inhibition of cells,

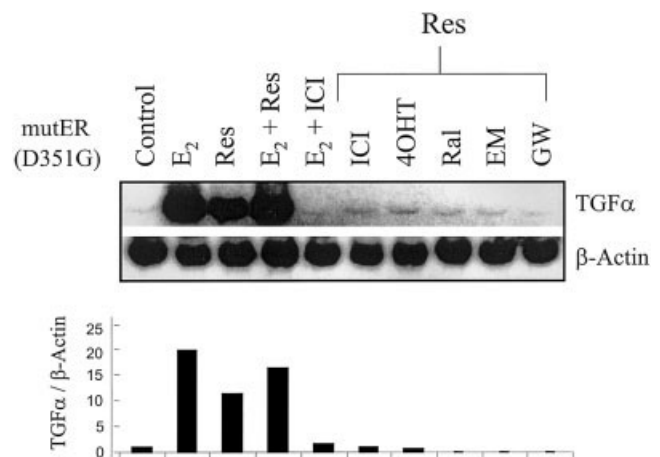


FIGURE 4 – The effect of Res on TGFα mRNA expression in cells expressing D351G ER. Res exhibits agonistic activity with D351G ER, and its effect was inhibited by ICI and various SERMs: 4OHT, Ral, EM and GW (all used at 10⁻⁶ M). Res was used at 5 × 10⁻⁵ M and E₂ was used at 10⁻⁹ M. Total RNA was isolated from cells following treatments for 24 hr as described in Materials and Methods. β-Actin was used as a loading control. A representative Northern blot is shown. Data are presented as the mean of 3 independent experiments.

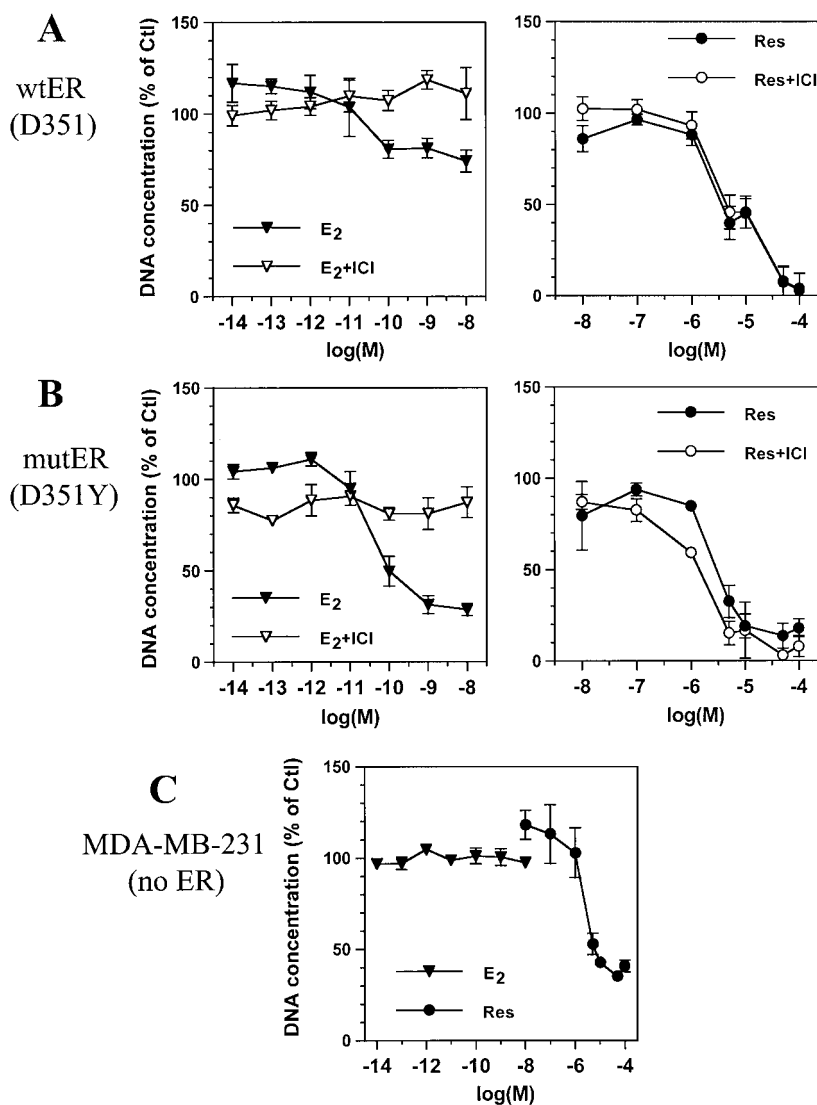


FIGURE 5 – Effects of E₂ and Res on the growth of cells stably expressing wtER (D351) (a); stably expressing mutER (D351Y) (b) and on MDA-MB-231 cells (c). Cells were plated at $4\text{--}5 \times 10^5$ cells per well in 24-well plates in appropriate media. The media was then removed and replaced with the same media in the presence of various concentrations of E₂, Res and combinations with ICI (10^{-6} M). Media with compounds was replaced every other day until day 6. The effect of the indicated concentrations of E₂ and Res on the proliferation was measured by the DNA assay procedure as described in Material and Methods. Data are presented as the mean \pm SE of 2 to 4 independent experiments in which each treatment was performed in triplicate.

were calculated for E₂ and Res. The IC₅₀ for E₂ and Res in cells expressing wtER were 2.5×10^{-11} M and 7.5×10^{-6} M, respectively. The IC₅₀ in cells expressing mutER were 5×10^{-11} M for E₂ and 5×10^{-6} M for Res.

A $\sim 97\%$ (for wtER) and $\sim 85\%$ (for D351Y ER) growth inhibition after a 6-day treatment with 5×10^{-5} M Res was observed vs. a $\sim 25\%$ and $\sim 65\%$ inhibition after E₂ (10^{-9} M) treatment, respectively. To determine whether the observed cell growth inhibition by Res was mediated through the ER, we performed combination experiments with a constant ICI concentration (10^{-6} M) and increasing concentrations of E₂ and Res. ICI was able to block the growth inhibitory effects of E₂ on these cells (also shown previously³⁰). In contrast, the growth inhibitory effects of Res were unaffected or slightly increased by ICI in both cell lines (Fig. 5a: IC₅₀ = 6.7×10^{-6} M and B: IC₅₀ = 1.5×10^{-6} M), indicating ER-independent events. Since both cell lines used were ER stable transfectants, we extended our study to parental MDA-MB-231 cells (Fig 5c). While E₂ did not affect the growth of these cells, Res was capable of significantly inhibiting the growth of these ER-negative cells (IC₅₀ = 2.3×10^{-6} M).

Resveratrol affects the level of ER protein expression

To further characterize the biological activity of Res, we determined how Res treatment affects ER protein expression in cells

transfected with wtER and D351Y ER. In confirmation of our previous results,^{31,43} E₂ decreases the level of ER protein in these cells, and the pure antiestrogen ICI causes substantial ER degradation. In Figure 6, a Western blot shows regulation of ER protein levels in ER-transfectants by E₂, Res, ICI alone and with co-treatments. Interestingly, all 3 ligands caused a significant decline in steady-state levels of wtER (Fig. 6a), although the extent to which ER levels were affected varied. E₂ and Res were similar in their ability to degrade wtER, while ICI alone was more efficient at lowering receptor levels. In combination experiments with ICI, both compounds showed further decreases in receptor levels. In contrast, Res stabilized and significantly elevated levels of mutER (D351Y) (Fig. 6b). These observations are consistent with the concept that mutations within the LBD of the ER make it more stable and resistant to ligand-specific regulation of ER proteolysis.^{44,45} As in the case with wtER, both E₂- and ICI-treatment of cells resulted in a significant decrease in levels of mutER. Interestingly, the Res-induced stabilization of mutER could be prevented by co-treatment with ICI, which brings the receptor level slightly lower than that of control. These results demonstrate that differences in the structure of the receptor can lead to the differences in the degree of ER degradation by Res and that stabilization of the mutant receptor by Res can be modulated by ICI.

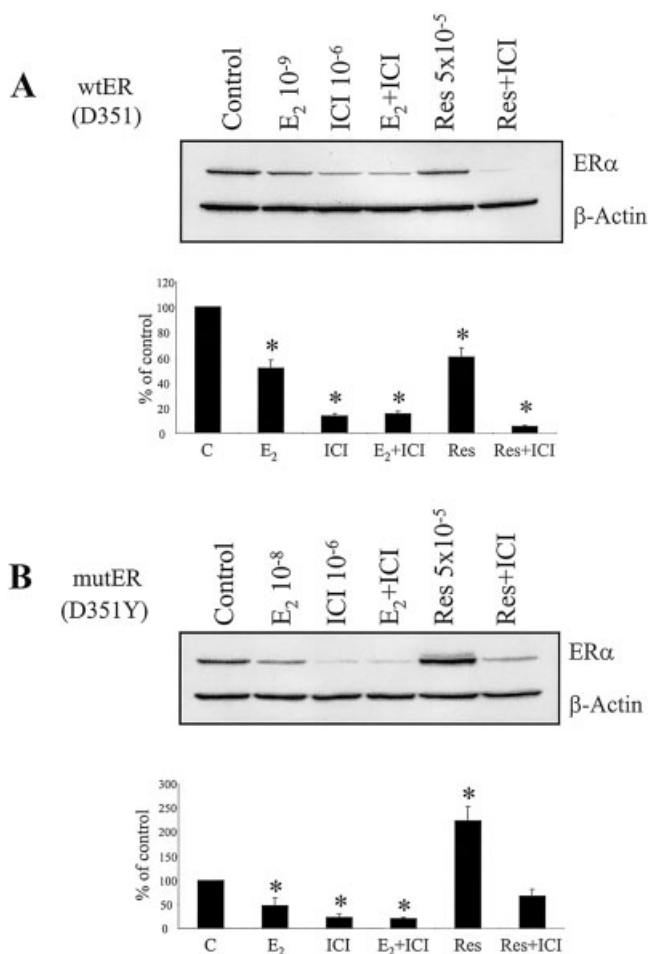


FIGURE 6 – The effects of Res on ER protein levels in cells expressing wtER (D351) (a) and mutER (D351Y) (b). Cells were treated with optimal concentrations of E₂ (10⁻⁹ M or 10⁻⁸ M), ICI (10⁻⁶ M), E₂ + ICI, Res (5 × 10⁻⁵ M) or Res + ICI for 48 hr and protein levels were analyzed by Western blotting as described in Material and Methods. Immunoreactive bands for ER were identified using ECL, were scanned and normalized to β -Actin. The control was set to 100% and the different treatment groups are expressed as a percentage of control levels. The resulting data were analyzed using ANOVA in the SPSS 9.0 statistical program. Data are presented as the mean \pm SE of 2–6 independent experiments and the asterisks indicate values that are significantly different (**p* < 0.05) from the control.

Resveratrol-induced gene expression profiling

We used Atlas cDNA Arrays (Clontech, Palo Alto, CA) to study gene expression profiles of cells expressing wtER and mutER (D351Y) after treatment with E₂ and Res for 24 hr. (Data sets for expression profiles are available at http://www.math.mtu.edu/~igor/Gene_index.html.) One of the striking features of the gene expression patterns seen in the above mentioned breast cancer cells was the profound effects on global gene activation by Res compared to the effects of several other ligands.⁴² The bivariate scatterplots of genes from the microarrays in Figure 7 show that both the number of modulated genes and the fold-changes of genes observed with Res-treatment were higher than with E₂-treatment. Analysis of expression data revealed that treatment with E₂ and Res altered the expression of a large group of genes compared to the control untreated cells. A set of 15 genes selected as overlapping up-regulated genes between the 2 treatments has been described elsewhere.⁴¹ There was a unique set of 60 genes altered only after treatment with Res (data not shown). Of these genes, 24

were expressed at levels of 4-fold or higher, 17 were expressed at 2.7–3.9-fold and 19 were expressed at 2–2.4-fold. The majority of genes (29%) expressed at levels higher than 4 represented transcription activators, intracellular transducers, effectors and modulators, and 26% from the same group belonged to basic transcription factors, growth factors, cytokines and chemokines. The highest induction in this group was observed for integrin beta 1 (ITGB1), which was increased 19.9-fold by Res.

Among genes responding to both E₂ and Res, the highest changes in the level of expression with Res-treatment was observed with p21^{cip1}/WAF1 (23-fold).⁴¹ The same tendency (up-regulation of p21^{cip1}/WAF1) was observed for cells expressing D351Y ER in response to both E₂ and Res, although to a lesser extent (8-fold increase for Res) (data not shown). Based on these observations, we evaluated protein levels of p21^{cip1}/WAF1 to address the possible involvement of this well-known cyclin-dependent kinase (CDK) inhibitor on the observed arrest in cell proliferation. Western blot analysis confirmed increased levels of p21^{cip1}/WAF1 after treatment with both E₂ and Res (Fig. 8). However, ICI was able to block the effects of both E₂ and Res on elevating the levels of p21^{cip1}/WAF1, indicating that there is no direct correlation between induction of p21^{cip1}/WAF1 and growth inhibition, at least by Res. The discrepancy between ICI-reversible effects in the case of E₂ and irreversible growth inhibition by Res (Fig. 5) suggest that while up-regulation of p21^{cip1}/WAF1 by both E₂ and Res is an ER-mediated event (Fig. 8), growth inhibitory effects induced by Res are not necessarily related to the elevated levels of p21^{cip1}/WAF1 and are predominantly ER-independent.

DISCUSSION

There is an increasing interest in phytoestrogens that might be valuable for cancer chemoprevention. Resveratrol has been characterized as a phytoestrogen based on its ability to bind and activate both ER α and ER β .^{8–10} The molecular mechanisms by which Res exerts its cancer-preventive effects are not known. Whether Res-occupied ER has agonist or antagonist activity has been controversial.^{8,9,20,27,28,46} The present study was designed to examine 1) whether Res has agonistic/antagonistic properties in breast cancer cells transfected with wtER α or mut D351Y and D351G; 2) whether Res's effects are mediated through the ER; 3) whether Res belongs to type I or type II estrogens; and 4) whether gene expression profiles of cells treated with E₂ and Res differ.

Our data demonstrate that like E₂, Res stimulated TGF α mRNA expression in a concentration-dependent manner in all 3 cell lines examined.^{31,34} This suggests that Res functions as an estrogen agonist in breast cancer cells stably transfected with the ER. Recently, Lu and Serrero²⁰ reported significant inhibition of TGF α mRNA expression by 10⁻⁵ M Res in MCF-7 cells and suggested a possible link between this inhibition and the antiproliferative effect of Res in these cells. The stimulation of TGF α mRNA and protein by E₂^{27,28,30,31,47} and by Res (current study) despite the growth inhibitory effects of both E₂ and Res on MDA-MB-231 cells stably expressing ER are in disagreement with this hypothesis.²⁰

The ability of the pure antiestrogen ICI to block activation of TGF α mRNA expression by E₂ and Res (Fig. 2b and Fig. 4) clearly indicates that this effect is mediated *via* the ER. Res acted as an ER agonist when applied alone, but studies from other laboratories suggested it might act as an antagonist when combined with E₂. Our studies showed that Res was an agonist in the presence of low concentrations of E₂ and displayed no antagonism at higher E₂ concentrations. This was observed with both wt and mutERs (Fig. 3). In addition, we conclude that Res is a type I estrogen because it is active with both wt and D351G ER (Fig. 4). Type I estrogens are the traditional estrogens that are sealed into the LBD of ER and whose agonist actions are amplified through a synergistic interaction of AF1 and AF2.³⁶ This knowledge may provide insight into possible carcinogenic potential of Res.

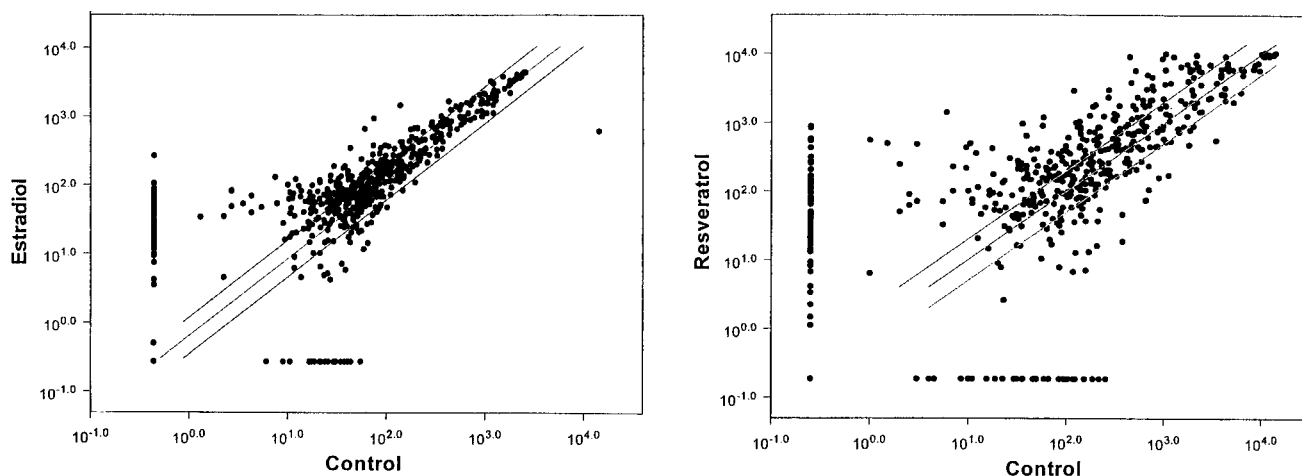


FIGURE 7 – Expression profiles of the untreated Control (X axis) and E₂-treated and Res-treated (Y axis) cells are shown as bivariate scatterplots of 588 genes from the microarray. The values are normalized adjusted intensities representing levels of expression. Genes with equal expression values lined up on the identity line (central diagonal), whereas outliers correspond to up- or down-regulated genes. The scale on each axis is logarithmic.

Information on the pharmacokinetics of Res and its potential agonist/antagonist efficacies are important to be established before any decision can be made on the practical utility of Res. Recently, the first study examining metabolism and disposition of Res *in vivo* was published.⁴⁸ The authors found that Res, in its aglycone form, has bioavailability as high as 38% in rats. For comparison, raloxifene, a polyhydroxylated SERM, has only a 2% bioavailability with a 60 mg daily dose but has clear-cut agonist and antagonist actions because of its high binding affinity for the ER.

The present study demonstrates that Res treatment of breast cancer cell lines stably expressing wtER and mutD351Y ER resulted in cell growth inhibition (Fig. 5). The apparently paradoxical growth inhibitory effect of E₂ in these cells has been reported previously.^{30,49} The observation that Res inhibited the growth of these cells is interesting for several reasons: 1) like E₂ and consistent with its agonistic properties, Res inhibited the growth of these cells in a concentration-dependent fashion and 2) while the growth inhibitory effects of E₂ were ER-mediated, it appeared that the antiproliferative effects of Res were not. This was evidenced by the inability of the pure antiestrogen ICI to block the effect and also by the growth inhibitory effect of Res on parental ER-negative MDA-MB-231 cells.

The antiproliferative effects of Res have been demonstrated in various cancer cell lines,^{13,18,50–54} including cultured human endometrial adenocarcinoma cells.¹⁰ However, the effects of Res on breast cancer cell proliferation have been the subject of conflicting reports. Gehm *et al.*⁸ reported that 10 μ M Res stimulated the growth of estrogen-dependent T47D cells to the same extent as a saturating (100 pM) concentration of E₂. The growth stimulation by either compound was blocked by ICI 182,780. However, higher (100 μ M) concentrations of Res inhibited T47D proliferation (Gehm and McAndrews, unpublished data). Similar results (growth stimulation at 10–25 μ M, declining at higher concentrations) were reported in MCF-7 cells by Basly *et al.*²⁶ In contrast, Mgbonyebi *et al.*²¹ reported that Res inhibited proliferation and decreased viability of both ER-positive (MCF-7) and ER-negative (MCF-10 and MDA-MB-231) cells, but the concentrations tested (22–180 μ M) were higher than those reported as growth-stimulatory by Gehm *et al.*⁸ Similarly, Hsieh *et al.*⁵⁵ reported that 25 μ M Res inhibited the growth of MCF-7 and ER-negative MDA-MB-435 cells. Inhibition was more pronounced in the latter cell line, suggesting that the growth inhibition was partially offset by ER agonism in the ER-positive cells. In addition to the higher Res dose, Mgbonyebi *et al.*²¹ and Hsieh *et al.*⁵⁵ used unstripped (es-

trogen containing) serum in the growth media, which may have masked the proliferative effect of Res, *i.e.*, the cells would already be stimulated maximally. Lu and Serrero²⁰ reported modest activation of MCF-7 cell proliferation by Res (0.1 to 5 μ M) in the absence of other estrogens but found that higher concentrations inhibited E₂-stimulated growth. Nakagawa *et al.*⁵⁶ also reported that low concentrations of Res stimulated growth of ER-positive breast cancer cell lines (MCF-7 and KPL-1), but concentrations greater than 44 μ M inhibited the growth of these and MDA-MB-231 cells. Overall, these studies suggest that low concentrations of Res exert an ER-mediated growth-promoting effect on ER-positive breast cancer cells, but that high concentrations have an ER-independent antiproliferative effect. However, Damianaki *et al.*¹⁹ have reported an inhibition of MCF-7, T47D and MDA-MB-231 cell growth by extremely low concentrations of Res (< 0.1 nM); the disparity between these results and the findings of other laboratories remains puzzling. Stimulation of proliferation by low-dose Res has also been reported in an ER-positive pituitary cell line,⁵⁷ so the effect may not be limited to breast cancer cells.

The role of the ER in the Res-mediated growth inhibition of cells remains unclear. Although Res's reported antiestrogenicity may account for some of its antiproliferative effects in ER-positive cells, it appears that this is not the only mechanism. The inhibition of several key enzymes involved in cell metabolism^{58–60} may explain some of the growth-inhibitory effects of Res, particularly those observed in ER-negative cells. Our data suggest ER-independent actions, in that expression of ectopic ER in an initially ER-negative environment did not affect much the growth-inhibitory effect of Res, and the ER antagonist ICI was unable to block this effect.

The ability of ligands to modulate intracellular levels of ER is important for ER pharmacology. The human ER has been shown to be a substrate for ubiquitination in the presence of E₂⁶¹ and the decline in ER levels upon exposure to different ligands has been shown to be mediated by the induction of proteasome-mediated proteolysis.^{44,62,63} Our own data (Timm Pearce *et al.*, in press) indicate that the proteasome is responsible for the degradation of ER by E₂ and ICI in cells stably transfected with the ER. Moreover, the ER stability is influenced by the nature of the bound ligand, mutations within the receptor and other factors such as ligand concentrations.^{44,45} Our results showed that the modulation of ER levels by Res was different in 2 cell lines: in cells expressing wtER, Res decreased expression of the receptor protein (Fig. 6) and the effect of E₂ was similar. In contrast, Res increased, but E₂

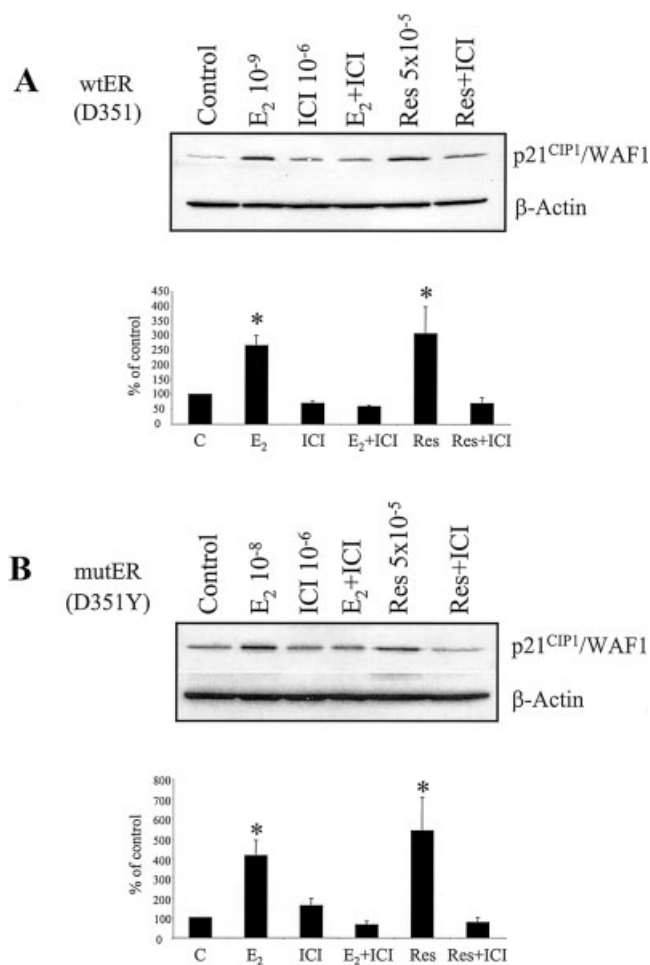


FIGURE 8 – The effects of Res on p21^{CIP1}/WAF1 protein levels in cells expressing wtER (D351) (a) and mutER (D351Y) (b). Cells were treated with optimal concentrations of E₂ (10⁻⁹ M or 10⁻⁸ M), ICI (10⁻⁶ M), E₂ + ICI, Res (5×10⁻⁵ M) or Res + ICI for 48 hr and protein levels were analyzed by Western blotting as described in Material and Methods. Immunoreactive bands for p21^{CIP1}/WAF1 were identified using ECL, were scanned and normalized to β-Actin. The control was set to 100% and the different treatment groups are expressed as a percentage of control levels. The resulting data were analyzed using ANOVA in the SPSS 9.0 statistical program. Data are presented as the mean ± SE of 2–6 independent experiments and the asterisks indicate values that are significantly different (**p* < 0.05) from the control.

decreased receptor protein levels in cells expressing D351Y mutER. The effect of different ligands on ER protein levels is not predictable on the basis of agonism; E₂ and the pure antiestrogen ICI decrease the levels of both wt and mutER, Res discriminates between them, whereas 4-OHT increases the levels of both ERs.³¹ Interestingly, stabilizing effects of Res and 4-OHT on the ER were reversible by ICI co-treatment (Fig. 6 and data not shown).

To begin to define patterns of gene expression relevant to Res-induced effects in breast cancer cells, we used cDNA Atlas Arrays to evaluate the gene expression profiles after the activation of the ER by Res, E₂ and other ligands.^{41,42} The analyses of Res-induced up-regulated genes indicated the most profound changes in the expression of cyclin dependent kinase inhibitor p21^{CIP1}/WAF1 in stably transfected MDA-MB-231 cells (23-fold increase for wtER and 8-fold increase for mutD351Y ER). Moreover, p21^{CIP1}/WAF1 was one of the “overlapping genes” found to be up-regulated by E₂ as well.⁴¹ We validated the changes of p21^{CIP1}/WAF1 after treatments with both E₂ and Res by real-time RT-PCR⁴¹ and by Western blot (Fig. 8). Our results clearly demonstrate ER-mediated E₂- and Res- induced up-regulation of p21^{CIP1}/WAF1 (Fig. 8a,b). Other studies have shown the up-regulation of p21^{CIP1}/WAF1 by Res,^{51,54,64,65} and some demonstrated that induction of p21^{CIP1}/WAF1 is a mechanism for Res-induced cell cycle arrest.^{51,54} Induction of p21^{CIP1}/WAF1 by E₂ in breast epithelial cells stably transfected with ER has been also proposed as a mechanism for growth inhibition by E₂.⁶⁶ However, our results suggest that the observed growth inhibitory effects induced by E₂ and Res may have different mechanisms: it may be related to the up-regulation of p21^{CIP1}/WAF1 in case of E₂ but probably is not related in case of Res, because of discrepancy between ICI-irreversible growth inhibition and ER-mediated character of elevated levels of p21^{CIP1}/WAF1.

In summary, we have described the concentration-dependent agonist actions of Res on the endogenous TGFα gene. The type I estrogenic actions of Res are mediated via the ER and produce high levels of p21^{CIP1}/WAF1. However, growth inhibitory effects of Res are mostly independent of the ER. We suggest that only extraordinarily high levels of Res in the cellular environment could produce anticancer activities but low levels would enhance endogenous estrogen action.

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