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Resveratrol Acts as a Mixed Agonist/Antagonist for Estrogen Receptors α and β*

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ABSTRACT

Epidemiological evidence indicates that phytoestrogens inhibit cancer formation and growth, reduce cholesterol levels, and show benefits in treating osteoporosis. At least some of these activities are mediated through the interaction of phytoestrogens with estrogen receptors α and β (ERα and ERβ). Resveratrol, *trans*-3,5,4′-trihydroxystilbene, is a phytoestrogen in grapes that is present in red wine. Resveratrol was shown to bind ER in cytosolic extracts from MCF-7 and rat uteri. However, the contribution of ERα *vs.* ERβ in this binding is unknown. Here we report that resveratrol binds ERβ and ERα with comparable affinity, but with 7,000-fold lower affinity than estradiol (E_2). Thus, resveratrol differs from other phytoestrogens that bind ERβ with higher affinity than ERα. Resveratrol acts as an estrogen agonist and stimulates ERE-driven reporter gene activity in CHO-K1 cells expressing either ERα or ERβ. The estrogen agonist activity of resveratrol depends on the ERE sequence and the type of ER. Resveratrol-liganded ERβ has higher transcriptional activity than E_2-liganded ERβ at a single palindromic ERE. This indicates that those tissues that uniquely express ERβ or that express higher levels of ERβ than ERα may be more sensitive to resveratrol's estrogen agonist activity. For the natural, imperfect EREs from the human c-*fos*, pS2, and progesterone receptor (PR) genes, resveratrol shows activity comparable to that induced by E_2. We report that resveratrol exhibits E_2 antagonist activity for ERα with select EREs. In contrast, resveratrol shows no E_2 antagonist activity with ERβ. These data indicate that resveratrol differentially affects the transcriptional activity of ERα and ERβ in an ERE sequence-dependent manner. (*Endocrinology* **141:** 3657–3667, 2000)

RESVERATROL is a bioflavonoid found naturally in grapes that has both chemopreventive (1–3) and cardioprotective activities (4) *in vitro* and in animal models. Red wine that contains 1–10 mg/liter and can be a major dietary source of resveratrol (5). Studies on the bioavailability of resveratrol in rats lead to the conclusion that even an average consumer of red wine, particularly over the long term, can absorb quantities of resveratrol that correlate with the beneficial health effects of red wine consumption observed in epidemiological studies (6–8).

Resveratrol is a stilbene that exists as *cis*- and *trans*-isomers. The *trans*-isomer appears to have greater anticancer and cardio-protective properties than the *cis*-isomer (9). Resveratrol has been characterized as a phytoestrogen based on its ability to bind to and activate estrogen receptor (ER) (10). ER is a nuclear steroid receptor that binds estrogens and regulates the transcription of estrogen-responsive genes by either binding directly to DNA, at particular sequences called estrogen response elements (EREs), or by interacting with other transcription factors, *e.g.* Sp1 (11), bound to their cognate sites on DNA. When activated by an agonist ligand, ERα interacts with coactivators, *e.g.* SRC-1 and CBP, that either acetylate lysine residues in histones to alter chromatin conformation and/or interact with components of the RNA polymerase II initiation complex to enhance target gene transcription (12).

There are two known ER subtypes, ERα and the more recently identified ERβ (8). Because ERα and ERβ exhibit different patterns of tissue distribution and have select differences in biochemical properties (13), it is important to determine which form mediates the effects of resveratrol. While both ER isoforms bind E_2 with comparable affinity, some phytoestrogens, *e.g.* genistein and coumestrol, show higher affinity for ERβ than ERα (5), suggesting that resveratrol may show selectivity for ERβ. Resveratrol was shown to compete with [^{125}I]E_2 for binding ER in an extract from MCF-7 human breast cancer cells with an IC_{50} value of ~10 μM (10). More recently, resveratrol was reported to compete with [^{3}H]E_2 for binding to rat uterine ER with an IC_{50} value of ~100 μM (14). Because MCF-7 cells and uterus reportedly express ERβ as well as ERα (15, 16), it is important to determine the affinity of resveratrol interaction with ERβ and with ERα.

Ligands that bind ER can act as agonists, antagonists, or mixed agonist/antagonists. The archetype mixed agonist/antagonist is tamoxifen (TAM), used clinically to prevent breast cancer promotion and recurrence. TAM has both estrogen agonist and antagonist activity depending on the cell type and gene promoter (reviewed in Ref. 17). Resveratrol showed estrogen agonist activity in MCF-7 cells, *i.e.* activating the expression of progesterone receptor (PR) and pS2 genes (2). However, resveratrol elicited only weak agonist activity in both a yeast hERα transcription assay and in transient transfections with ERα in COS-1 cells. In another study, resveratrol antagonized E_2-stimulated growth and inhibited transcription of PR in MCF-7 cells (18). In animal

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studies, oral administration of resveratrol to weanling rats had no significant effect on estrogenic responses such as serum cholesterol or messenger RNA (mRNA) for insulin-like growth factor I, but gave a slight increase in uterine wet weight (19). The same study showed that resveratrol antagonized the effect of E_2 on serum cholesterol (19). Thus, the relative estrogen agonist/antagonist activity of resveratrol remains to be determined.

This study examined the relative agonist/antagonist activity of resveratrol in defined assays using ERα and ERβ. We examined whether resveratrol preferentially binds ERα or ERβ, the effect of resveratrol on the proliferation of cells expressing ERα or ERβ, how resveratrol impacts ER-ERE binding *in vitro*, and how resveratrol affects the expression of reporter gene activity from a consensus and naturally occurring EREs from estrogen- responsive genes in transiently transfected CHO-K1 cells. Our results show that resveratrol binds ERα and ERβ with comparable affinity, but with much lower affinity than E_2. Resveratrol-occupied ERα and ERβ bind an ERE *in vitro*, but resveratrol inhibits ER-ERE binding in a concentration-dependent manner. Resveratrol exhibits agonist activity in transiently transfected cells using a variety of ERE-driven reporter constructs and shows differences in activity depending on the ERE sequence and on which ER is expressed. With ERα, but not ERβ, resveratrol shows E_2 antagonist activity from certain EREs, including a consensus ERE. Thus, the mechanism of action of resveratrol is unique for ERα and ERβ.

Materials and Methods

Cell maintenance

Chinese Hamster Ovary cells (CHO-K1) were purchased from ATCC (Manassas, VA) and maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Life Technologies, Inc., Grand Island, NY) supplemented with 10% calf serum (CS). All other cell culture reagents were purchased from Life Technologies, Inc.

Plasmid preparation

The sequences of EREs used are:

EREc38: 5′-CCAGGTCAGAGTGACCTGAGCTAAAATAACACATT-3′;

PR1148: 5′-AGCCCTCCCTCCTGCGAGGTCACCA**GCTCTT**GGTGCCTG TTT-3′;

pS2: 5′-CTTCCCCCTGCAAGGTCAGCGTG**G**CC**A**CCCCGTGAGCCA-CT-3′; and

Fos-1211: 5′-AGCTTGGGCTGAGCCG**G**CAGCGTGACCCCGCATG-3′.

The *underlined* nucleotides correspond to the minimal core consensus ERE. The nucleotides in *bold* indicate an alteration in the consensus ERE. EREc38, PR-1148, pS2, and Fos-1211 were cloned into the pGL3-promoter luciferase reporter vector (Promega Corp., Madison, WI) as described (20). A mammalian expression vector containing the sequence for recombinant human (rh) rhERα was generously supplied by Dr. Benita Katzenellenbogen (21). An expression vector containing the sequence for recombinant rat (rr) ERβ was generously provided by Dr. J.-A. Gustafsson (22).

Nuclear extract preparation

Baculovirus-expressed rhERα and rrERβ were prepared as nuclear extracts (NE) from Sf-21 cells as previously described (23, 24).

Competition binding experiments

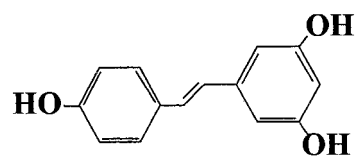
Aliquots of NE from Sf-21 cells containing 6 nM (monomer) rhERα or rrERβ were incubated in TEGK100 buffer (40 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% (vol/vol) glycerol, 100 mM KCl, 0.5 mM PMSF, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.75 μg/ml pepstatin, 1 mM DTT) with 15.5 nM [^{3}H]E_2 (NEN Life Science Products, Boston, MA) and the indicated concentrations of *trans*-resveratrol (generously provided by Pharma Science, Montréal, Québec, Canada or purchased from Sigma, St. Louis, MO), or E_2 (Sigma). Unless specifically stated, all references to resveratrol in this manuscript indicate *trans*-resveratrol (see Fig. 1). NE isolated form Sf-21 cells expressing ERα or ERβ were used as the source of receptors and nonspecific ligand binding was determined using a NE Sf-21 cells expressing alkaline phosphatase. Nonspecific binding varied between 7.6 and 15.5%. ER-bound and unbound [^{3}H]E_2 were separated by hydroxyapatite (HAP) (Bio-Rad Laboratories, Inc., Hercules, CA) (25). Radioactivity in the HAP pellet was counted in a liquid scintillation counter (Wallac, Inc. 1409, Turku, Finland). Specific [^{3}H]E_2 binding was calculated, graphed, and analyzed using a GraphPad Software, Inc. prism (San Diego, CA). K_i was estimated by the formula described in (26).

Determination of resveratrol solubility

The solubility of resveratrol has not been published. We determined resveratrol solubility in 5% ethanol/95% H_2O at room temperature by dissolving different amounts of resveratrol in the solvent, followed by centrifugation and measuring the resulting absorbance at 217 and 304 nm. No additional increase in the absorbance was observed when the resveratrol concentration exceeded 250 μM, indicating that 250 μM is the limit of resveratrol solubility.

Electrophoretic mobility shift assay (EMSA)

An EREc38 oligomer, 77 nucleotides in length, which includes sequences flanking the ERE between the *Eco*RI and *Hin*dIII sites in pGEM-7Zf(+), which do not bind ER (27), was prepared and fill-in labeled with [^{32}P]α-dATP (800 Ci/mmol) (NEN Life Science Products) using Klenow large fragment DNA polymerase I (New England Biolabs, Inc., Beverly, MA) (20). Unincorporated nucleotides were removed by centrifugation through a Centri-Spin 20 column (Princeton Separations, Adelphia, NJ). Labeled EREc38 (50,000 cpm) was incubated for 2 h at 4 C with a nuclear extract of baculovirus-expressed rhERα or rrERβ. Binding reactions were performed in TDPEKG buffer (40 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.5 mM PMSF, 1 mM EDTA, 111 mM KCl, 10% vol/vol glycerol) and included 5 μg poly(deoxyinosine-deoxycytidine) (Midland Certified Reagent Co., Midland, TX), 10 μg purified BSA (New England Biolabs, Inc.)/reaction, in a total reaction volume of 30 μl, with a final salt concentration of 92 mM KCl. An ERα-specific antibody H222, generously provided by Abbott Laboratories (Abbott Park, IL), was diluted 1:10 in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and 1 μl of the diluted H222 was added to selected samples in each experiment to confirm the identity of ERα protein in the shifted ER-ERE complexes. ERβ-specific antibodies PA1–310 (Affinity BioReagents, Inc., Golden, CO) and Y-19 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used undiluted in select samples to confirm the identity of ERβ protein in shifted ER-ERE complexes. After incubation, the protein-DNA mixture was loaded onto 4% nondenaturing polyacrylamide gels and electrophoresed as described (20). Gels were dried under vacuum and autoradiographed on Kodak X-Omat film with an intensifying screen (Lightning Plus from DuPont Co., Wilmington, DE). The ratio of ER-bound to free DNA was determined using a Packard Instruments InstantImager and associated software, Packard Imager for Windows v 2.04 (Packard Instrument Co., Meriden, CT).



***trans*-Resveratrol**

FIG. 1. The chemical structure of *trans*-resveratrol.

Transient transfection experiments

CHO-K1 cells were plated in 12-well plates at 2×10^5 cells/well with IMDM (-phenol red) supplemented with 10% charcoal-stripped CS. The cells were transfected with 0.6 μg reporter construct containing the ERE, 0.1 μg pCMV β-gal, 10 ng pCMV-ERα or pCMV-ERβ, and 0.49 μg pGEM-7Zf(+) (Promega Corp., Madison, WI) when 80% confluent. The transient transfection was performed using Transfast (Promega Corp.) according to directions supplied by Promega Corp. Cells were treated, in triplicate, 24 h later with resveratrol, E_2 (Sigma), or 4-hydroxytamoxifen (4-OHT) (Research Biochemicals International, Natick, MA) diluted in phenol-red-free IMDM (−). The cells were harvested 30 h later, and the luciferase and β-galactosidase (β-gal) activities were assayed (20). All data for transient transfections were normalized by β-gal to account for transfection efficiency. Statistical analyses were performed using Student's *t* test in Microsoft Corp. Excel '97.

Cell proliferation

Cell proliferation was determined using the Cell Proliferation Kit 1 (MTT) according to the directions provided by the supplier (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, 2×10^4 CHO-K1 cells/well were plated in 96-well plates and treated as described above for the transient transfection experiments. Mock transfected cells were incubated with Transfast without added plasmid DNA and then treated with hormones or vehicle as described above. Cell proliferation experiments were conducted concomitantly with transient transfection experiments. Cells were transfected with 60 ng 1EREc38, 10 ng β-gal, 1 ng rhERα or rrERβ, and 49 ng of pGEM-7Zf(+). Cells cotransfected with ERα or ERβ and treated with 0.1 nM E_2, 50 μM resveratrol, 0.1 nM E_2 and 50 μM resveratrol, 100 μM resveratrol, 1 μM 4-OHT, 1 μM 4-OHT and 1 nM E_2, 1 μM 4-OHT and 50 μM resveratrol, and EtOH for control. At the time that the transfection was harvested, the MTT assay was performed according to manufacturer's instructions. The absorbance of solubilized crystals was measured at 595 nm in a Molecular Devices SpectraMAX250 plate reader. The means from three separate experiments were analyzed using Student's *t* test for two samples assuming unequal variances in GraphPadPrism.

Results

Resveratrol binds ERα and ERβ with comparable affinity

Resveratrol was shown to bind ER in MCF-7 (10) and rat uterine cytosol extracts (14). However, the contribution of ERα *vs.* ERβ for this binding is unknown. Competition binding experiments were used to determine the relative binding affinity of resveratrol for ERα and ERβ. Resveratrol binds to rhERα (Fig. 2A) and rrERβ (Fig. 2B). The relative binding affinities (RBA) of resveratrol for ERα and ERβ are not statistically different (Table 1). The equilibrium dissociation constants (K_I) are different, reflecting higher affinity of E_2 binding to ERα than ERβ (Table 1). However, since the 95% confidence intervals for K_I overlap, the difference in K_I for resveratrol between ERβ and ERα is not statistically significant. This is the first demonstration of resveratrol interaction with ERβ.

The IC_{50} values obtained from these experiments indicate that resveratrol binds both ERα and ERβ with lower affinity than E_2 (Table 1). The estimated IC_{50} values for resveratrol of 58.5 μM and 130 μM for ERα and ERβ, respectively, are in agreement with the value of 100 μM reported for ER in MCF-7 cell extract (14). Together, these data indicate that resveratrol binds ERα and ERβ with comparable binding affinity.

Resveratrol has no effect on CHO-K1 cell proliferation unless cells are cotransfected with ERα or ERβ

Resveratrol has been reported to inhibit the proliferation of ER positive and negative cultured human breast cancer cells (10, 28). To determine the effect of resveratrol on CHO-K1 cell proliferation in a receptor-isoform-dependent assay, cells were transfected with expression vectors for either ERα or ERβ. The proliferation of untransfected, "mock-transfected," and ERα or ERβ transfected CHO-K1 cells treated with EtOH, E_2, or resveratrol was determined using the MTT assay (Fig. 3 and data not shown). The mock transfected cells showed no alteration in proliferation regardless of cell treatment, indicating that TransFast is not toxic to the cells (data not shown). Cells transfected with ERα or ERβ showed no alteration in cell proliferation with or without treatment with E_2 or 4-OHT (Fig. 3 and data not shown). However, cells transfected with ERα or ERβ and treated with 100 μM resveratrol showed decreased proliferation. Untransfected or mock-transfected cells showed no decrease in proliferation with 100 μM resveratrol treatment, indicating the effect is dependent upon ER expression (Fig. 3 and data not

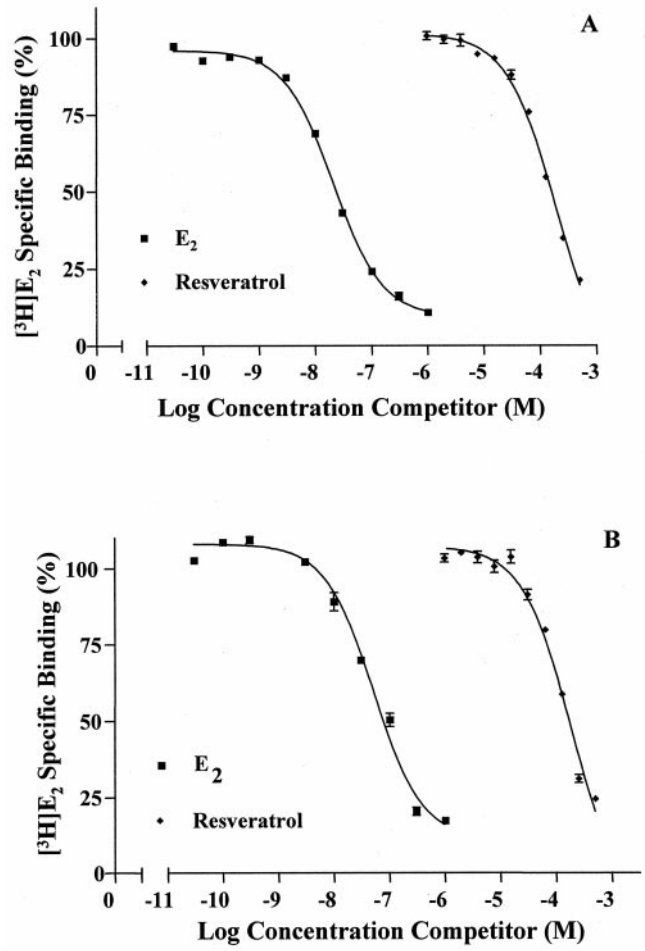


FIG. 2. Resveratrol competes with [^{3}H]E2 for binding rhERα and rrERβ. Competition binding experiments were performed with baculovirus expressed rhERα (A) and rrERβ (B) as described in *Materials and Methods*. Increasing concentrations of either E_2 (*closed squares*) or resveratrol (*closed diamonds*) were mixed with 15.5 nM [^{3}H]E_2, and incubated in triplicate with 6 nM monomer rhERα or rrERβ. [^{3}H]E_2-ER binding was determined by HAP assay (25). Data were graphed as percent of saturation of the specific [^{3}H]E_2 binding capacity *vs.* competitor concentration. These data indicate that resveratrol binds ERα and ERβ with an affinity approximately 117,000- and 108,000-fold lower than E_2, respectively.

TABLE 1. Resveratrol binds ERα and ERβ

Receptor	Log_{10} (IC_{50})	95% C.L.[a] RBA ($\times 10^{-5}$)[b]	95% C.L.[b] K_I (μM)	r^2
rhERα	−3.720 ± 0.055	6.11–11.20	4.33–7.90[c]	0.999
rrERβ	−3.735 ± 0.111	4.70–15.66	7.12–23.73[d]	0.995

ERα and ERβ ligand binding competition experiments were performed and analyzed as described in *Materials and Methods*. The values are the mean from two experiments in which individual samples were performed in triplicate. The values for the 95% confidence interval limits (C.L.) are given. The correlation coefficient (r^2) is indicated.

[a] SE cannot be calculated because of antilogarithm transformation of the confidence intervals for log_{10} (IC_{50}).

[b] Assuming the RBA = 1.0 for E_2.

[c] Assuming K_D = 0.5 nM for interaction between E_2 and rhERα.

[d] Assuming K_D = 1.2 nM for interaction between E_2 and rrERβ.

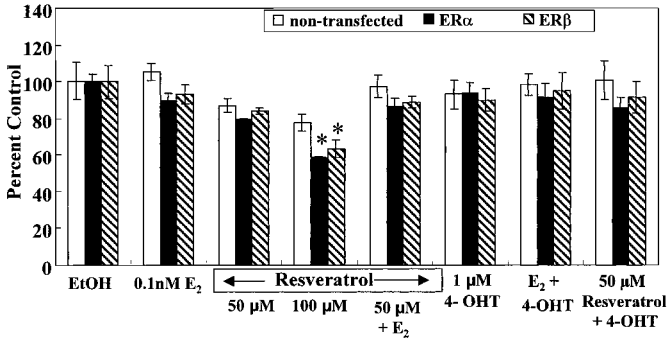


FIG. 3. Effect of E_2 or resveratrol on the proliferation of ERα or ERβ-expressing CHO-K1 cells. The effect of the indicated concentrations of E_2 or resveratrol on the proliferation of CHO-K1 cells transfected with ERα or ERβ *vs.* nontransfected (treatment only) cells was measured by the tetrazolium dye (MTT) assay as described in *Materials and Methods*. Data are presented as the mean ± SEM of three different experiments in which each treatment was performed in triplicate. *Asterisks* indicate values that are statistically different ($P < 0.001$) from control (EtOH) values.

shown). Similarly, cells transfected with pCMV-β-gal and treated 100 μM resveratrol show no decrease in cell proliferation. Cells treated with E_2 showed no decrease in viability. These data indicate that 100 μM resveratrol decreases cell proliferation only when the CHO-K1 cells are transfected with ERα or ERβ.

Resveratrol-liganded ER binds EREs

To determine if resveratrol affects the interaction of ERα or ERβ with an ERE, ERα or ERβ were incubated with E_2, 4-OHT, or resveratrol and ERE binding was measured by EMSA (Fig. 4). ERα bound specifically to EREc38, as indicated by the supershift of the entire bound complex with ERα-specific antibody H222 (Fig. 4A). The multiple ERα bands are due to truncated ERα present in the NE. As anticipated from previous reports (29–31), preincubation of ERα with E_2 accelerated the migration of the ERα-EREc38 complex, while addition of 4-OHT slowed the migration of the 4-OHT-ERα-EREc38 complex (compare Fig. 4, lanes 2, 3, and 4). Resveratrol had no apparent effect on the migration of the ERα-EREc38 complex. These data indicate differences in ERα conformation in the presence of these ligands. Likewise, resveratrol did not affect the binding of H222 to ERα-EREc38, generating a supershifted complex similar to that of the E_2-ERα-EREc38 complexed with H222 (Fig. 4, lanes 6 and 1).

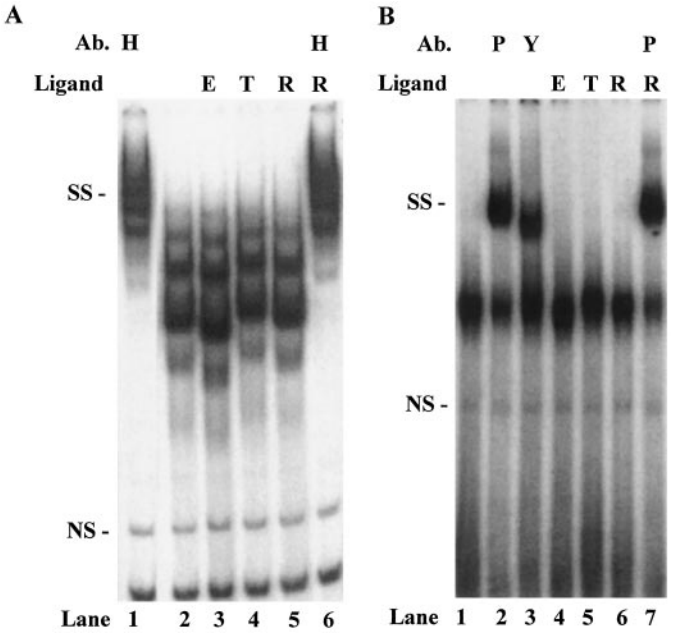


FIG. 4. ERα and ERβ bind an ERE in the presence of resveratrol. A, A nuclear extract of rh ERα (6.67 nM dimer, final) was incubated with 30 μM E_2 (E), 4-OHT (T), or resveratrol (R). The reactions in lanes 1 and 6 included H222 (H) antibody to ERα. B. A nuclear extract of rh ERβ (1.9 nM dimer, final) was incubated with 16.7 μM E_2 (E), 4-OHT (T), or resveratrol (R). The reactions in lanes 2 and 7 included 1 μl of PA1–310 and in lane 3, 1 μl of Y-19 anti-ERβ antibodies. For the reaction in both gels, 50,000 cpm (3.5 nM) of [^{32}P]EREc38 was added. Incubation and EMSA reaction conditions are described in *Materials and Methods*.

Similar results were detected for ERβ. Preincubation of ERβ with E_2 accelerated the migration of the ERβ-EREc38 complex, while addition of 4-OHT slowed the migration of the 4-OHT-ERβ-EREc38 complex (compare Fig. 4B, lanes 1, 4, and 5). Resveratrol had no apparent effect on the migration of the ERβ-EREc38 complex (compare Fig. 4B, lanes 1 and 6). These data indicate differences in ERβ conformation in the presence of these ligands. Resveratrol did not affect the interaction of antibody PA1–310 with the resveratrol-ERβ-EREc38 complex (Fig. 4B, lane 7).

While the concentration of resveratrol added was expected to fully occupy ERα or ERβ, based on the data shown in Fig. 2, we cannot exclude the possibility that the receptor was not fully saturated with resveratrol in this experiment. Because resveratrol was recently reported to inhibit the binding of the arylhydrocarbon receptor (AHR)/AHR-nuclear translocator (ARNT) heterodimer to a xenobiotic response element in EMSA (32), we examined the effect of increasing concentrations of resveratrol and E_2 on ER-ERE binding *in vitro* using EMSA (Fig. 5 and data not shown). Quantitation of the amount of ERα-EREc38 or ERβ-EREc38 complexes shows that resveratrol inhibits ER-ERE binding at concentrations > 10 μM, but that identical concentrations of E_2 resulted in less inhibition of ER-EREc38 binding, until the final concentra-

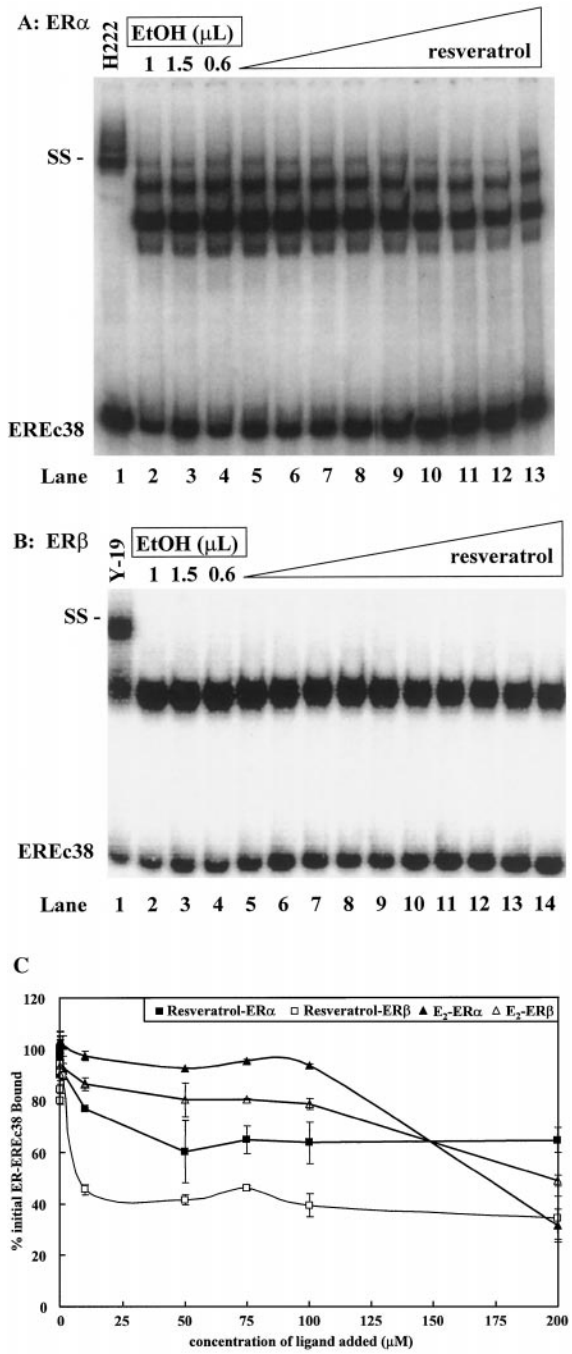


FIG. 5. Resveratrol inhibits ERα and ERβ-EREc38 binding *in vitro*. Nuclear extracts of rh ERα (A) and rr ERβ (B) (147 fmol) were incubated with the indicated volumes or concentrations of ethanol (EtOH), as vehicle control, or resveratrol (range 100 pM–200 μM final). ERα antibody H222 and ERβ antibody Y-19 were added to lane 1 in A and B, respectively. EMSA was performed as described in Fig. 4 and *Materials and Methods*. C, The total amount of ER-EREc38 complex (all retarded ERα-ERE bands in A) formed with increasing concentrations of resveratrol or E_2 was quantitated. There was no significant difference in the amount of either ERα or ERβ bound to EREc38 when incubated with 0.6–1.5 μl of EtOH. Thus, the amount of ER-EREc38 complex formed in the presence of EtOH was set to 100% and the amounts of ER-EREc38 complexes formed with increasing concentrations of resveratrol or E_2 was calculated relative to that amount. The data are the average ± SEM of 2–4 independent EMSAs. The fills and symbols are indicated at the top of panel C.

tion reached 200 μM (Fig. 5C). This inhibition of ER-ERE binding by resveratrol can also be observed by noting the increased amount of free EREc38 at the bottom of the gel (Fig. 5, A and B). Resveratrol had a more pronounced inhibitory effect on ERβ-EREc38 binding compared with ERα-EREc38 binding. Like resveratrol, E_2 had more of an inhibitory effect on ERβ-EREc38 binding compared with ERα-EREc38 binding. This observation indicates that ERβ-EREc38 binding is more labile than ERα-EREc38 binding.

Resveratrol induces reporter gene activity with ERα from a consensus ERE

We used cotransfection assays in CHO-K1 cells to compare the transcriptional activities and ligand responsiveness of ERα and ERβ. This cell line was selected because it requires exogenous ERα or ERβ to activate ERE-driven reporter gene expression and thus allows evaluation of the transcriptional response of each ER isoform in isolation with each ERE. First, we examined the effect of resveratrol on reporter gene activity driven by one copy of a consensus ERE called EREc38 (sequence in *Materials and Methods*) with ERα. Resveratrol stimulated luciferase activity in a concentration-dependent manner up to 50 μM, but 100 μM resveratrol inhibited luciferase activity (Fig. 6A). Because results from the MTT assay indicate that 100 μM resveratrol decreased cell proliferation in ERα-expressing cells (Fig. 3), this is likely the explanation for the decreased reporter activity at this resveratrol concentration. E_2, at concentrations of 1 or 10 nM, has higher agonist activity with ERα than 50 μM resveratrol. Resveratrol at concentrations of 1, 10, or 50 μM had no effect on basal luciferase activity from the pGL3-pro-luciferase parental vector (data not shown). Addition of 100 nM 4-OHT blocked resveratrol activity with ERα (Fig. 6A and data not shown). While the fold induction of E_2-stimulated luciferase detected is low, we note that others have reported that single EREs show significantly lower levels of transcriptional activation in response to E_2 compared with commonly used multiple tandem EREs (24, 33, 34). These results indicate that the estrogen agonist activity of resveratrol is mediated by ERα-ERE interaction in transiently transfected CHO-K1 cells.

Resveratrol induces reporter gene activity with ERα from natural, imperfect EREs

Most estrogen-responsive genes contain one or more imperfect EREs and/or multiple ERE half-sites (35). Genes containing imperfect EREs, *i.e.* EREs that differ in one or more nucleotides in the inverted repeat of the consensus ERE, usually exhibit lower responsiveness to E_2 in reporter gene assays in transfected cells, compared with a consensus ERE (24, 33). Furthermore, ERα binds these EREs with reduced affinity compared with binding to EREc38 (Refs. 36, 37) and data not shown). Therefore, to determine whether resveratrol activates ERα-mediated reporter gene activity from imperfect EREs, we performed transient transfection assays using luciferase reporter plasmids containing the imperfect EREs and their native flanking regions from the human c-*fos*, pS2, and PR genes (Fig. 6B, sequences in *Materials and Methods*).

E_2 did not induce as much luciferase activity from the natural, imperfect EREs from the human pS2 and PR genes

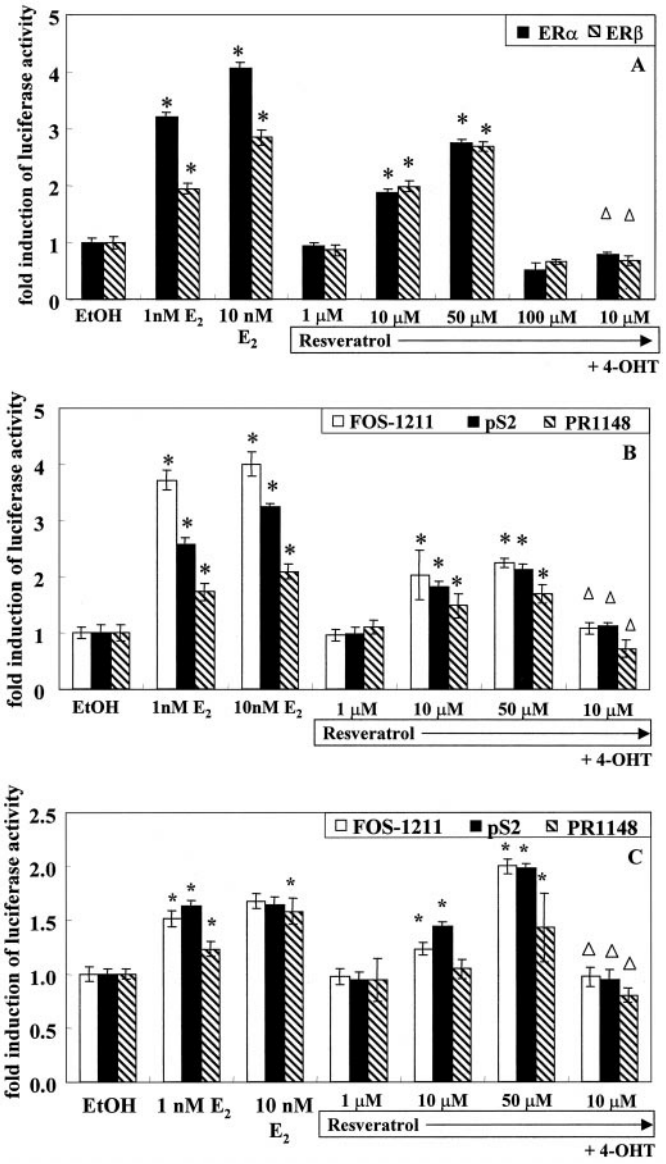


FIG. 6. Resveratrol acts as an ERα and ERβ agonist in transient transfection assay. A, CHO-K1 cells were cotransfected with pGL3–1(EREc38) luciferase, pCMV-βgal, and either pCMV-ERα or pCMV-ERβ, indicated as different *filled boxes*, and treated with EtOH, as the vehicle control, or the indicated concentrations of E_2, resveratrol, or resveratrol and 4-OHT. B, CHO-K1 cells were cotransfected with pGL3-luciferase reporter plasmid bearing the EREs from the human c-*fos* (FOS-1211, *open bars*), pS2 (*closed bars*), and PR (PR1148, *hatched bars*) genes, pCMV-βgal, and pCMV-ERα and treated with EtOH, E_2, or resveratrol at the indicated concentration. C, CHO-K1 cells were cotransfected with pGL3-luciferase reporter plasmid bearing the EREs from the human c-*fos* (FOS-1211), pS2, and PR (PR1148) genes, pCMV-βgal, and pCMV-ERβ and treated with EtOH, E_2, resveratrol or resveratrol and 4-OHT at the indicated concentrations. The cells were transiently transfected and treated as described in *Materials and Methods*. Cells were harvested 30 h after starting treatment, and the cell extracts were assayed for luciferase and β-gal activities. In each panel, the fold induction of luciferase activity was normalized for β-gal and is expressed as the ratio of RLU between treatment groups and the ethanol control (which was set to 1). Data are the mean ± SEM from three to five different experiments in which each treatment was performed in triplicate within the experiment. *Asterisks* and *open triangles* indicate values that are significantly different ($P < 0.05$) from the control and 10 μM resveratrol values, respectively.

as from the consensus EREc38 (compare Fig. 6, A and B). However, the luciferase activity induced by E_2 from the imperfect ERE from the human c-*fos* gene was similar than that induced from EREc38. While there was a significant increase in luciferase activity from pS2 when cells were treated with 10 nM E_2, no significant dose-response relationship was detected for induction from Fos-1211 or PR1148. As seen for EREc38, resveratrol induced significantly lower luciferase expression from Fos-1211 and pS2 compared with E_2. The luciferase activity induced by 50 μM resveratrol from PR1148 was comparable to that induced by 1 nM E_2.

The induction of luciferase activity from the natural EREs by resveratrol was blocked by cotreatment with 4-OHT, indicating that ERα is responsible for resveratrol-induced reporter activity. As seen for EREc38, treatment of the CHO-K1 cells with 100 μM resveratrol inhibited luciferase activity from the pS2, FOS-1211, and PR1148 EREs (data not shown). In conclusion, the data from these transient transfection assays indicate that resveratrol acts as an estrogen agonist with ERα. These results are similar to those detected in transiently transfected, ERα-expressing COS-1 cells with either a vitellogenin ERE or LH-β promoter-luciferase reporter plasmid (14).

Resveratrol induces reporter gene activity with ERβ from a consensus ERE

ERβ has been shown, in transient transfection assays using a single or multiple tandem copies of a consensus ERE, to have lower activity in response to E_2 than ERα (31). However, in COS-1 cells, ERβ induced higher reporter activity from the vitellogenin ERE than ERα in response to concentrations of E_2 ranging from 0.01–1 μM (14). Here, we observed that cotransfection of CHO-K1 cells with ERβ and EREc38 generated lower luciferase expression in response to E_2 compared with ERα (Fig. 6A). Comparable protein expression levels of ERα and ERβ were achieved in these cells (Western blot data not shown). ERβ induced 61% of the activity of ERα with 1 nM E_2. These data are consistent with the 59, 52, and 62% of ERβ activity relative to ERα detected using reporters bearing one or 3 tandem consensus EREs in CEF, HeLa, and HepG2 cells, respectively (31, 38).

With ERβ, resveratrol stimulated luciferase expression from EREc38 in a concentration-dependent manner up to 50 μM, whereas 100 μM resveratrol inhibited luciferase activity. These results are similar to those reported for ERβ with a vitellogenin ERE reporter in transiently transfected COS-1 cells (14). In CHO-K1 cells, resveratrol-stimulated activity was inhibited by cotreatment with 4-OHT (Fig. 6A), which also blocked E_2-induced activity from ERβ (data not shown), indicating that direct interaction of resveratrol with ERβ is responsible for the induction of luciferase activity from EREc38. Interestingly, in contrast to the differences in luciferase activity induced by E_2 with ERα and ERβ, resveratrol induced nearly identical levels of luciferase activity from EREc38 with either ERα or ERβ (Fig. 6A). This indicates that resveratrol-liganded ERβ has similar transcriptional activity to E_2-ERβ at a single perfect, palindromic ERE. These data differ from those for ERβ expression in COS-1 cells in which 500 μM resveratrol showed higher induction of reporter ac-

tivity from two tandem copies of the vitellogenin ERE than any of the concentrations of E_2 (0.01–1 mM) examined (14). These findings indicate that cell-specific factors influence the agonist activity of resveratrol with ERβ.

Resveratrol induces reporter gene activity with ERβ from natural, imperfect EREs

Next we examined the induction of luciferase activity from the EREs from the human c-*fos*, pS2, and PR genes with ERβ (Fig. 6B). Please note that the scale for fold-induction of luciferase activity for revised Fig. 6C is one half the scale used for Fig. 6, A and B. As seen for ERα, E_2 induced lower activity from each of the imperfect EREs than from EREc38 (compare Fig. 6, A and C). Unlike ERα for which E_2 stimulated more activity from Fos-1211 than the other natural EREs, there was no difference in the luciferase activity induced by ERβ with 10 nM E_2 from the imperfect EREs. These data indicate that ERα and ERβ transactivate reporter gene expression differentially in response to E_2 from natural imperfect EREs in CHO-K1 cells.

Resveratrol stimulated ERβ-driven reporter activity from each natural-occurring ERE in a concentration-dependent manner, although the response with PR1148 was not statistically different between resveratrol concentrations. For both ERα and ERβ, PR1148 was least responsive to resveratrol. As anticipated, the luciferase activity from the three imperfect EREs was lower than that induced from EREc38 (compare Fig. 6, A and C). However, for Fos-1211 and pS2, the luciferase activity induced by 50 μM resveratrol was greater than that stimulated by 1 or 10 nM E_2. As seen with ERα, treatment of the ERβ-transfected CHO-K1 cells with 100 μM resveratrol inhibited luciferase activity from all EREs due to decreased CHO-K1 cell proliferation (Fig. 3 and data not shown). The induction of luciferase activity from each of the natural EREs by resveratrol with ERβ was blocked by cotreatment with 4-OHT, indicating that ERβ is responsible for resveratrol-induced reporter activity.

At 50 μM, resveratrol induced identical levels of reporter activity from EREc38, Fos-1211, and pS2 with ERα and ERβ. However, E_2 induced higher reporter activity from all EREs with ERα than ERβ. For PR1148, ERβ was less active than ERα with resveratrol and E_2. Taken together, these results imply that resveratrol-liganded ERα and ERβ are equivalently transcriptionally active with the Fos-1211 and pS2 EREs. In contrast, E_2-liganded ERβ interacts with EREc38 and the imperfect EREs from the human c-*fos*, pS2, and PR genes less productively than ERα. Finally, these data indicate that the ER agonist activity of resveratrol is not identical for ERα and ERβ and varies with ERE sequence.

Resveratrol antagonizes E_2 activity at select EREs with ERα

Resveratrol was reported to display "superagonist" activity in MCF-7 cells transfected with an ERE-driven luciferase reporter plasmid, *i.e.* greater activity than with 0.1 nM E_2 treatment (9, 10). This phenomenon is not understood because these two reports show different definitions of superagonist activity. In the first report (10), "superagonist" activity was not the equivalent of synergy since the responses of E_2 and resveratrol were additive (10). In the more recent report, the reporter activity detected in MCF-7 cells treated with 0.1 nM E_2 plus 25 μM resveratrol was 2-fold greater than the anticipated additive activity of the two ligands (9). We evaluated how resveratrol impacted E_2-stimulated luciferase activity from EREc38 or the natural EREs from the human c-*fos*, pS2, and PR genes in CHO-K1 cells expressing either ERα (Fig. 7A) or ERβ (Fig. 7B). No additive activity was detected with ERα and 0.1 nM E_2. Interestingly, 50 μM resveratrol had E_2 antagonist activity for EREc38 and PR1148, but not Fos-1211 or pS2. Thus, variations in the ERE sequence appear to influence the agonist/antagonist activity of resveratrol with ERα in E_2-treated CHO-K1 cells. We note that the relatively low fold induction that we observed with single EREs is similar to that reported by other investigators (33, 34).

In contrast to ERα, with ERβ the luciferase activity stimulated by 1 nM E_2 from EREc38 and PR1148 was not antagonized by cotreatment with 50 μM resveratrol. As seen for ERα, resveratrol had no effect on E_2-induced reporter activity from pS2. The results for Fos-1211 were similar for ERα and ERβ: increased activity with E_2 plus resveratrol. In no case

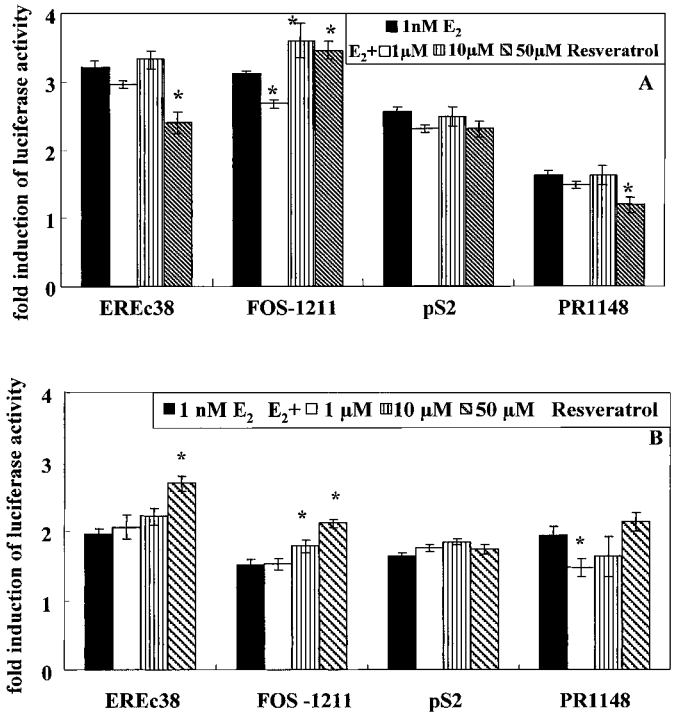


FIG. 7. Resveratrol exhibits little or no antagonist activity with ERα and additive agonist activity with ERβ at various EREs. CHO-K1 cells were cotransfected with pGL3-luciferase reporter plasmid bearing 1(EREc38) or the EREs from the human c-*fos* (FOS-1211), pS2, and PR (PR1148) genes, pCMV-βgal, and pCMV-ERα (A) or pCMV-ERβ (B). In both panels, cells were treated with 0.1 nM E_2 or 0.1 nM E_2, plus the indicated concentrations of resveratrol. The transient transfection, treatment, and assay conditions were as described in *Materials and Methods* and in Fig. 6. The fold induction of luciferase activity was normalized for β-gal and is expressed as the ratio of RLU between treatment groups and the vehicle control (which was set to 1). Data are the mean ± SEM from three different experiments in which each treatment was performed in triplicate within the experiment. In both panels, *asterisks* indicate values that are significantly different ($P < 0.05$) from the values observed with 1 nM E_2 for the cognate ERE.

was the activity of E_2 and resveratrol additive. The activity induced by E_2 plus resveratrol for each ERE with ERβ was blocked by 4-OHT, indicating that it is dependent on ERβ AF-2 (data not shown).

To examine the relative agonist/antagonist activity with a stronger ERE promoter, we evaluated the induction of luciferase activity from two tandem, head-to-tail, copies of EREc38, called 2(EREc38), with 10 nM E_2, resveratrol, or E_2 + resveratrol in CHO-K1 cells transfected with ERα or ERβ (Fig. 8). As seen with the single EREs (Figs. 6 and 7), ERα had higher transcriptional activity than ERβ in response to E_2. Resveratrol showed weak agonist activity for both ERα and ERβ. Similar to the data for a single copy of EREc38, resveratrol suppressed E_2-ERα-stimulated luciferase activity from 2(EREc38) in a concentration-dependent manner. In contrast, resveratrol did not inhibit E_2-ERβ activity. This result indicates that ERα and ERβ respond differently to resveratrol at the same ERE. Resveratrol, when combined with E_2 exhibits antagonist activity with ERα, but no antagonist activity with ERβ.

Discussion

Epidemiological evidence indicates that phytoestrogens have biological activities including inhibition of cancer initiation and growth, reduction of serum cholesterol levels, and benefits in treating osteoporosis (39, 40). Resveratrol is a bioflavonoid that occurs naturally in grapes and is especially prevalent in red wine that contains 1–10 mg/liter (5). This gives a concentration of *trans*-resveratrol of 0.1–10 μM and cis-resveratrol of 0.5–10 μM. Given the daily recommended dose of wine is 250 ml (41), it is estimated that a person would ingest 0.025–2.5 μmol (6 μg-0.6 mg) of *trans*-resveratrol daily (9). Based on pharmacokinetic studies performed in rats (7), these amounts can provide serum concentrations that correspond to the concentrations at which the estrogenic/antiestrogenic effects of resveratrol were observed here.

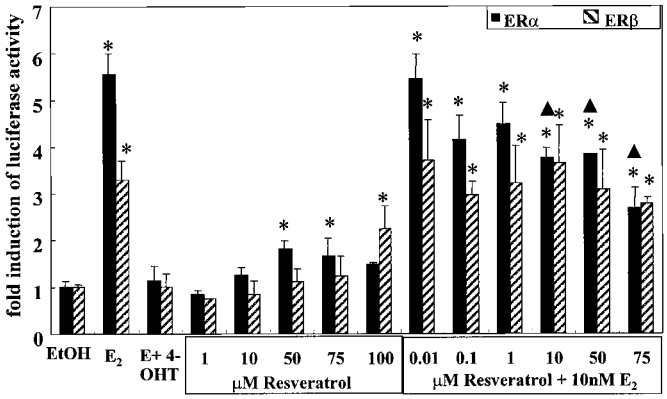


FIG. 8. Combined E_2 and resveratrol have different effects on ERα and ERβ. CHO-K1 cells were cotransfected with pGL3-luciferase reporter plasmid bearing 2(EREc38), pCMV-βgal, and pCMV-ERα (*black bars*) or pCMV-ERβ (*hatched bars*). The cells were treated with 10 nM E_2, 10 nM E_2 + 100 nM 4-OHT, the indicated concentrations of resveratrol (μM), or 10 nM E_2 plus resveratrol at the indicated concentrations. The transient transfection, treatment, and assay conditions were as described in *Materials and Methods* and in Fig. 6. The fold induction of luciferase activity was normalized for β-gal and is expressed as the ratio of RLU between treatment groups and the vehicle control (which was set to 1). Data are the mean ± SEM from three to five different experiments in which each treatment was performed in triplicate within the experiment. The *asterisks* and the *closed triangles* indicate values that are significantly different from the EtOH control and stimulated by E_2 alone for ERα and ERβ, respectively ($P < 0.05$).

Resveratrol has chemopreventive and chemotherapeutic activities (1, 2) and has been classified as a phytoestrogen because it binds to ERα with low affinity (10). Here we evaluated the estrogen agonist/antagonist activity of resveratrol with ERα and ERβ *in vitro* and in transiently transfected cells. Our results show that resveratrol binds ERα and ERβ with comparable affinity, but with much lower affinity than E_2. This finding contrasts with data showing that several phytoestrogens bind ERβ with higher affinity than ERα (42). One possible explanation for the lower affinity of resveratrol binding to ERβ compared with other phytoestrogens is its structural similarity with diethylstilbestrol (DES) (10), which binds ERβ with lower affinity than ERα (43).

Whether resveratrol-occupied ERα has agonist or antagonist activity has been controversial (10, 14, 18). Resveratrol was first reported to be a relatively weak ER ligand but showed "superagonist" activity, *i.e.* higher reporter activity than E_2, in ERα-expressing MCF-7 cells (9, 10). However, another report found no evidence of superagonism in COS cells with either ERα or ERβ (14). Data from our transient transfection assays in CHO-K1 cells using a consensus ERE or the natural imperfect EREs from the human c-*fos*, pS2, or PR genes indicate that resveratrol acts as an estrogen agonist with ERα and ERβ. However, resveratrol does not display superagonist activity either alone or in combination with E_2. These results are similar to those detected in COS-1 cells transfected with ERα and either a vitellogenin ERE or LH-β promoter-luciferase reporter plasmid (14).

Our results demonstrate that the agonist activity of resveratrol with ERβ is fundamentally different from E_2 agonist activity because, in contrast to E_2 which induces higher activity of ERα than ERβ, resveratrol activated equal reporter activity from EREc38 with both ERα and ERβ. In contrast, 500 μM resveratrol was reported to induce higher ERE-driven reporter activity by ERβ than any concentration of E_2 (0.01–1 mM) examined in COS-1 cells (14). Because both we and Ashby *et al.* (14) used human ERα and rat ERβ expression vectors and pGL3-luciferase vectors in our experiments, we conclude that the difference between our findings and those reported by Ashy *et al.* (14) is likely due to differences in the expression of coregulators between CHO-K1 and COS-1 cells. Importantly, our data indicate that resveratrol-liganded ERβ has higher transcriptional activity than E_2- liganded ERβ at a single palindromic ERE. This indicates that those tissues that uniquely express ERβ or that express more ERβ than ERα may be more sensitive to resveratrol's estrogen agonist activity.

Biochemical (29, 30, 44, 45) and crystal structure (46, 47) studies indicate ligand-specific differences in ERα conformation that impact interaction with coactivators. We observed clear differences in the migration of ERE-bound ERα and ERβ either unoccupied or occupied by E_2, 4-OHT, or resveratrol. These data indicate differences in ER conformation in the presence of resveratrol compared with E_2 or

4-OHT. The concentrations at which resveratrol inhibited ERE binding by ERα and ERβ *in vitro* are concentrations at which resveratrol exhibited agonist activity in transiently transfected cells. Further experiments, *e.g. in vivo* DNase I footprinting, are needed to determine *in vivo* effects of resveratrol on ER-ERE binding. The crystal structure of the ERβ LBD occupied by the phytoestrogen genistein showed that helix 12 did not adopt the distinctive "agonist" position seen with E_2 binding to the ERα LBD, but lay in an orientation similar to that observed when the ERβ LBD was occupied by the select estrogen receptor modulator (SERM) raloxifene (48). The authors concluded that this positioning of the transactivational helix 12 was consistent with the partial agonist activity of genistein (48). Given the pharmacological activities of resveratrol observed here, we predict that resveratrol-bound ERβ LBD may show a structure similar to that of the genistein-occupied ERβ. Because alterations in LBD conformation impact the interaction of ERα and ERβ with coactivators, further experiments are needed to assess coactivator effects on resveratrol-liganded ERβ activity.

While resveratrol reportedly gave a dose-dependent increase in reporter activity with concentrations as high as 500 μM with both ERα and ERβ in transiently transfected COS cells (14), we observed that 100 μM resveratrol inhibited ERE-driven reporter activity in CHO-K1 cells. Because the limit of resveratrol solubility is 250 μM in 5% EtOH, we are uncertain as to the soluble concentration of resveratrol in the COS cell assays (14). Moreover, we observed decreases cell proliferation in CHO-K1 cells expressing ERα or ERβ and treated with 100 μM resveratrol. There was no significant decrease in CHO-K1 cell proliferation in cells treated with 100 μM resveratrol and expressing β-galactosidase or in mock-transfected cells under the same conditions. While others have reported that E_2 treatment of cells stably overexpressing ERα inhibits cell proliferation (49–53), E_2 at concentrations that stimulate reporter activity in transiently transfected CHO-K1 cells had no effect on cell proliferation in the presence or absence of transfected ERα or ERβ. Therefore, stable expression of ERα in ERα negative cells appears to be required for E_2-induced inhibition of cell proliferation in response to E_2, but not resveratrol. We conclude that expression of ERα or ERβ is involved in the decrease in CHO-K1 cell proliferation with 100 μM resveratrol. Others reported that 100 μM resveratrol inhibited the growth of ERα-expressing MCF-7 cells (18). However, the mechanism for inhibition of MCF-7 cell proliferation may not be ERα-mediated since resveratrol also inhibited the growth of ER-negative breast cancer cells (28).

In addition to its agonist activity, resveratrol exhibited antiestrogenic activity in MCF-7 cells (18). Resveratrol decreased the levels of transcription of PR, insulin-like growth factor-receptor, and transforming growth factor-α genes and stimulated the expression of transforming growth factor-β2, results similar to those elicited by tamoxifen in these cells (18). While the authors concluded that the most likely mechanism for the antiestrogenic effect of resveratrol is its direct competition with E_2 for ER binding, they also suggested that resveratrol might prevent ER binding to EREs (18). Our data support both suggestions because we observed that resveratrol competes with E_2 for ERα and ERβ binding and inhibited ERα and ERβ binding to EREc38 *in vitro*. The concentrations at which resveratrol inhibited ER-ERE binding *in vitro* are those at which resveratrol exhibits agonist/antagonist activity in CHO-K1 cells.

We observed both ER isoform-specific and ERE-specific differences in the agonist activity induced by resveratrol in CHO-K1 cells. For example with ERα, the activity induced by E_2 was greater than that stimulated by any concentration of resveratrol for EREc38, Fos-1211, and pS2, but not PR1148. In contrast, resveratrol and E_2 were equally transcriptionally active with ERβ at all EREs tested. With ERα and PR1148, although the induction levels are low, they are significantly above the ethanol control values, and resveratrol-induced luciferase activity was comparable to that induced by E_2. This result implies that, when bound to the PR1148 ERE, resveratrol-liganded and E_2-liganded ERα and ERβ are equally effective at recruiting components of the coactivator/RNA polymerase II preinitiation complex.

In addition to estrogen agonist activity, we also report that resveratrol has estrogen antagonist activity in CHO-K1 cells. However, resveratrol's antagonist activity was only observed with ERα and not ERβ. These data are reminiscent of the lack of 4-OHT agonist activity with ERβ (54). We speculate that the antagonist activity of resveratrol may be mediated by AF-1, which appears to be absent in ERβ. Interestingly, the antagonist activity of resveratrol was only observed with EREc38, whether as a single or two tandem copies, and PR1148. These data indicate that the ERE alters the pharmacological properties of resveratrol mediated by ERα. This result agrees with our postulate that the ERE sequence acts as an allosteric modulator of ER activity (29). Further experiments are needed to define exactly what regions of ERα and ERβ are necessary for resveratrol agonist and antagonist activity and to define the exact ERE sequence requirements for resveratrol antagonist activity with ERα.

In addition to its direct ER binding, resveratrol has many non-ER-mediated cellular activities that may influence ER transcriptional activation through "cross-talk" mechanisms. For example, resveratrol induces phosphorylation of the mitogen-activated protein (MAP) kinase family members, extracellular regulated kinase 1 (ERK1), and ERK2, in neuroblastoma SH-SY5Y cells (55) Activation of the MAP kinase pathway has been shown to activate unliganded ERα through phosphorylation of serine 118 in AF-1 (56). However, this ligand-independent pathway does not appear to be important in our experiments because 4-OHT blocked both E_2 and resveratrol activity, indicating that direct activation of ERα through the LBD was responsible for the activity reported here. Moreover, because the transcriptional effects of resveratrol vary with alterations of the ERE sequence, resveratrol's activity appears to be mediated by direct interaction of the resveratrol-occupied ER with EREs.

While the pharmacokinetics of resveratrol metabolism have not yet been examined in humans, results from rodent studies indicate that two servings of red wine may provide two-digit micromolar serum concentrations of resveratrol (1), *i.e.* concentrations identical to those at which the pharmacological activities of resveratrol were observed here as well as reported by others (10, 14, 18). The cell-type and ERE-sequence dependence of the transcriptional activity of

resveratrol with ERα and ERβ may be related to cell-specific differences in the activity of enzymes that modulate ER function, *e.g.* protein kinases, and in the expression of coactivator or corepressor proteins. Continued analysis of ERα and ERβ interaction with estrogenic ligands, estrogen-regulated genes, and coregulator proteins is necessary to gain a better understanding of how these receptors regulate estrogenic activity at the cellular and molecular level and whether the anticancer and cardioprotective activities of resveratrol are mediated by ER.

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