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Estrogenic effects of resveratrol in breast cancer cells expressing mutant and wild-type estrogen receptors: role of AF-1 and AF-2

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Abstract

Resveratrol, a hydroxystilbene found in grapes and wine, has previously been shown to be a non-flavonoid phytoestrogen, and to act as an estrogen receptor (ER) superagonist in MCF-7 cells transiently transfected with estrogen-responsive reporter constructs. Several additional hydroxystilbenes, including diethylstilbestrol (DES) and piceatannol, were tested, and all showed ER agonism or partial agonism, but superagonism was specific to resveratrol. Moreover, superagonism was observed in cells carrying a stably integrated reporter gene, indicating that this phenomenon is not a result of transient transfection.

To examine the role of the transcriptional activation function (AF) domains of ERα in resveratrol agonism, we compared the effects of resveratrol and estradiol (E2) on expression of exogenous reporter genes and an endogenous estrogen-regulated gene (TGFα) in MDA-MB-231 cells stably transfected with wild-type (wt) ERα or mutants with deleted or mutated AF domains. In reporter gene assays, cells expressing wtERα showed a superagonistic response to resveratrol. Deletion of AF-1 or mutation of AF-2 attenuated the effect of resveratrol disproportionately compared to that of E2, while deletion of AF-2 abrogated the response to both ligands. In TGFα expression assays, resveratrol acted as a full agonist in cells expressing wtERα. Deletion of AF-1 attenuated stimulation by E2 more severely than that by resveratrol, as did deletion of AF-2. In contrast, mutation of AF-2 left both ligands with a limited ability to induced TGFα expression. In summary, the effect of modifying or deleting AF domains depends strongly on the ligand and the target gene.
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Keywords: Resveratrol; Phytoestrogens; Estrogen receptor; Activation function; Transforming growth factor α; Reporter gene assay

1. Introduction

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) was discovered by Michio Takaoka more than 60 years ago, in the resin of *Veratrum grandiflorum* (false hellebore) [1]. Resveratrol attracted attention when it was found to be present in grapes and red wine [2]; its anti-oxidant, anti-inflammatory and anti-thrombotic properties have been suggested to play a role in the health benefits of wine and the “French paradox” [3,4]. In addition, it is present at substantial concentrations in some traditional Asian medicinal plants [5,6]. Interest in resveratrol was further heightened when Jang et al. [7] reported that it has anti-carcinogenic effects in a variety of models of tumor initiation, promotion and progression.

Resveratrol has some structural similarity to the synthetic estrogen DES, which led us to consider the possibility that it would modulate the activity of ERs. As previously reported [8], we found that it acts as an ER agonist in human breast cancer cell lines. Specifically, we observed that it stimulates the growth of estrogen-dependent T47D cells; it also induces the expression of endogenous estrogen-regulated genes and exogenous estrogen-responsive reporter genes in MCF-7 cells. Reporter gene activation is also observed in ER-negative MDA-MB-231 breast cancer cells but only when cotransfected with a plasmid expressing

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Abbreviations: AF, activation function; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; MEM, minimal essential medium; PR, progesterone receptor; SERM, selective estrogen receptor modulator; TGFα, transforming growth factor alpha; tk, thymidine kinase; wt, wild-type

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ERα. These effects are blocked by the ER antagonist ICI 182780. On the basis of these findings, we identified resveratrol as a phytoestrogen. Its stilbene backbone makes it structurally distinct from previously reported dietary phytoestrogens, which are typically flavonoids or lignans [9].

ER ligands are functionally categorized by their effect on the transcriptional activity of the receptor. Ligands that activate transcription at EREs are agonists; those that inhibit agonist-induced transcription are antagonists. The differential effects of these compounds on receptor activity have been ascribed to different conformations of the receptor–ligand complex, with concomitant differences in interaction with transcriptional co-activator and co-repressor proteins. Some ligands activate transcription but to an extent significantly less than that produced by classical agonists such as E2; these have been referred to as partial agonists, or partial agonist/antagonists if they inhibit the effects of co-administered E2. Many if not all such compounds show varying degrees of agonism/antagonism depending on the cell type and target gene, and are referred to as selective estrogen receptor modulators (SERMs; for a recent review of this class of compounds see [10]).

Because the expression of natural estrogen-regulated genes may also be regulated by a wide variety of additional factors, artificial reporter genes (consisting of one or more EREs, a minimal promoter, and the coding sequence for some easily-assayed gene product) are widely used to study the effects of ER agonists and antagonists. Using such a system in the ER-positive breast cancer cell line MCF-7, we previously found [8] that the grape compound resveratrol displayed a remarkable property: at optimal concentrations (typically 10–30 μM), resveratrol induced two–three-fold higher reporter activity than a maximally effective dose of estradiol (E2). This phenomenon, which we dubbed "superagonism", has since been confirmed by other workers [11]. In contrast to the superagonism observed in MCF-7 cells, we observed only partial agonism in BG-1 cells [8]. Subsequent reports from other laboratories have reported a range of ER agonism and/or antagonism in various cell types [11–19].

On the basis of these cell type-specific effects, resveratrol may be considered a natural SERM. In gene expression profiling analysis it clusters with the SERMs idoxifene and GW 5638 [20], but according to the molecular classification proposed by Jordan et al. [21] it is a class I estrogen like E2 and DES [19]. Type I estrogens are thought to be completely enclosed within the ligand-binding pocket by the repositioning of helix 12, which also results in the classical active conformation of the carboxy-terminal AF-2 domain and permits maximal interaction of AF-2 with the amino-terminal AF-1. In contrast, with type II estrogenic ligands, such as tamoxifen, helix 12 is blocked from folding completely over the ligand-binding site, and a different conformation of AF-2 is produced. These categories are distinguished functionally by their ability to activate expression of TGFα, an estrogen target gene, in MDA-MB-231 cells stably expressing wtERα or a D351G mutant.

In this paper we further examine resveratrol's ability to act as an ERα agonist, antagonist and superagonist. Experiments in MCF-7 cells confirm the specificity of superagonism, and the roles of the AF-1 and AF-2 domains are studied using MDA-MB-231 cells stably expressing mutant ERαs in which these domains are deleted or mutated. Ligand-stimulated receptor activity is assessed based on both TGFα expression and reporter gene activity.

2. Materials and methods

2.1. Chemicals

Resveratrol was the kind gift of Pharmascience Inc. (Montreal, Que., Canada). E2, DES, piceatannol, and additional resveratrol were purchased from Sigma (St. Louis, MO); no differences were found in the resveratrol from the two sources. Other hydroxystilbenes (*trans*-3,3′,4-trihydroxystilbene; *trans*-3,3′,5-trihydroxystilbene; and *trans*-3,4-dihydroxystilbene) were synthesized as previously described [22].

2.2. Plasmids and adenoviral vectors

The reporter plasmids used in this study have been described previously [8,23]. Adenoviral reporter vectors were constructed from plasmids tk18-luc, ERE-tk81-luc, ERE2-tk81-luc, tk109-luc, ERE-tk109-luc and ERE2-tk109-luc following the procedure previously published [24,25].

2.3. Cell lines and transfections

MCF-7 cells were grown and transfected as described in [8]. The E2TL1 stable transfectant was generated by liposome-mediated transfection of MCF-7 cells with ERE2-tk109-luc and pSV2neo, followed by selection with 250 μg/ml G418. Individual clones were selected and expanded over a period of 3 months. Due to poor survival when cells were plated at high dilution, only one clonal line was obtained. These cells were maintained in MEM supplemented with 5% calf serum and non-essential amino acids, and switched to estrogen-depleted medium (containing charcoal-stripped serum and lacking phenol red) 3–5 days before treatment. After 24 h incubation in treatment media, cells were assayed for luciferase.

The generation of MDA-MB-231 subclone 10A [26] and the stably transfected derivatives used in the present work has previously been described. S30 cells [26] express wtERα. ERαΔAF1 (designated D351ΔAF1 in [27]) expresses an N-terminal truncation mutant in which amino acids 1-180, including AF-1, are deleted. ERα537X (D351Δ537 in [27]) expresses a C-terminal

truncation mutant in which amino acids distal to residue 536, which include AF-2, are deleted. G3m cells [27,28] express a mutant with three amino acid substitutions (D538A/E542A/D545A) in the E domain, resulting in the loss of AF-2 activity [29,30]. Levels of receptor protein expression are similar in all four stable transfectant lines [27]. These cells and the MDA-MB-231 parental line were maintained in phenol red-free MEM supplemented with non-essential amino acids, 5% charcoal-stripped calf serum, 6 ng/ml bovine insulin, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.4. Adenoviral gene transfer

MDA-MB-231 cells and the derivative cell lines were seeded in 24-well plates at a density of $\sim 8.5 \times 10^4$ cells per well in estrogen-depleted medium (described above). Twenty-four hours later, adenoviral vectors were added to the cells at a multiplicity of infection (MOI) of five plaque-forming units (PFU) per cell. After ∼16 h, virus-containing medium was removed and replaced with treatment media. Cells were treated for 24 h, lysed and assayed for luciferase activity, using triplicate wells for each treatment.

2.5. TGFα mRNA assays

TGFα mRNA expression was assayed by Northern blotting as described previously [19]. Because of the low level of expression in untreated cells and the concomitant high relative variation, TGFα mRNA levels were normalized to E2-treated cells rather than untreated controls when combining data from independent experiments.

3. Results

Resveratrol is unusual among dietary phytoestrogens in having a hydroxystilbene structure, and is also highly unusual in its ability to stimulate the ERα-mediated reporter gene expression to levels greater than those obtained with saturating doses of E2 (superagonism). To determine if this is a general property of these compounds, we used the reporter gene system to compare the estrogenic activities of E2 and resveratrol with five other hydroxystilbenes: DES, a well-known synthetic estrogen; piceatannol, an antileukemic phytochemical [31]; and three synthetic hydroxystilbenes [22] (structures shown in Fig. 1). As shown, all the tested compounds stimulated expression of the estrogen-responsive reporter to some extent, but only resveratrol displayed superagonism. DES and E2, which are maximally effective at ∼0.1 nM, were tested at concentrations up to 10 μM but produced no additional reporter activity (data not shown). These results indicate that superagonism is not a general property of hydroxystilbene estrogens, but a specific property of resveratrol, dependent on the number and position of the hydroxyl substituents. In view of this, it is noteworthy that computer modeling of resveratrol binding to the ERα ligand-binding domain predicts that all three hydroxyl groups participate in hydrogen bond formation, creating a bonding pattern distinct from that produced by E2 or DES [32].

Although resveratrol has a superagonistic effect on reporter constructs such as ERE-tk109-luc in transiently transfected MCF-7 cells, it stimulates expression of the endogenous estrogen-regulated genes for progesterone receptor (PR) and pS2 only to the same extent as E2, i.e. full agonism but not superagonism [8]. This might reflect

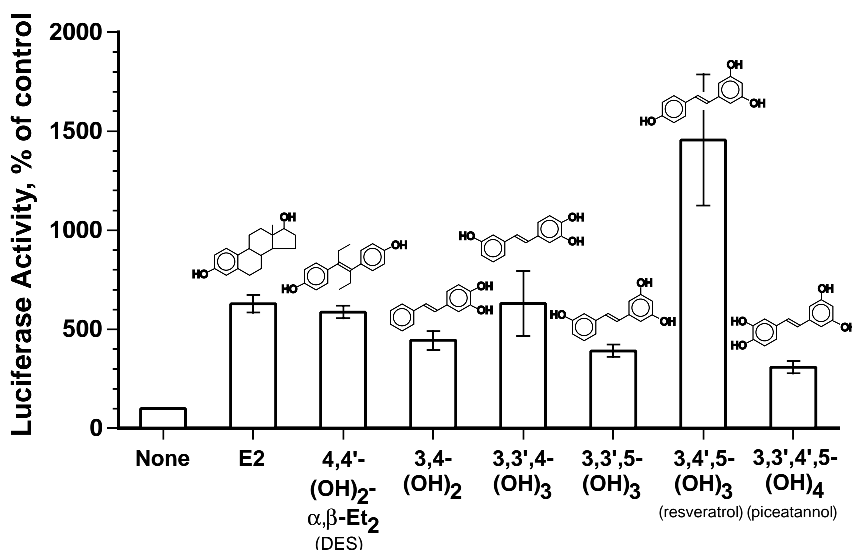

Fig. 1. Comparing ERα agonism of various hydroxystilbenes. MCF-7 cells were transfected with ERE-tk109-luc as described in Section 2, and treated with E2 or DES (1 nM) or the other indicated compounds (20 μM). In separate experiments (not shown), the optimal concentration for all the non-ethylated hydroxystilbenes shown was found to be in the 10–20 μM range. The results are expressed as a percentage of the activity of untreated control cells, and are plotted as means ± S.E. for two–four independent experiments.

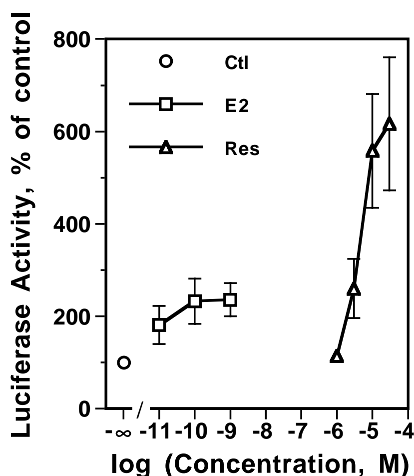

Fig. 2. Resveratrol acts as an ERα superagonist on a stably integrated target gene. E2TL1 cells (MCF-7 cells stably transfected with the ERE2-tk109-luc reporter plasmid) were treated with E2 and resveratrol (Res) as described in Section 2. Results are expressed as % of control (untreated cells) and plotted as means ± S.E. for three independent experiments. Luciferase expression in 10 and 30 μM Res-treated cells was significantly ($P < 0.05$) higher than in 1 nM E2-treated cells in each experiment.

gene-specific effects or differences between the regulation of simple artificial promoters and more complex natural ones, but could also indicate that superagonism is limited to the expression of transiently transfected genes. To address this possibility, we stably transfected MCF-7 cells with the reporter plasmid ERE2-tk109-luc. The resulting cell line, E2TL1, exhibits a lower fold induction by both E2 and resveratrol than is seen in transiently transfected cells, but resveratrol consistently produces luciferase activity several times greater than that obtained from a saturating dose of E2 (Fig. 2). This indicates that superagonism can occur on genes stably integrated into the cellular genome and is not a phenomenon limited to transiently transfected cells.

In order to better understand the mechanism of resveratrol's effects on ER transactivation, we initially attempted to use wt and mutant ERαs transiently transfected into ER-negative cell lines. However, resveratrol agonism relative to E2 was highly variable with transiently expressed receptors. In order to obtain more consistent results, we employed lines of MDA-MB-231 cells that were stably transfected with wtERα and various mutants containing deleted or altered activation function (AF) domains. These are illustrated schematically in Fig. 3. Because these cell lines are refractory to further transfection, we used adenovirus-based constructs instead of plasmids to introduce reporter genes. This enabled us to examine the activity of the wt and mutants ERαs on consensus EREs in the context of a simple heterologous promoter. In addition, the effects of resveratrol and E2 on an endogenous estrogen-regulated gene, *TGFA*, which codes for TGFα, were examined in each cell line.

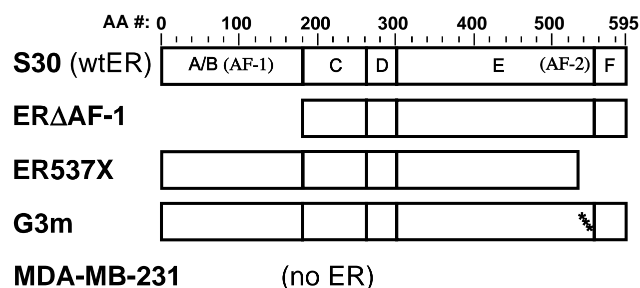

Fig. 3. ERα variants stably expressed by cell lines used in this study. Schematic diagrams show domains and deletions. Asterisks indicate point mutations.

3.1. MDA-MB-231 cells

To control for non-ER-mediated effects, the ER-negative parental cell line, MDA-MB-231, was tested for response to E2 and resveratrol using the reporter gene and TGFα mRNA assays. Results are shown in Fig. 4. E2 (1 nM) had no effect on reporter activity. Resveratrol at low concentrations (≤5 μM) had a modest (∼50%) stimulatory effect on luciferase expression, which appears to be independent of ER expression in the cells or the presence of EREs in the reporter construct. At higher concentrations, this stimulation was diminished or reversed.

Northern blots indicate that neither resveratrol nor E2 had any significant effect on TGFα expression in these cells.

3.2. S30 cells (wtER)

S30 cells were derived from MDA-MB-231 by stable transfection with a wtER expression vector [26]. In these cells, E2 strongly stimulated the expression of luciferase by the ERE-containing reporter constructs (Fig. 5, top row). Consistent with previous reports in other systems [33–35], the increase in activity was more marked when two EREs were present in tandem (>20-fold) than in the presence of a single ERE (>10-fold).

In MCF-7 breast cancer cells, which are naturally ER-positive, we have previously observed that resveratrol acts as a superagonist on some estrogen-responsive reporter genes [8]. This phenomenon also occurs in S30 cells, as shown in Fig. 5. Luciferase activity in cells infected with single- or double-ERE reporter vectors and treated with 50 μM resveratrol was about twice that of cells treated with a saturating dose of E2. In cells infected with an ERE-less control vector, E2 had no effect and resveratrol induced only a slight stimulation that declined at higher concentrations, similar to the results obtained in ER-negative cells. Superagonism was also observed in S30 cells infected with the reporter construct Ad-2ERE-TATA-luc (data not shown), indicating that this phenomenon is not specific to the thymidine kinase (tk) promoter.

It has previously been shown that TGFα expression in S30 cells is upregulated in a dose-dependent fashion by E2

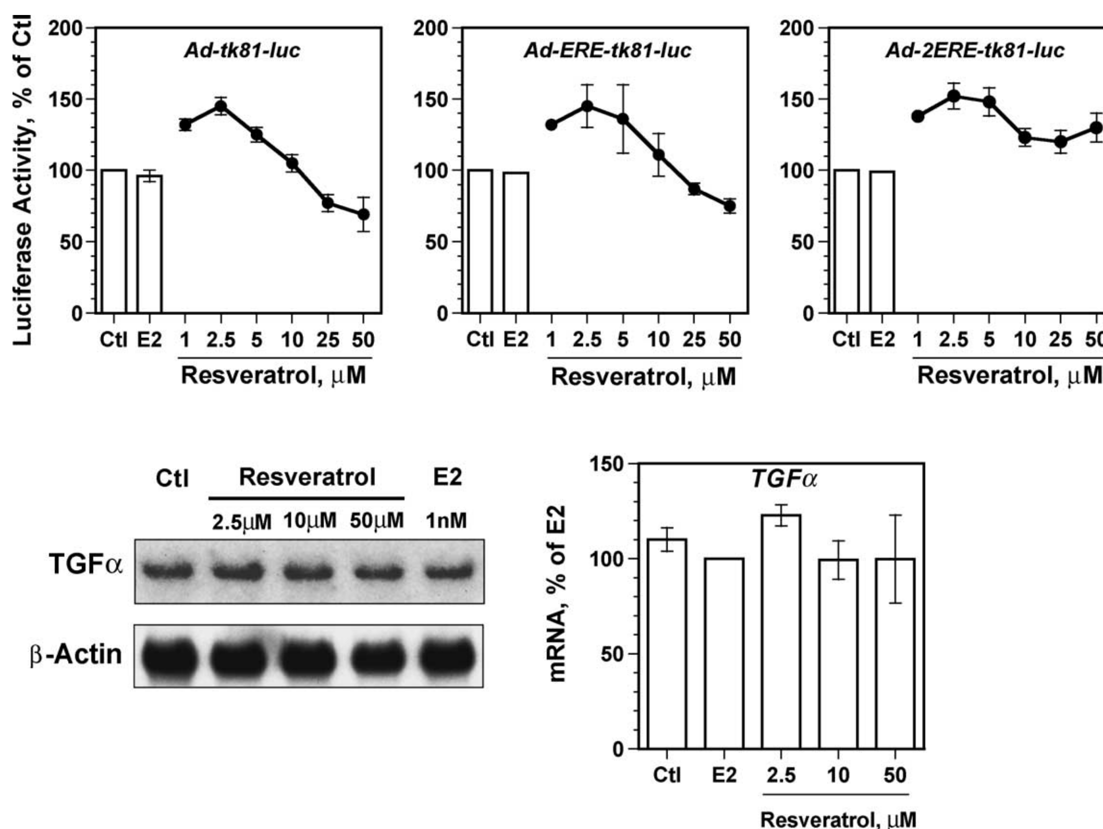

Fig. 4. Effect of E2 and resveratrol on estrogen targets in ER-negative MDA-MB-231 cells. Top row: Cells were infected with adenoviral reporter vectors, treated and assayed as described in Section 2. Luciferase activity is expressed as % of control (cells treated with 0.1% ethanol) and plotted as mean and range for two independent experiments. Bottom row: RNA was prepared from control and treated cells and assayed as described in Section 2. Northern blot bands from a typical experiment are shown on the left. TGFα mRNA was quantitated, normalized to β-actin, and plotted as % of E2-treated cells. Graph shows means ± S.E. for four independent experiments.

[36] and resveratrol [19]. As shown in Fig. 5 (lower row) resveratrol's effects on this estrogen target gene were similar to those of E2. The dose–response relationship is similar to that for the luciferase reporters, except that fold induction in response to both agonists is lower, and the maximal response to resveratrol is approximately equal to that produced by E2. Thus, as assessed by TGFα expression, resveratrol is a full ER agonist but not a superagonist in S30 cells.

Subsequent to our initial report [8] on the ER-agonist activity of resveratrol in breast cancer (MCF-7, MDA-MB-231 and T47D) and ovarian cancer (BG-1) cell lines, other workers reported that, in the presence of E2, resveratrol could act as an ER antagonist in MCF-7 and ER-transfected CHO-K1 cells [11,14,15]. To determine if resveratrol has anti-estrogenic activity in S30 cells, we examined the effects of E2 and resveratrol in combination. As shown in Fig. 6, resveratrol had no antagonistic effects on estrogen induction of reporter activity in these cells. In the presence of 1 nM E2, increasing resveratrol concentrations further increased luciferase activity until it reached the same maximum obtained in the presence of resveratrol alone. We have elsewhere [19] shown a similar lack of antagonism in S30 cells as assayed by TGFα expression. Resveratrol appears to be a pure agonist (or superagonist) in this cell line.

3.3. Effect of AF-1 deletion on resveratrol agonism

To assess the role of AF-1 in resveratrol agonism, we used ERαΔAF1cells, which stably express an N-terminal truncation mutant of ERα that lacks the AF-1 domain [27]. As shown in Fig. 7 (upper row), induction of the ERE reporter constructs by E2 was strongly decreased in these cells compared to the wtER-expressing S30 cells (e.g. ∼2.5-fold versus ∼11-fold for the single-ERE reporter). It is noteworthy that the response to resveratrol was diminished even more strongly than that to E2 (at 50 μM, <2-fold versus >20-fold in S30). Thus, resveratrol was only a partial agonist in ERαΔAF1 cells. In addition, it was observed that in these cells the double-ERE reporter produced no higher fold response to E2 than the single ERE, and only a marginally greater response to resveratrol. This is in contrast to the usual behavior seen in wtERα-expressing cells, as shown in Fig. 5 and previously reported [33–35], in which multiple EREs enhance the response to E2 and partial agonists. It appears that the AF-1 region of the receptor is required

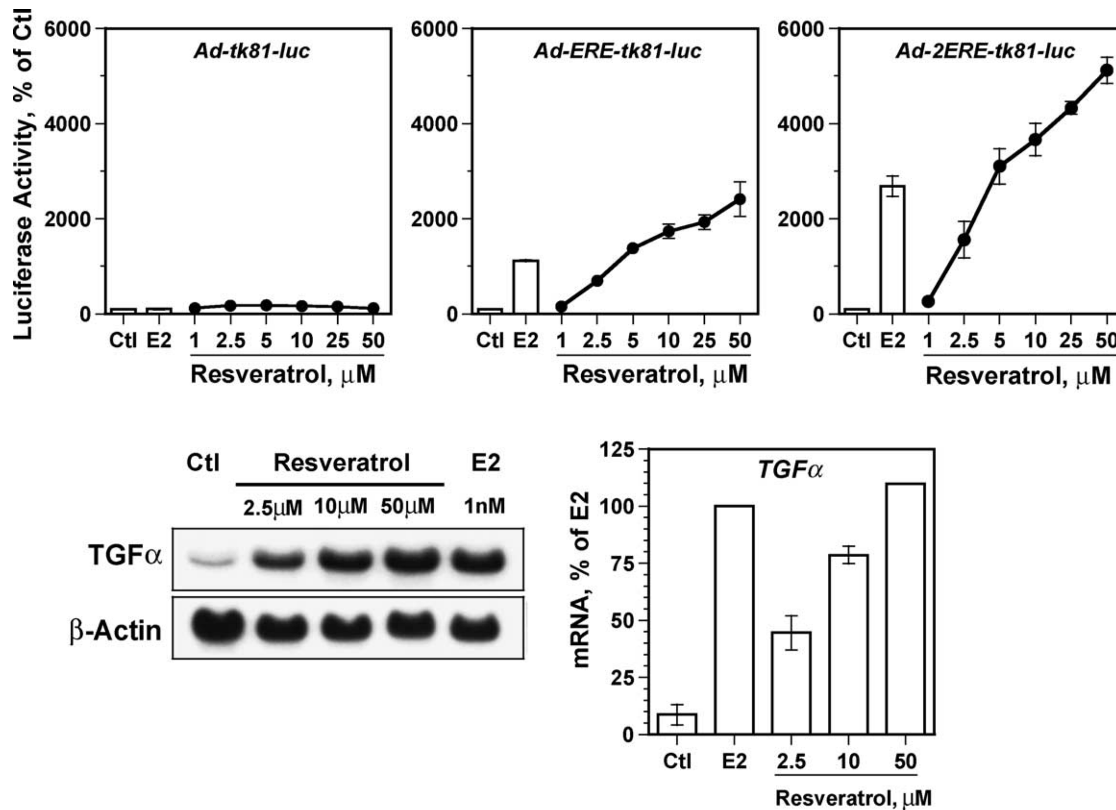

Fig. 5. Effect of E2 and resveratrol on estrogen targets in cells stably expressing wtERα. Top row: S30 cells were infected with adenoviral reporter vectors, treated and assayed as described in Section 2. Luciferase activity is expressed as % of control (cells treated with 0.1% ethanol) and plotted as mean and range for two independent experiments. Bottom row: RNA was prepared from control and treated cells and assayed as described in Section 2. Northern blot bands from a typical experiment are shown on the left. TGFα mRNA was quantitated, normalized to β-actin, and plotted as % of E2-treated cells. Graph shows mean and range for two independent experiments. Some error bars were too small to be shown at the plotted scale.

for this synergistic action of multiple EREs, and also for the superagonistic effect of resveratrol.

In the absence of EREs, E2 had no effect, and resveratrol at low concentrations produced a modest, apparently

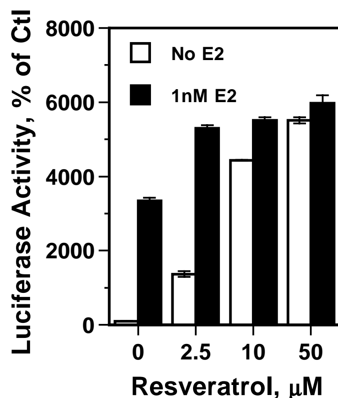

Fig. 6. Effect of resveratrol in the presence of E2 in cells expressing wtERα. S30 cells were infected with Ad-ERE-tk81-luc and treated with the indicated concentrations of resveratrol in the presence (solid bars) or absence (open bars) of 1 nM E2. Luciferase activity is expressed as % of control (cells treated with 0.1% ethanol) and plotted as means ± S.E. for triplicate wells. Some error bars were too small to be shown at the plotted scale.

ER-independent, activation similar to that observed in the ER-negative parental cell line (MDA-MB-231).

Reduced induction of TGFα expression by E2 in ERαΔAF1cells (compared to wtERα-expressing S30 cells) was observed, as previously reported [27]. Induction by resveratrol was also diminished. In contrast to the reporter gene assays, however, the response to E2 was more severely affected than that to resveratrol, which retained more ability to induce TGFα mRNA expression.

3.4. Effect of C-terminal deletion on resveratrol and E2 agonism

To examine the effects of AF-2 deletion on resveratrol and E2 action, we used the cell line ERα537X, which stably expresses a C-terminal truncation mutant ER in which helix 12 and all distal portions of the receptor have been deleted. Hormone binding affinity is similar to that of wtERα [27]. As shown in Fig. 8, the responses of the luciferase reporter vectors to E2 and resveratrol in these cells were very similar to those in untransfected MDA-MB-231 cells, indicating virtually complete ablation of receptor activity. Induction of TGFα expression by E2 was also severely curtailed by the C-terminal truncation, as previously reported

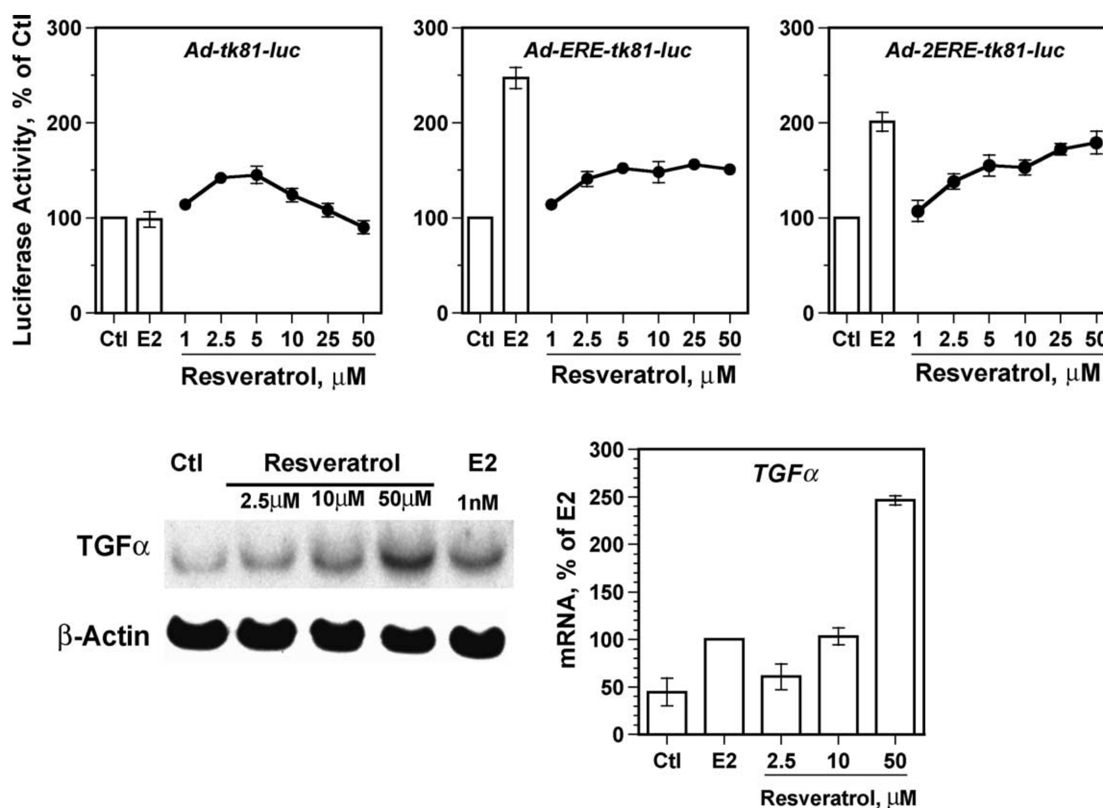

Fig. 7. Effect of E2 and resveratrol on estrogen targets in cells stably expressing AF-1-deficient ERα. Top row: ERαΔAF1 cells were infected with adenoviral reporter vectors, treated and assayed as described in Section 2. Luciferase activity is expressed as % of control (cells treated with 0.1% ethanol) and plotted as mean and range for two independent experiments. Bottom row: RNA was prepared from control and treated cells and assayed as described in Section 2. Northern blot bands from a typical experiment are shown on the left. TGFα mRNA was quantitated, normalized to β-actin, and plotted as % of E2-treated cells. Graph shows mean and range for two independent experiments. Some error bars were too small to be shown at the plotted scale.

[27]. However, induction by resveratrol was still substantial (∼5-fold).

3.5. Effect of AF-2 inactivating mutation on resveratrol agonism

G3m cells [27,28] stably express a mutant ER containing three point mutations in the AF-2 region, which have been reported to eliminate the ability of this domain to activate gene expression [29,30]. Surprisingly, in these cells the ERE reporter constructs showed an enhanced response to E2, compared to cells expressing the wtERα (Fig. 9; cf. Fig. 5). Because this mutant has been reported to require higher E2 concentrations to produce full estrogenic response in some reporter systems [30] and the TGFα assay (Liu, unpublished results), higher E2 concentrations were tested. Ten nanomolar E2 produced substantially greater reporter activity than 1 nM, but 100 nM was only marginally more effective than 10 nM. The maximum resveratrol concentration tested was increased to 100 μM but this produced little or no increase over 50 μM. Higher concentrations were not used due to cytotoxicity and solubility considerations. In contrast to E2, resveratrol was much less effective at inducing ERE reporter activity in these cells than in those expressing wtERα. The response induced by resveratrol in G3m cells was substantially less than that induced by E2 and also less than that induced by resveratrol in S30 cells. Thus, this mutation enhanced the effect of E2 on the reporter activity but diminished the effect of resveratrol, converting resveratrol from a superagonist to a partial agonist.

In these cells, as in those expressing wtERα, the response of the double-ERE reporter to E2 was two– to three-fold greater than that of the single ERE. However, both reporters showed about the same response to resveratrol. This is in contrast to cells expressing the AF-1 deletion mutant, in which the double ERE did not enhance the response to either ligand.

In the TGFα mRNA assay (Fig. 9, lower panel), G3m cells showed a considerably diminished response to both E2 and resveratrol compared to cells expressing wtERα. However, both ligands were still able to induce a several-fold increase in TGFα expression, and the magnitude of the response was similar for E2 and resveratrol (i.e. resveratrol acted as a full agonist). The response to E2 is consistent with previously published experiments in these cells [27,28].

Because resveratrol exhibited only partial agonism in the reporter assay when administered to G3m cells by itself, we tested its ability to act as an estrogen antagonist in this

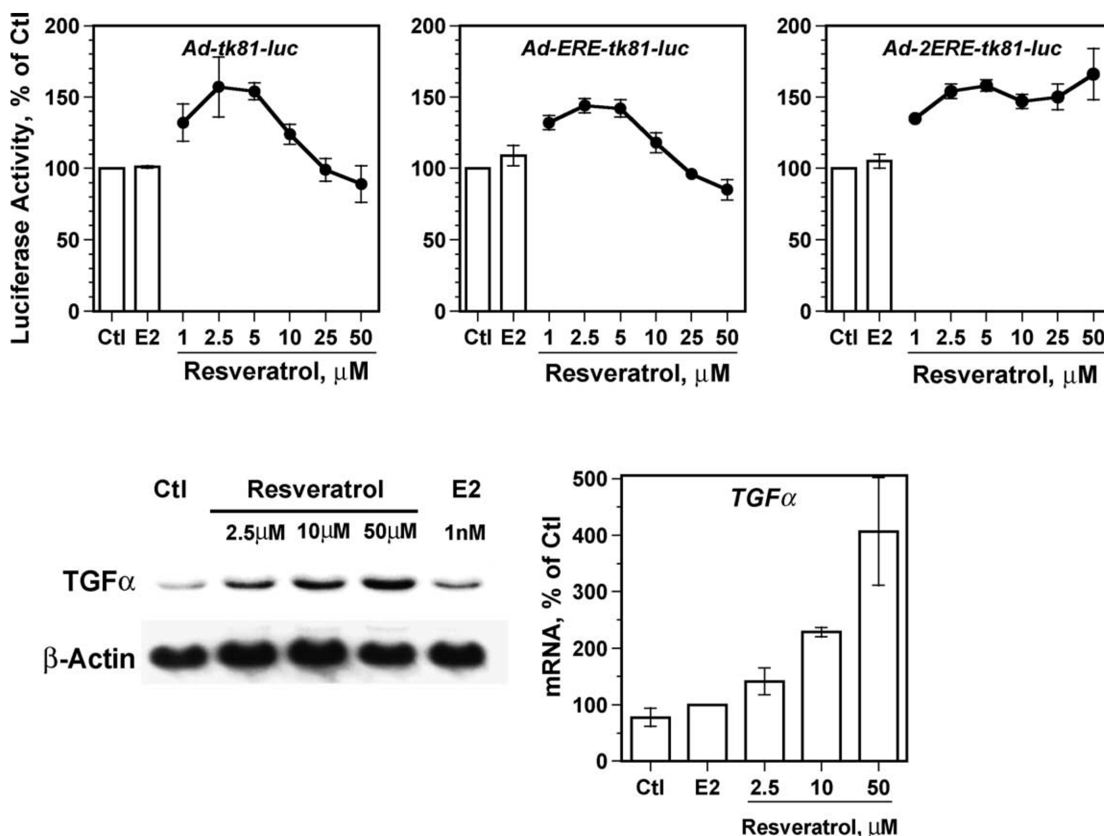

Fig. 8. Effect of E2 and resveratrol on estrogen targets in cells stably expressing C-terminal truncated ERα. Top row: ERα537X cells were infected with adenoviral reporter vectors, treated and assayed as described in Section 2. Luciferase activity is expressed as % of control (cells treated with 0.1% ethanol). Each point represents the means ± S.E. for three independent experiments (Ad-2ERE-tk81-luc) or mean and range of two independent experiments (other vectors). Bottom row: RNA was prepared from control and treated cells and assayed as described in Section 2. Northern blot bands from a typical experiment are shown on the left. TGFα mRNA was quantitated, normalized to β-actin, and plotted as % of E2-treated cells. Graph shows mean and range for two independent experiments. Some error bars were too small to be shown at the plotted scale.

system. As shown in Fig. 10 (left panel), resveratrol antagonized reporter activation by E2 when the two compounds were administered together. The inhibitory effect of resveratrol was diminished at higher E2 concentrations (data not shown), consistent with competition by the two ligands for the same binding site. We also examined the effect of resveratrol on E2-induced TGFα expression. To avoid overwhelming possible antagonism with a high E2 concentration, 1 nM E2 rather than the maximally activating 10–100 nM was used. As shown in Fig. 10 (right panel), resveratrol did not antagonize E2-induced TGFα expression, but instead further increased that expression in a roughly additive fashion. This is consistent with the full-agonist effect of resveratrol when tested alone in this assay.

4. Discussion

Although our initial report of ER superagonism by resveratrol [8] was confirmed by Basly et al. [11], many conflicting reports have also appeared, and the effect of resveratrol on ERs is controversial. Ashby et al. [13] reported partial agonism in Cos-1 cells transfected with either ERα or ERβ. Bhat et al. [17] found resveratrol to be a partial agonist/antagonist, based on assays of reporter gene activity and expression of PR and pS2 in MCF-7, T47D, S30 and LY2 cells. The same laboratory reported antagonism in Ishikawa cells [16]. Bowers et al. [15] observed partial to full agonism in CHO-K1 cells transfected with ERα or ERβ and reporters based on various EREs. Lu and Serrero [14], examining the effect of resveratrol on PR expression in MCF–7 cells, reported ER antagonism in the presence of E2 and partial agonism in its absence. Stahl et al. [12] found that resveratrol, like E2, stimulates the synthesis and secretion of prolactin by cultured pituitary cells; ICI 182,780 antagonizes both effects.

Based on these reports, it appears that resveratrol's ability to act as an ER agonist varies between different cell types. This is consistent with our prior observation that it displays only partial agonism in BG-1 cells, using the same reporters with which it displays superagonism in MCF-7 cells. The degree of agonism also depends upon the promoter context of the ERE: we found that, even in MCF-7 cells, resveratrol acts as a partial agonist on reporter constructs based on the SV40 promoter (unpublished results). However, superagonism was still observed using reporters containing a TATA

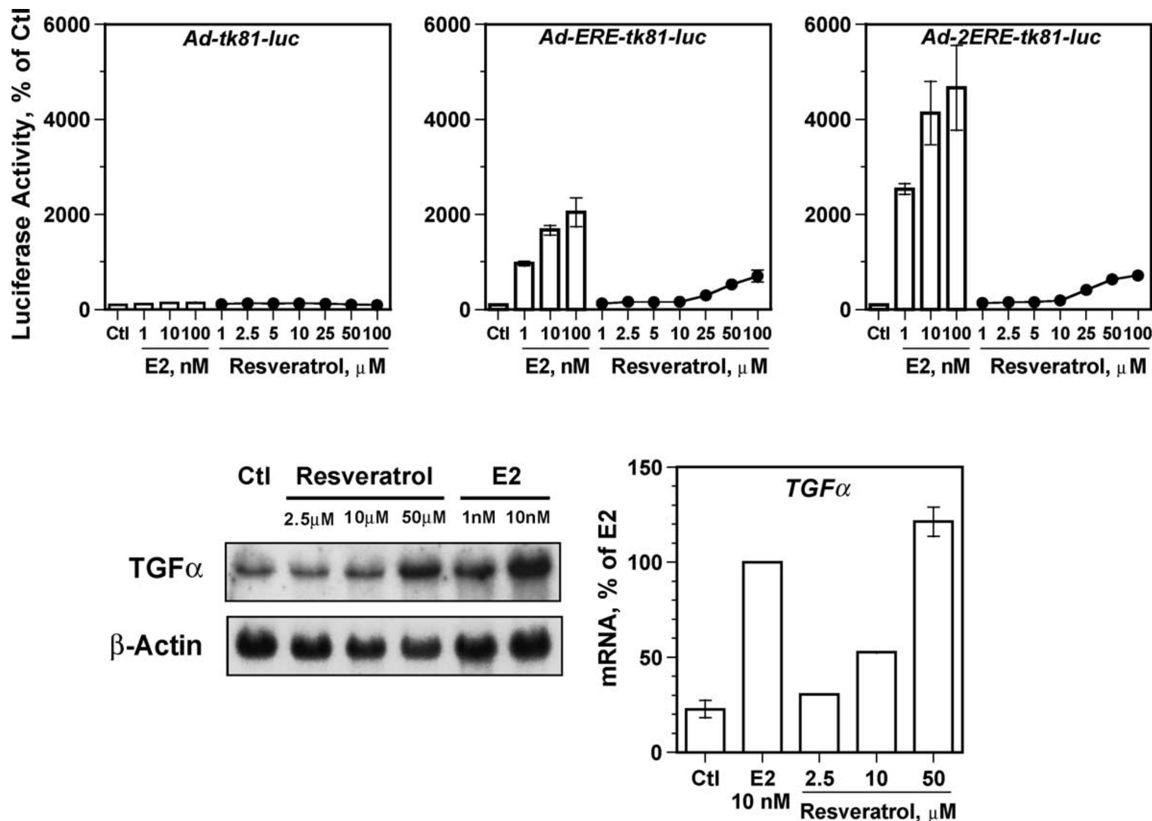

Fig. 9. Effect of E2 and resveratrol on estrogen targets in cells stably expressing ERα with mutations in AF-2. Top row: G3m cells were infected with adenoviral reporter vectors, treated and assayed as described in Section 2. Luciferase activity is expressed as % of control (cells treated with 0.1% ethanol). Each point represents the mean and S.E. for two–four independent experiments (some experiments did not include all concentrations). Bottom row: RNA was prepared from control and treated cells and assayed as described in Section 2. Northern blot bands from a typical experiment are shown on the left. TGFα mRNA was quantitated, normalized to β-actin, and plotted as % of E2-treated cells. Graph shows mean and range for two independent experiments. Some error bars were too small to be shown at the plotted scale.

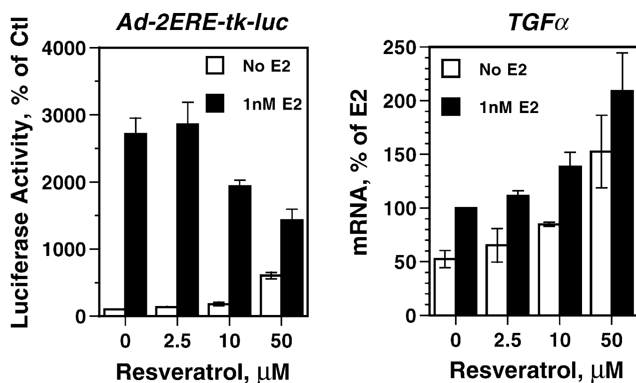

Fig. 10. Estrogen antagonism by resveratrol in G3m4 cells. Left panel: Cells were infected with Ad-2ERE-tk81-luc and treated with the indicated concentrations of resveratrol in the presence (filled bars) or absence (open bars) of 1 nM E2. Luciferase activity is expressed as % of control (cells treated with 0.1% ethanol). Each point represents the mean and range of two independent experiments. Similar results were obtained in an additional experiment using Ad-2ERE-tk81-luc. Right panel: RNA was prepared from control and treated cells and assayed as described in Section 2. TGFα mRNA was quantitated, normalized to β-actin, and plotted as % of E2-treated cells. Graph shows mean and range for two independent experiments. Some error bars were too small to be shown at the plotted scale.

box instead of a minimal tk promoter; this indicates that superagonism is not due to some peculiarity of the tk promoter.

To confirm that superagonism was not limited to transiently transfected genes, we generated an MCF-7-derived cell line carrying a stably integrated estrogen-responsive reporter. Resveratrol acted as a superagonist in these cells, consistent with the results obtained by Basly et al. [11] in a similar cell line. We also tested a panel of other hydroxystilbenes to determine if superagonism is a general property of these compounds. The other hydroxystilbenes tested behaved as full or partial agonists, but only resveratrol acted as a superagonist. These findings indicate that ERα superagonism is a specific property of resveratrol, although it occurs only in certain cell and promoter contexts.

The use of mutant ERαs expressed in an ER-negative background has been a powerful technique for studying the function of various domains in the receptor. Because resveratrol superagonism was variable in transiently transfected cells, we utilized a set of cell lines derived from ER-negative MDA-MB-231 by stable transfection with wt or mutant receptors. In this system, resveratrol consistently displayed superagonism on wtERα similar to that previously observed in MCF-7 cells.

ERα contains two activation function domains that mediate estrogen-dependent gene transcription. AF-2, in the C-terminal region, is made active by conformational changes in the receptor protein that result from ligand binding. AF-1, in the N-terminal domain, does not depend on ligand binding but is regulated by phosphorylation and may interact synergistically with AF-2 in a ligand-dependent fashion [30,37,38]. Among potential mechanisms for resveratrol superagonism we considered the following three non-exclusive possibilities: (1) resveratrol produces a more active conformation of AF-2 than does E2; (2) resveratrol enhances the activity of AF-1, e.g. by altering the activity of a kinase or phosphatase; (3) resveratrol produces a more effective interaction of AF-1 and AF-2 than obtains in the E2-liganded receptor.

To evaluate the role of the AF domains in resveratrol agonism, we used other MDA-MB-231-derived cell lines that express mutant ERαs in which the AF-1 and AF-2 domains are deleted or mutated. Estrogenic signaling in these cells had previously been studied using TGFα expression as an endpoint, but reporter gene experiments had not previously been done, and the estrogenic effects of resveratrol or other phytoestrogens had not been characterized.

Interestingly, the effects of the mutations on resveratrol and E2 agonism were quite distinct, and also differed depending on whether reporter activity or TGFα expression was used as an endpoint.

In reporter gene assays, the wtERα responded strongly to E2, and even more strongly to resveratrol (superagonism). The fold induction by both agonists was increased when two EREs in tandem, rather than a single ERE, were attached to the minimal promoter of the reporter construct. Deletion of the AF-1 domain substantially reduced the fold induction by E2, and even more severely reduced induction by resveratrol. Another striking consequence of AF-1 deletion was that the enhanced response of the double-ERE reporter was eliminated.

Unsurprisingly, deletion of AF-2 completely eliminated the effect of E2 on the reporters, and the effect of resveratrol was also ablated. Since the AF-1 domain in this mutant is intact, the absence of a receptor-specific effect of resveratrol suggests that superagonism in wtERα is not due to unusual activation of AF-1, e.g. by altered phosphorylation.

In striking contrast to the effect of deleting the AF-2 region, mutation of this domain by replacing three acidic residues in helix 12 with alanines resulted in an increased fold response to E2 compared to that in cells expressing wt receptor, yet response to resveratrol was greatly diminished. The presence of tandem EREs enhanced the response to E2 but not to resveratrol with this mutant.

The pattern of results for TGFα expression was different from that seen with the reporter genes. In cells expressing wtERα, E2 and resveratrol both produced a maximal induction of about 10-fold. Resveratrol thus acted as a full agonist. Deletion of the AF-1 domain reduced the stimulatory effect of both ligands on TGFα expression, but much more so for E2 than resveratrol, in contrast to the reporter gene experiments.

Deletion of AF-2 virtually eliminated the ability of E2 to stimulate TGFα expression, similar to the results in the reporter gene assays. But unexpectedly, resveratrol had a substantial ability to stimulate TGFα expression via the AF-2-deleted mutant, presumably acting via AF-1. Resveratrol also stimulated TGFα expression in cells expressing ERα with a triple point mutation in AF-2 (G3m cells), but in these cells E2 was also effective.

Our results differ substantially from those of Yoon et al. [39], who examined the ability of various estrogenic compounds, including resveratrol, to activate wtERα and mutants corresponding to ERαΔAF1 and G3m transiently expressed in three ER-negative cell lines, including MDA-MB–231. Using a reporter plasmid with three EREs, they observed no agonism by resveratrol on the wt receptor, and partial agonism on the mutants with deleted AF-1 or mutated AF-2 domains. The differences between their results and ours may result from the different reporter constructs, the use of transiently versus stably expressed receptors, or their use of a single resveratrol concentration (100 μM) which we have found to be above that which produces maximal stimulation of wtERα.

Because resveratrol has been reported to be an estrogen antagonist in some systems, we examined the effect of combined treatment with resveratrol and E2 in the two cell lines in which E2 showed strong agonism (S30 and G3m). In S30 cells (expressing wtERα), resveratrol showed no antagonist effect in either the reporter gene (Fig. 6) or TGFα expression assays [19]. This is consistent with its activity as a superagonist and full agonist in these assays when administered alone. In G3m cells (expressing ERα with a mutated AF-2), resveratrol acted as an antagonist to E2. This is consistent with its partial agonism when administered alone. In the TGFα expression assay, in contrast, resveratrol did not antagonize the effect of E2 and acted as a full agonist when administered alone.

To summarize, in reporter gene assays resveratrol acts as a superagonist on wtERα stably expressed on an MDA-MB-231 background, but deletion or mutation of the AF-1 or AF-2 domains severely impairs its ability to activate gene transcription, rendering it completely ineffective (ERα537X) or less agonistic than E2 in the same cells (ERαΔAF1, G3m). Resveratrol agonism in this system is thus more dependent than E2 agonism on the presence and integrity of both AF domains.

In the TGFα expression assay, however, resveratrol acts as a full agonist on the wtERα, and deletion or mutation of the AF-1 or AF-2 domains impairs resveratrol agonism less than (in ERαΔAF1 and ERα537X), or no more than (in G3m), E2 agonism.

It therefore appears that the role of the AF-1 and AF-2 domains in ERα-mediated gene transcription varies depending on both the activating ligand and the target gene, and that these dependencies interact in a complex, non-linear fashion.

The disproportionate sensitivity of resveratrol superagonism to deletion of AF-1 or mutation of AF-2 suggests that the greater activity of the resveratrol-liganded (as compared to E2-liganded) receptor may result from enhanced interaction of these two domains, which are thought to be able to synergize in some promoter contexts [30]. It may be significant that deletion of AF-1 eliminated both superagonism and the enhanced response associated with tandem EREs. Possibly both phenomena depend on interactions between AF-1 and AF-2 domains on adjacent DNA-bound receptor molecules. We attempted to determine if resveratrol was more effective than E2 at inducing transcriptionally productive interaction of AF-1 and AF-2 when these domains are expressed in separate polypeptides, using expression plasmids kindly provided by Benita Katzenellenbogen [40]. However, we were unable to demonstrate any stimulation of reporter gene transcription by either ligand (data not shown). Explanation of the superagonism phenomenon may have to await a clearer understanding of the structures of the AF-1 domain and the ERα-resveratrol complex.

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