

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 (ER α and ER β). 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 ER α vs. ER β in this binding is unknown. Here we report that resveratrol binds ER β and ER α with comparable affinity, but with 7,000-fold lower affinity than estradiol (E₂). Thus, resveratrol differs from other phytoestrogens that bind ER β with higher affinity than ER α . Resveratrol acts as an estrogen agonist and stimulates ERE-driven reporter gene activity in

CHO-K1 cells expressing either ER α or ER β . The estrogen agonist activity of resveratrol depends on the ERE sequence and the type of ER. Resveratrol-liganded ER β has higher transcriptional activity than E₂-liganded ER β at a single palindromic ERE. This indicates that those tissues that uniquely express ER β or that express higher levels of ER β than ER α 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 E₂. We report that resveratrol exhibits E₂ antagonist activity for ER α with select EREs. In contrast, resveratrol shows no E₂ antagonist activity with ER β . These data indicate that resveratrol differentially affects the transcriptional activity of ER α and ER β 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, ER α 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, ER α and the more recently identified ER β (8). Because ER α and ER β 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 E₂ with comparable affinity, some phytoestrogens, *e.g.* genistein and coumestrol, show higher affinity for ER β than ER α (5), suggesting that resveratrol may show selectivity for ER β . Resveratrol was shown to compete with [¹²⁵I]E₂ for binding ER in an extract from MCF-7 human breast cancer cells with an IC₅₀ value of ~10 μ M (10). More recently, resveratrol was reported to compete with [³H]E₂ for binding to rat uterine ER with an IC₅₀ value of ~100 μ M (14). Because MCF-7 cells and uterus reportedly express ER β as well as ER α (15, 16), it is important to determine the affinity of resveratrol interaction with ER β and with ER α .

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 hER α transcription assay and in transient transfections with ER α in COS-1 cells. In another study, resveratrol antagonized E₂-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 E₂ 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 ER α and ER β . We examined whether resveratrol preferentially binds ER α or ER β , the effect of resveratrol on the proliferation of cells expressing ER α or ER β , 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 ER α and ER β with comparable affinity, but with much lower affinity than E₂. Resveratrol-occupied ER α and ER β 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 ER α , but not ER β , resveratrol shows E₂ antagonist activity from certain EREs, including a consensus ERE. Thus, the mechanism of action of resveratrol is unique for ER α and ER β .

Materials and Methods

Cell maintenance

Chinese Hamster Ovary cells (CHO-K1) were purchased from ATCC (Manassas, VA) and maintained in Scovell'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'-CCAGGTCAGAGTACCTGAGCTAAAATAACACATT-3';
PR1148: 5'-AGCCCTCCCTCCTGCGAGGTCACCAAGCTCTTGGTGCCCTGTT-3';
pS2: 5'-CTTCCCCTGCAAGGTCAGCGTGGCCACCCCGTGAGCCACT-3'; and
Fos-1211: 5'-AGCTTGGCTGAGCCGGCAGCGTGACCCCGCATG-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) rhER α was generously supplied by Dr. Benita Katzenellenbogen (21). An expression vector containing the sequence for recombinant rat (rr) ER β was generously provided by Dr. J.-A. Gustafsson (22).

Nuclear extract preparation

Baculovirus-expressed rhER α and rrER β 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) rhER α or rrER β were incubated in TEGK100 buffer (40 mM Tris-HCl (pH 7.4), 1

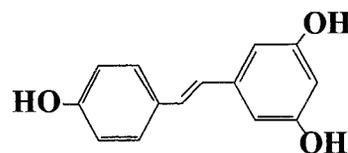
mM EDTA, 10% (vol/vol) glycerol, 100 mM KCl, 0.5 mM PMSF, 2 μ g/ml aprotinin, 0.5 μ g/ml leupeptin, 0.75 μ g/ml pepstatin, 1 mM DTT) with 15.5 nM [³H]E₂ (NEN Life Science Products, Boston, MA) and the indicated concentrations of *trans*-resveratrol (generously provided by Pharma Science, Montréal, Québec, Canada or purchased from Sigma, St. Louis, MO), or E₂ (Sigma). Unless specifically stated, all references to resveratrol in this manuscript indicate *trans*-resveratrol (see Fig. 1). NE isolated from Sf-21 cells expressing ER α or ER β 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 [³H]E₂ 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 [³H]E₂ 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% H₂O 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 μ M, indicating that 250 μ M 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 EcoRI and HindIII sites in pGEM-7Zf(+), which do not bind ER (27), was prepared and fill-in labeled with [³²P] 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 rhER α or rrER β . 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 μ g poly(deoxyinosine-deoxycytidine) (Midland Certified Reagent Co., Midland, TX), 10 μ g purified BSA (New England Biolabs, Inc.)/reaction, in a total reaction volume of 30 μ l, with a final salt concentration of 92 mM KCl. An ER α -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 μ l of the diluted H222 was added to selected samples in each experiment to confirm the identity of ER α protein in the shifted ER-ERE complexes. ER β -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 ER β 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 μ g reporter construct containing the ERE, 0.1 μ g pCMV β -gal, 10 ng pCMV-ER α or pCMV-ER β , and 0.49 μ g 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 rhER α or rrER β , and 49 ng of pGEM-7Zf(+). Cells cotransfected with ER α or ER β and treated with 0.1 nM E $_2$, 50 μ M resveratrol, 0.1 nM E $_2$ and 50 μ M resveratrol, 100 μ M resveratrol, 1 μ M 4-OHT, 1 μ M 4-OHT and 1 nM E $_2$, 1 μ M 4-OHT and 50 μ M 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 ER α and ER β with comparable affinity

Resveratrol was shown to bind ER in MCF-7 (10) and rat uterine cytosol extracts (14). However, the contribution of ER α vs. ER β for this binding is unknown. Competition binding experiments were used to determine the relative binding affinity of resveratrol for ER α and ER β . Resveratrol binds to rhER α (Fig. 2A) and rrER β (Fig. 2B). The relative binding affinities (RBA) of resveratrol for ER α and ER β are not statistically different (Table 1). The equilibrium dissociation constants (K_i) are different, reflecting higher affinity of E $_2$ binding to ER α than ER β (Table 1). However, since the 95% confidence intervals for K_i overlap, the difference in K_i for resveratrol between ER β and ER α is not statistically significant. This is the first demonstration of resveratrol interaction with ER β .

The IC $_{50}$ values obtained from these experiments indicate that resveratrol binds both ER α and ER β with lower affinity than E $_2$ (Table 1). The estimated IC $_{50}$ values for resveratrol of 58.5 μ M and 130 μ M for ER α and ER β , respectively, are in agreement with the value of 100 μ M reported for ER in MCF-7 cell extract (14). Together, these data indicate that resveratrol binds ER α and ER β with comparable binding affinity.

Resveratrol has no effect on CHO-K1 cell proliferation unless cells are cotransfected with ER α or ER β

Resveratrol has been reported to inhibit the proliferation of ER positive and negative cultured human breast cancer

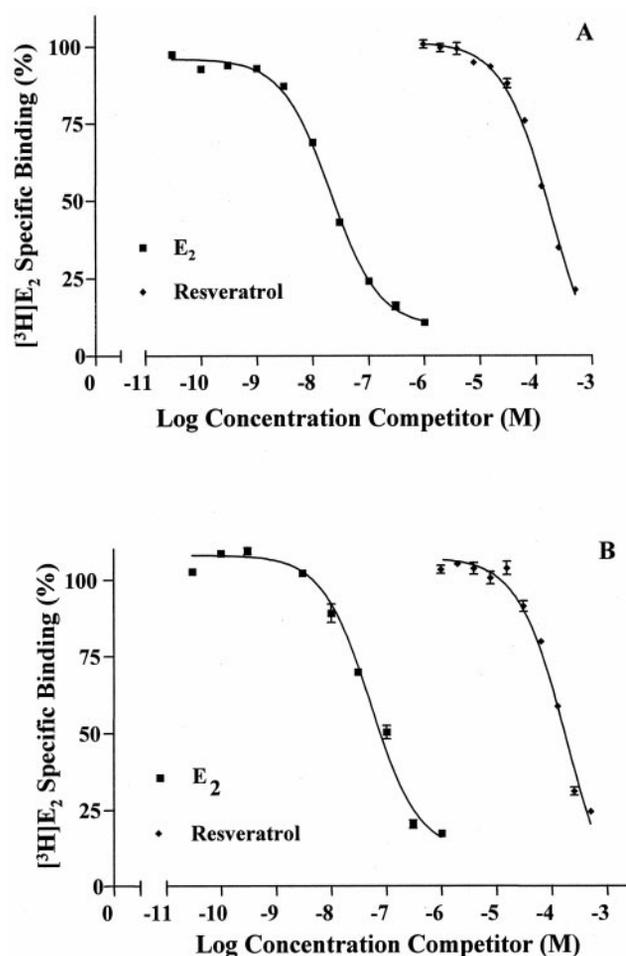


FIG. 2. Resveratrol competes with [3 H]E $_2$ for binding rhER α and rrER β . Competition binding experiments were performed with baculovirus expressed rhER α (A) and rrER β (B) as described in *Materials and Methods*. Increasing concentrations of either E $_2$ (closed squares) or resveratrol (closed diamonds) were mixed with 15.5 nM [3 H]E $_2$, and incubated in triplicate with 6 nM monomer rhER α or rrER β . [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 ER α and ER β with an affinity approximately 117,000- and 108,000-fold lower than E $_2$, respectively.

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 ER α or ER β . The proliferation of untransfected, "mock-transfected," and ER α or ER β 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 ER α or ER β 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 ER α or ER β and treated with 100 μ M resveratrol showed decreased proliferation. Untransfected or mock-transfected cells showed no decrease in proliferation with 100 μ M resveratrol treatment, indicating the effect is dependent upon ER expression (Fig. 3 and data not

TABLE 1. Resveratrol binds ER α and ER β

Receptor	Log ₁₀ (IC ₅₀)	95% C.L. ^a RBA ($\times 10^{-5}$) ^b	95% C.L. ^b K _i (μ M)	r ²
rhER α	-3.720 \pm 0.055	6.11–11.20	4.33–7.90 ^c	0.999
rrER β	-3.735 \pm 0.111	4.70–15.66	7.12–23.73 ^d	0.995

ER α and ER β 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 (r²) is indicated.

^a SE cannot be calculated because of antilogarithm transformation of the confidence intervals for log₁₀ (IC₅₀).

^b Assuming the RBA = 1.0 for E₂.

^c Assuming K_D = 0.5 nM for interaction between E₂ and rhER α .

^d Assuming K_D = 1.2 nM for interaction between E₂ and rrER β .

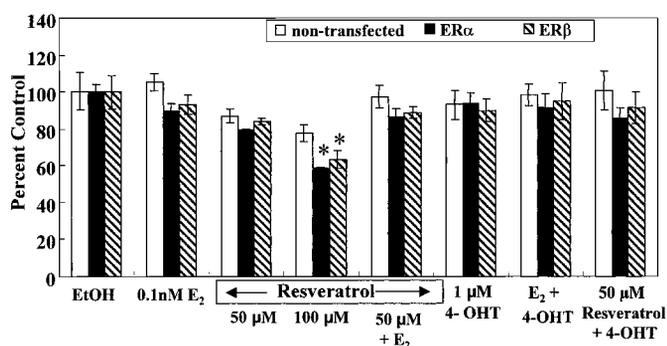


FIG. 3. Effect of E₂ or resveratrol on the proliferation of ER α or ER β -expressing CHO-K1 cells. The effect of the indicated concentrations of E₂ or resveratrol on the proliferation of CHO-K1 cells transfected with ER α or ER β vs. nontransfected (treatment only) cells was measured by the tetrazolium dye (MTT) assay as described in *Materials and Methods*. Data are presented as the mean \pm 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 μ M resveratrol show no decrease in cell proliferation. Cells treated with E₂ showed no decrease in viability. These data indicate that 100 μ M resveratrol decreases cell proliferation only when the CHO-K1 cells are transfected with ER α or ER β .

Resveratrol-liganded ER binds EREs

To determine if resveratrol affects the interaction of ER α or ER β with an ERE, ER α or ER β were incubated with E₂, 4-OHT, or resveratrol and ERE binding was measured by EMSA (Fig. 4). ER α bound specifically to EREc38, as indicated by the supershift of the entire bound complex with ER α -specific antibody H222 (Fig. 4A). The multiple ER α bands are due to truncated ER α present in the NE. As anticipated from previous reports (29–31), preincubation of ER α with E₂ accelerated the migration of the ER α -EREc38 complex, while addition of 4-OHT slowed the migration of the 4-OHT-ER α -EREc38 complex (compare Fig. 4, lanes 2, 3, and 4). Resveratrol had no apparent effect on the migration of the ER α -EREc38 complex. These data indicate differences in ER α conformation in the presence of these ligands. Like-

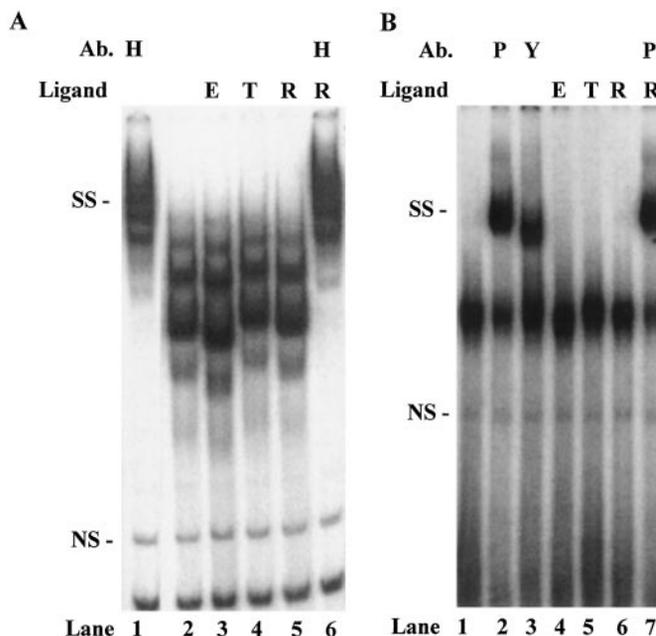


FIG. 4. ER α and ER β bind an ERE in the presence of resveratrol. A, A nuclear extract of rh ER α (6.67 nM dimer, final) was incubated with 30 μ M E₂ (E), 4-OHT (T), or resveratrol (R). The reactions in lanes 1 and 6 included H222 (H) antibody to ER α . B, A nuclear extract of rh ER β (1.9 nM dimer, final) was incubated with 16.7 μ M E₂ (E), 4-OHT (T), or resveratrol (R). The reactions in lanes 2 and 7 included 1 μ l of PA1–310 and in lane 3, 1 μ l of Y-19 anti-ER β antibodies. For the reaction in both gels, 50,000 cpm (3.5 nM) of [³²P]EREc38 was added. Incubation and EMSA reaction conditions are described in *Materials and Methods*.

wise, resveratrol did not affect the binding of H222 to ER α -EREc38, generating a supershifted complex similar to that of the E₂-ER α -EREc38 complexed with H222 (Fig. 4, lanes 6 and 1).

Similar results were detected for ER β . Preincubation of ER β with E₂ accelerated the migration of the ER β -EREc38 complex, while addition of 4-OHT slowed the migration of the 4-OHT-ER β -EREc38 complex (compare Fig. 4B, lanes 1, 4, and 5). Resveratrol had no apparent effect on the migration of the ER β -EREc38 complex (compare Fig. 4B, lanes 1 and 6). These data indicate differences in ER β conformation in the presence of these ligands. Resveratrol did not affect the interaction of antibody PA1–310 with the resveratrol-ER β -EREc38 complex (Fig. 4B, lane 7).

While the concentration of resveratrol added was expected to fully occupy ER α or ER β , 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 E₂ on ER-ERE binding *in vitro* using EMSA (Fig. 5 and data not shown). Quantitation of the amount of ER α -EREc38 or ER β -EREc38 complexes shows that resveratrol inhibits ER-ERE binding at concentrations $> 10 \mu$ M, but that identical concentrations of E₂ resulted in less inhibition of ER-EREc38 binding, until the final concentra-

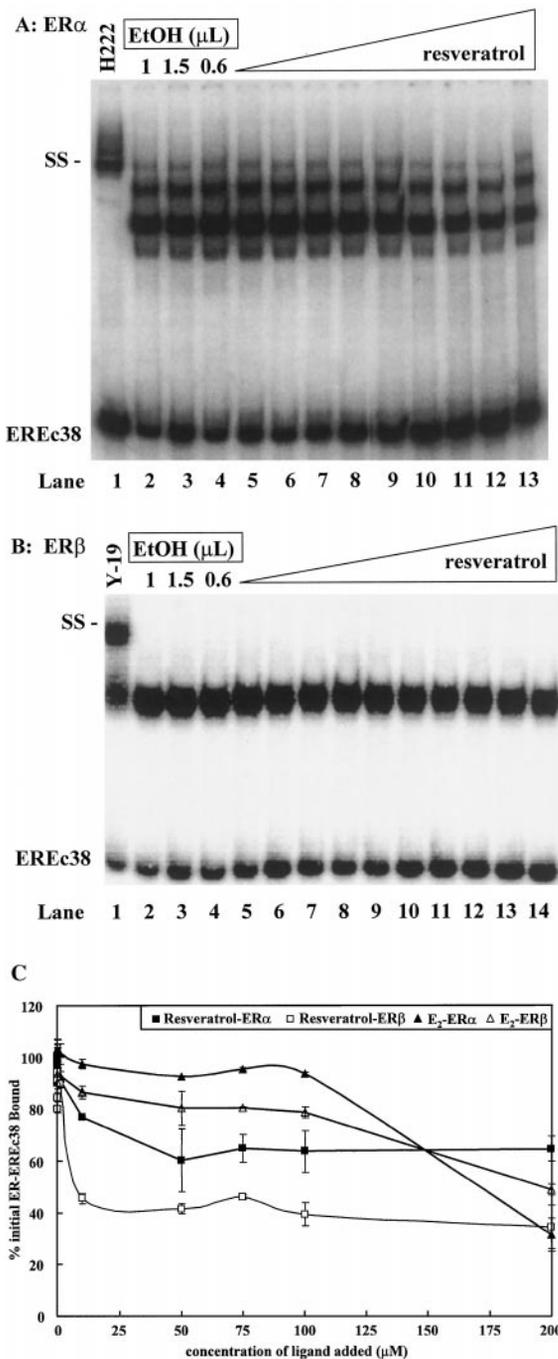


FIG. 5. Resveratrol inhibits ER α and ER β -EREc38 binding *in vitro*. Nuclear extracts of rh ER α (A) and rr ER β (B) (147 fmol) were incubated with the indicated volumes or concentrations of ethanol (EtOH), as vehicle control, or resveratrol (range 100 pM–200 μ M final). ER α antibody H222 and ER β 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 ER α -ERE bands in A) formed with increasing concentrations of resveratrol or E₂ was quantitated. There was no significant difference in the amount of either ER α or ER β bound to EREc38 when incubated with 0.6–1.5 μ l 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 E₂ was calculated relative to that amount. The data are the average \pm SEM of 2–4 independent EMSAs. The fills and symbols are indicated at the top of panel C.

tion reached 200 μ M (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 ER β -EREc38 binding compared with ER α -EREc38 binding. Like resveratrol, E₂ had more of an inhibitory effect on ER β -EREc38 binding compared with ER α -EREc38 binding. This observation indicates that ER β -EREc38 binding is more labile than ER α -EREc38 binding.

Resveratrol induces reporter gene activity with ER α from a consensus ERE

We used cotransfection assays in CHO-K1 cells to compare the transcriptional activities and ligand responsiveness of ER α and ER β . This cell line was selected because it requires exogenous ER α or ER β 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 ER α . Resveratrol stimulated luciferase activity in a concentration-dependent manner up to 50 μ M, but 100 μ M resveratrol inhibited luciferase activity (Fig. 6A). Because results from the MTT assay indicate that 100 μ M resveratrol decreased cell proliferation in ER α -expressing cells (Fig. 3), this is likely the explanation for the decreased reporter activity at this resveratrol concentration. E₂, at concentrations of 1 or 10 nM, has higher agonist activity with ER α than 50 μ M resveratrol. Resveratrol at concentrations of 1, 10, or 50 μ M 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 ER α (Fig. 6A and data not shown). While the fold induction of E₂-stimulated luciferase detected is low, we note that others have reported that single EREs show significantly lower levels of transcriptional activation in response to E₂ compared with commonly used multiple tandem EREs (24, 33, 34). These results indicate that the estrogen agonist activity of resveratrol is mediated by ER α -ERE interaction in transiently transfected CHO-K1 cells.

Resveratrol induces reporter gene activity with ER α 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 E₂ in reporter gene assays in transfected cells, compared with a consensus ERE (24, 33). Furthermore, ER α binds these EREs with reduced affinity compared with binding to EREc38 (Refs. 36, 37) and data not shown). Therefore, to determine whether resveratrol activates ER α -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*).

E₂ did not induce as much luciferase activity from the natural, imperfect EREs from the human *pS2* and *PR* genes

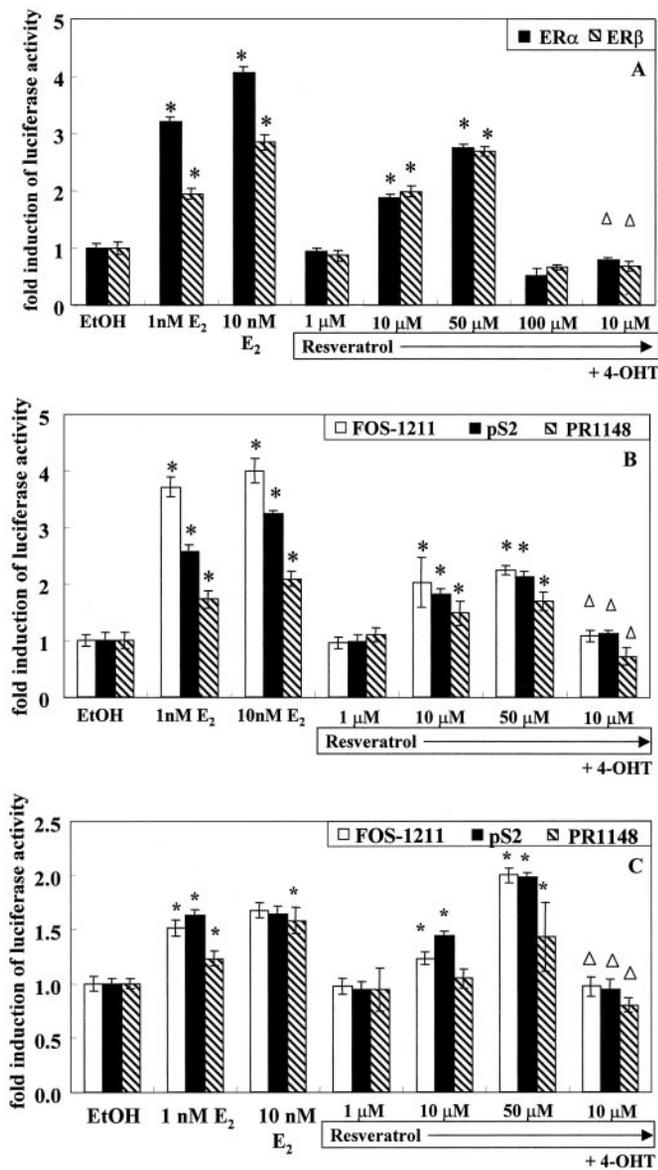


FIG. 6. Resveratrol acts as an ER α and ER β agonist in transient transfection assay. A, CHO-K1 cells were cotransfected with pGL3-1(EREc38) luciferase, pCMV- β gal, and either pCMV-ER α or pCMV-ER β , indicated as different filled boxes, and treated with EtOH, as the vehicle control, or the indicated concentrations of E₂, 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- β gal, and pCMV-ER α and treated with EtOH, E₂, 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- β gal, and pCMV-ER β and treated with EtOH, E₂, 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 \pm 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 μ M resveratrol values, respectively.

as from the consensus EREc38 (compare Fig. 6, A and B). However, the luciferase activity induced by E₂ from the imperfect ERE from the human *c-fos* gene was similar to that induced from EREc38. While there was a significant increase in luciferase activity from pS2 when cells were treated with 10 nM E₂, 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 E₂. The luciferase activity induced by 50 μ M resveratrol from PR1148 was comparable to that induced by 1 nM E₂.

The induction of luciferase activity from the natural EREs by resveratrol was blocked by cotreatment with 4-OHT, indicating that ER α is responsible for resveratrol-induced reporter activity. As seen for EREc38, treatment of the CHO-K1 cells with 100 μ M 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 ER α . These results are similar to those detected in transiently transfected, ER α -expressing COS-1 cells with either a vitellogenin ERE or LH- β promoter-luciferase reporter plasmid (14).

Resveratrol induces reporter gene activity with ER β from a consensus ERE

ER β 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 E₂ than ER α (31). However, in COS-1 cells, ER β induced higher reporter activity from the vitellogenin ERE than ER α in response to concentrations of E₂ ranging from 0.01–1 μ M (14). Here, we observed that cotransfection of CHO-K1 cells with ER β and EREc38 generated lower luciferase expression in response to E₂ compared with ER α (Fig. 6A). Comparable protein expression levels of ER α and ER β were achieved in these cells (Western blot data not shown). ER β induced 61% of the activity of ER α with 1 nM E₂. These data are consistent with the 59, 52, and 62% of ER β activity relative to ER α detected using reporters bearing one or 3 tandem consensus EREs in CEF, HeLa, and HepG2 cells, respectively (31, 38).

With ER β , resveratrol stimulated luciferase expression from EREc38 in a concentration-dependent manner up to 50 μ M, whereas 100 μ M resveratrol inhibited luciferase activity. These results are similar to those reported for ER β 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 E₂-induced activity from ER β (data not shown), indicating that direct interaction of resveratrol with ER β is responsible for the induction of luciferase activity from EREc38. Interestingly, in contrast to the differences in luciferase activity induced by E₂ with ER α and ER β , resveratrol induced nearly identical levels of luciferase activity from EREc38 with either ER α or ER β (Fig. 6A). This indicates that resveratrol-liganded ER β has similar transcriptional activity to E₂-ER β at a single perfect, palindromic ERE. These data differ from those for ER β expression in COS-1 cells in which 500 μ M resveratrol showed higher induction of reporter ac-

tivity from two tandem copies of the vitellogenin ERE than any of the concentrations of E₂ (0.01–1 nM) examined (14). These findings indicate that cell-specific factors influence the agonist activity of resveratrol with ER β .

Resveratrol induces reporter gene activity with ER β 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 ER β (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 ER α , E₂ induced lower activity from each of the imperfect EREs than from EREc38 (compare Fig. 6, A and C). Unlike ER α for which E₂ stimulated more activity from Fos-1211 than the other natural EREs, there was no difference in the luciferase activity induced by ER β with 10 nM E₂ from the imperfect EREs. These data indicate that ER α and ER β transactivate reporter gene expression differentially in response to E₂ from natural imperfect EREs in CHO-K1 cells.

Resveratrol stimulated ER β -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 ER α and ER β , 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 μ M resveratrol was greater than that stimulated by 1 or 10 nM E₂. As seen with ER α , treatment of the ER β -transfected CHO-K1 cells with 100 μ M 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 ER β was blocked by cotreatment with 4-OHT, indicating that ER β is responsible for resveratrol-induced reporter activity.

At 50 μ M, resveratrol induced identical levels of reporter activity from EREc38, Fos-1211, and pS2 with ER α and ER β . However, E₂ induced higher reporter activity from all EREs with ER α than ER β . For PR1148, ER β was less active than ER α with resveratrol and E₂. Taken together, these results imply that resveratrol-liganded ER α and ER β are equivalently transcriptionally active with the Fos-1211 and pS2 EREs. In contrast, E₂-liganded ER β interacts with EREc38 and the imperfect EREs from the human *c-fos*, pS2, and PR genes less productively than ER α . Finally, these data indicate that the ER agonist activity of resveratrol is not identical for ER α and ER β and varies with ERE sequence.

Resveratrol antagonizes E₂ activity at select EREs with ER α

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 E₂ 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 E₂ and resveratrol were additive (10). In the more recent report, the reporter activity detected in MCF-7 cells treated with 0.1 nM E₂ plus 25 μ M resveratrol was 2-fold greater than the anticipated additive activity of the two ligands (9). We evaluated how resveratrol impacted E₂-stimulated luciferase activity from EREc38 or the natural EREs from the human *c-fos*, pS2, and PR genes in CHO-K1 cells expressing either ER α (Fig. 7A) or ER β (Fig. 7B). No additive activity was detected with ER α and 0.1 nM E₂. Interestingly, 50 μ M resveratrol had E₂ 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 ER α in E₂-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 ER α , with ER β the luciferase activity stimulated by 1 nM E₂ from EREc38 and PR1148 was not antagonized by cotreatment with 50 μ M resveratrol. As seen for ER α , resveratrol had no effect on E₂-induced reporter activity from pS2. The results for Fos-1211 were similar for ER α and ER β : increased activity with E₂ plus resveratrol. In no case

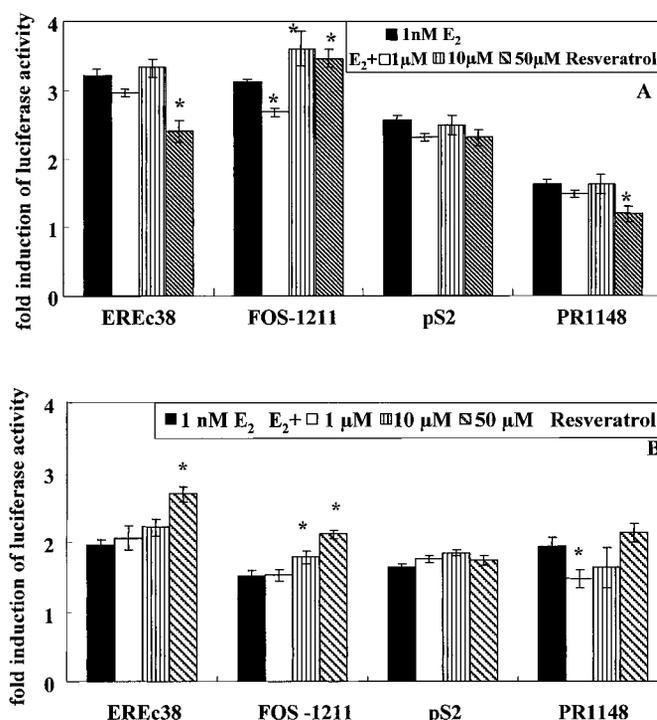


FIG. 7. Resveratrol exhibits little or no antagonist activity with ER α and additive agonist activity with ER β at various EREs. CHO-K1 cells were cotransfected with pGL3-luciferase reporter plasmid bearing 1(EREc38) or the EREs from the human *c-fos* (FOS-1211), pS2, and PR (PR1148) genes, pCMV- β gal, and pCMV-ER α (A) or pCMV-ER β (B). In both panels, cells were treated with 0.1 nM E₂ or 0.1 nM E₂ 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 \pm 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 E₂ for the cognate ERE.

was the activity of E₂ and resveratrol additive. The activity induced by E₂ plus resveratrol for each ERE with ER β was blocked by 4-OHT, indicating that it is dependent on ER β 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 E₂, resveratrol, or E₂ + resveratrol in CHO-K1 cells transfected with ER α or ER β (Fig. 8). As seen with the single EREs (Figs. 6 and 7), ER α had higher transcriptional activity than ER β in response to E₂. Resveratrol showed weak agonist activity for both ER α and ER β . Similar to the data for a single copy of EREc38, resveratrol suppressed E₂-ER α -stimulated luciferase activity from 2(EREc38) in a concentration-dependent manner. In contrast, resveratrol did not inhibit E₂-ER β activity. This result indicates that ER α and ER β respond differently to resveratrol at the same ERE. Resveratrol, when combined with E₂ exhibits antagonist activity with ER α , but no antagonist activity with ER β .

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 μ M and *cis*-resveratrol of 0.5–10 μ M. Given the daily recommended dose of wine is 250 ml (41), it is estimated that a

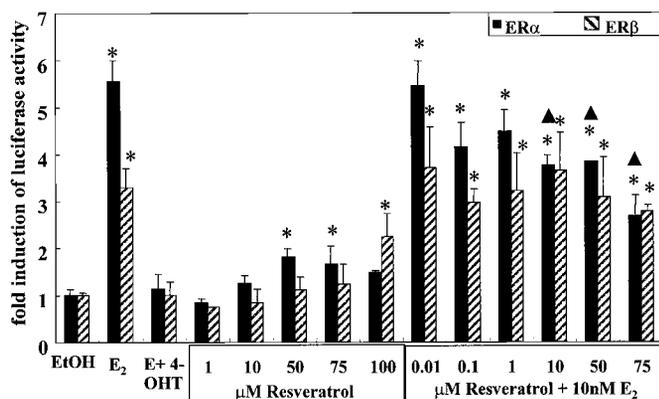


FIG. 8. Combined E₂ and resveratrol have different effects on ER α and ER β . CHO-K1 cells were cotransfected with pGL3-luciferase reporter plasmid bearing 2(EREc38), pCMV- β gal, and pCMV-ER α (black bars) or pCMV-ER β (hatched bars). The cells were treated with 10 nM E₂, 10 nM E₂ + 100 nM 4-OHT, the indicated concentrations of resveratrol (μ M), or 10 nM E₂ 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 \pm 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 E₂ alone for ER α and ER β , respectively ($P < 0.05$).

person would ingest 0.025–2.5 μ mol (6 μ g–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.

Resveratrol has chemopreventive and chemotherapeutic activities (1, 2) and has been classified as a phytoestrogen because it binds to ER α with low affinity (10). Here we evaluated the estrogen agonist/antagonist activity of resveratrol with ER α and ER β *in vitro* and in transiently transfected cells. Our results show that resveratrol binds ER α and ER β with comparable affinity, but with much lower affinity than E₂. This finding contrasts with data showing that several phytoestrogens bind ER β with higher affinity than ER α (42). One possible explanation for the lower affinity of resveratrol binding to ER β compared with other phytoestrogens is its structural similarity with diethylstilbestrol (DES) (10), which binds ER β with lower affinity than ER α (43).

Whether resveratrol-occupied ER α 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 E₂, in ER α -expressing MCF-7 cells (9, 10). However, another report found no evidence of superagonism in COS cells with either ER α or ER β (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 ER α and ER β . However, resveratrol does not display superagonist activity either alone or in combination with E₂. These results are similar to those detected in COS-1 cells transfected with ER α and either a vitellogenin ERE or LH- β promoter-luciferase reporter plasmid (14).

Our results demonstrate that the agonist activity of resveratrol with ER β is fundamentally different from E₂ agonist activity because, in contrast to E₂ which induces higher activity of ER α than ER β , resveratrol activated equal reporter activity from EREc38 with both ER α and ER β . In contrast, 500 μ M resveratrol was reported to induce higher ERE-driven reporter activity by ER β than any concentration of E₂ (0.01–1 nM) examined in COS-1 cells (14). Because both we and Ashby *et al.* (14) used human ER α and rat ER β expression vectors and pGL3-luciferase vectors in our experiments, we conclude that the difference between our findings and those reported by Ashby *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 ER β has higher transcriptional activity than E₂-liganded ER β at a single palindromic ERE. This indicates that those tissues that uniquely express ER β or that express more ER β than ER α 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 ER α conformation that impact interaction with coactivators. We observed clear differences in the migration of ERE-bound ER α and ER β either unoccupied or occupied by E₂, 4-OHT, or resveratrol. These data indicate differences in ER conformation in the presence of resveratrol compared with E₂ or

4-OHT. The concentrations at which resveratrol inhibited ERE binding by ER α and ER β *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 ER β LBD occupied by the phytoestrogen genistein showed that helix 12 did not adopt the distinctive "agonist" position seen with E₂ binding to the ER α LBD, but lay in an orientation similar to that observed when the ER β LBD was occupied by the select estrogen receptor modulator (SERM) raloxifene (48). The authors concluded that this positioning of the trans-activational 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 ER β LBD may show a structure similar to that of the genistein-occupied ER β . Because alterations in LBD conformation impact the interaction of ER α and ER β with coactivators, further experiments are needed to assess coactivator effects on resveratrol-liganded ER β activity.

While resveratrol reportedly gave a dose-dependent increase in reporter activity with concentrations as high as 500 μ M with both ER α and ER β in transiently transfected COS cells (14), we observed that 100 μ M resveratrol inhibited ERE-driven reporter activity in CHO-K1 cells. Because the limit of resveratrol solubility is 250 μ M 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 ER α or ER β and treated with 100 μ M resveratrol. There was no significant decrease in CHO-K1 cell proliferation in cells treated with 100 μ M resveratrol and expressing β -galactosidase or in mock-transfected cells under the same conditions. While others have reported that E₂ treatment of cells stably overexpressing ER α inhibits cell proliferation (49–53), E₂ 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 ER α or ER β . Therefore, stable expression of ER α in ER α negative cells appears to be required for E₂-induced inhibition of cell proliferation in response to E₂, but not resveratrol. We conclude that expression of ER α or ER β is involved in the decrease in CHO-K1 cell proliferation with 100 μ M resveratrol. Others reported that 100 μ M resveratrol inhibited the growth of ER α -expressing MCF-7 cells (18). However, the mechanism for inhibition of MCF-7 cell proliferation may not be ER α -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- β 2, 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 E₂ 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 E₂ for ER α and ER β binding and inhibited

ER α and ER β 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 ER α , the activity induced by E₂ was greater than that stimulated by any concentration of resveratrol for EREc38, Fos-1211, and pS2, but not PR1148. In contrast, resveratrol and E₂ were equally transcriptionally active with ER β at all EREs tested. With ER α 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 E₂. This result implies that, when bound to the PR1148 ERE, resveratrol-liganded and E₂-liganded ER α and ER β 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 ER α and not ER β . These data are reminiscent of the lack of 4-OHT agonist activity with ER β (54). We speculate that the antagonist activity of resveratrol may be mediated by AF-1, which appears to be absent in ER β . 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 ER α . 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 ER α and ER β are necessary for resveratrol agonist and antagonist activity and to define the exact ERE sequence requirements for resveratrol antagonist activity with ER α .

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 ER α 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 E₂ and resveratrol activity, indicating that direct activation of ER α 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 ER α and ER β 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 ER α and ER β 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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Call for Papers for the Special June 2001 Issue

At the request of Endocrine Society president Dr. Benita Katzenellenbogen, *Endocrinology* and the other Endocrine Society journals will devote their June 2001 issues to topics in Reproductive Hormones and Human Health. This theme will also be emphasized at the 83rd Annual Meeting of The Endocrine Society in Denver, Colorado, June 20–23.

The editors of *Endocrinology* are seeking submissions for this special issue. Manuscripts reporting investigations of male or female reproduction in a variety of organ systems, including the skeleton, gastrointestinal tract, cardiovascular, mammary, immune and neuroendocrine systems, as well as the gonads and reproductive tracts, are welcome. Of special interest will be papers that describe new mouse and animal models of human diseases involving reproductive hormones, including mammary, prostate, and other reproductive tract cancers.

The deadline for submissions is NOVEMBER 30, 2000. Please indicate explicitly in your cover letter that you wish to have the manuscript considered for the June 2001 issue.