ELSEVIER

Biochemical Pharmacology 62 (2001) 1113–1124

Biochemical Pharmacology

Involvement of a post-transcriptional mechanism in the inhibition of *CYP1A1* expression by resveratrol in breast cancer cells

Jeong-Eun Lee, Stephen Safe*

Department of Veterinary Physiology & Pharmacology, Texas A&M University, 4466 TAMU, College Station, TX 77843-4466, USA

Received 15 December 2000; accepted 19 March 2001

Abstract

Resveratrol decreases basal and induced CYP1A1 mRNA/protein levels in both *in vitro* and *in vivo* models, and some studies suggest that resveratrol acts as an aryl hydrocarbon receptor (AhR) antagonist. Treatment of T47D or MCF-7 cells with 10 μM resveratrol inhibited induction of CYP1A1 mRNA and CYP1A1-dependent activity after treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), as previously reported. In contrast, resveratrol did not inhibit TCDD-induced reporter gene activity in cells transfected with an Ah-responsive construct containing a human *CYP1A1* gene promoter insert, whereas 3′-methoxy-4′-nitroflavone, a "pure" AhR antagonist, inhibited this response. Resveratrol induced transformation of the rat cytosolic AhR and, after treatment of T47D and MCF-7 cells with resveratrol, a transformed nuclear AhR complex was observed. In contrast to 3′-methoxy-4′-nitroflavone, resveratrol did not block TCDD-induced AhR transformation *in vitro* or nuclear uptake of the AhR complex in breast cancer cells. Thus, the action of resveratrol on the AhR was consistent with that of an AhR agonist; however, resveratrol did not exhibit functional AhR agonist or antagonist activities in breast cancer cells. Actinomycin D chase experiments in T47D cells showed that resveratrol and dehydroepiandrosterone both increased the rate of CYP1A1 mRNA degradation, whereas resveratrol did not affect CYP1A1-dependent activity in cells pretreated with TCDD for 18 hr. These data suggest that resveratrol inhibits CYP1A1 via an AhR-independent post-transcriptional pathway.

Keywords: Resveratrol; Ah receptor; Inhibition; CYP1A1

1. Introduction

Resveratrol is a polyphenolic phytoalexin that is found in both free and conjugate forms in high concentrations in grapes, grape juice, and red wine and in other plant extracts [1,2]. For example, average concentrations of *trans*-resveratrol plus the glucoside conjugate are 3.88 mg/L in red grape juices, whereas lower levels of the corresponding *cis*-isomers (0.85 mg/L) have been observed [2]. Extracts containing resveratrol have been used in traditional Chinese and Japanese medicine for treating inflammation and cardiovascular disease, and these applications are consistent with many of the biochemical properties observed for resveratrol [3–7]. For example, resveratrol inhibits the oxidation of low-density lipoprotein, platelet aggregation, and eicosanoid synthesis, and protects isolated rat hearts from ischemia reperfusion injury [3–7]. A role for resveratrol in cardiovascular disease has been proposed [8], but the estrogenic activity of this compound is inconsistent between studies [9–11].

Resveratrol also inhibits tumorigenesis in mouse skin and the development of preneoplastic lesions in carcinogen-induced mouse mammary glands [12], and these chemoprotective responses may be related to other biochemical effects of resveratrol that include antioxidant responses and inhibition of cyclooxygenase activity [13–15]. Resveratrol also inhibits CYP1A1 expression/levels and CYP1A1-dependent activity in both *in vivo* and *in vitro* models [16–19], and it has been suggested that resveratrol may be an AhR antagonist, preventing induction of CYP1A1 by agonists such as TCDD. Interestingly, other hydroxylated phytochemicals [20,21], such as flavones and the flavonols quer-

* Corresponding author. Tel.: +1-979-845-5988; fax: +1-979-862-4929.

E-mail address: ssafe@cvm.tamu.edu (S. Safe).

Abbreviations: AhR, aryl hydrocarbon receptor; CAT, chloramphenicol acetyltransferase; DHEA, dehydroepiandrosterone; DIM, diindolylmethane; DME F-12, Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham; DRE, dioxin responsive element; DTT, dithiothreitol; EROD, ethoxyresorufin O-deethylase; FBS, fetal bovine serum; MEM, minimum essential medium, and TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00763-8

cetin and kaempferol, also interact with the AhR, but the former compound is an agonist, whereas kaempferol is an AhR antagonist and blocks CYP1A1 induction in MCF-7 human breast cancer cells [20]. Previous studies on resveratrol as an AhR agonist [16,18,19] showed that although inhibition of CYP1A1 was commonly observed, resveratrol's mechanism of action and its interaction with the AhR were inconsistent. For example, Ciolino and coworkers [18] reported that resveratrol does not competitively bind the AhR but blocks TCDD-induced formation of the nuclear AhR complex; in contrast, Casper and coworkers [16] showed that resveratrol bound the AhR receptor but did not block formation of a nuclear AhR complex in T47D cells cotreated with TCDD. Our studies confirm that resveratrol inhibits CYP1A1-dependent EROD activity in both MCF-7 and T47D breast cancer cells, induces transformation and nuclear uptake of the AhR complex, but does not inhibit induction of reporter gene activity in cells transfected with an Ah-responsive construct containing the –1142 to +2434 region of the *CYP1A1* gene promoter. Actinomycin D chase experiments demonstrated that resveratrol decreased CYP1A1 mRNA stability by post-transcriptional processes, thereby decreasing cellular CYP1A1 via AhR-independent pathways.

2. Materials and methods

2.1. Cells, chemicals, and biochemicals

TCDD was prepared in this laboratory (>98% pure by chromatographic analysis), and resveratrol (99% pure) was commercially available from the Sigma Chemical Co. Trimethoxyresveratrol was prepared quantitatively from resveratrol by the methylation of resveratrol, using diazomethane in ether/methanol, and by monitoring the reaction progress by thin-layer or gas-liquid chromatography. The resulting product was >99% pure by gas chromatographic analysis, and the molecular weight was confirmed by gas chromatography–mass spectrometry. 3′-Methoxy-4′-nitroflavone was prepared as described [22]. Actinomycin D, DHEA, ethoxyresorufin, DME F-12 without phenol red, αMEM, PBS, acetyl-CoA, and 100× antibiotic/antimycotic solution were purchased from Sigma. MCF-7 and T47D human breast cancer cells were obtained from the American Type Culture Collection (ATCC). MEM was purchased from Life Technologies. [γ-^{32}P]ATP (3000 Ci/mmol) and [^{14}C]chloramphenicol (53 mCi/mmol) were obtained from NEN Research Products. Poly[d(I-C)] and T4-polynucleotide kinase were purchased from Boehringer Mannheim. MG132 was purchased from Calbiochem. DRE and mutant DRE were synthesized by the Gene Technologies Laboratory at Texas A&M University. The murine CYP1A1 cDNA probe was obtained from ATCC, and the plasmid pGMB1.1 was a gift from Dr. Don Cleveland (Johns Hopkins University) and carries the mouse β-tubulin cDNA into the *Eco*RI site of pGMB1.1. Digestion of the plasmid yielded a 1.3-kb fragment that was used to detect β-tubulin mRNA. RNA extraction solution (RNA STAT-60^{TM}) was purchased from Tel-Test. All other chemicals and biochemicals used in these studies were the highest quality available from commercial sources. The plasmid pRNH11c contains the human CYP1A1 regulatory region from –1142 to +2434 fused to the bacterial *CAT* reporter gene (provided by Dr. R. Hines, University of Wisconsin).

2.2. Cell growth

MCF-7 cells were grown as monolayer cultures in MEM supplemented with 10% FBS (Intergen) plus $NaHCO_3$ (0.026 M), gentamycin (2.5 mg/L), penicillin/streptomycin (10,000 units/L and 10 mg/L), amphotericin B (1.25 mg/L), and 10 μg insulin. T47D cells were grown in αMEM supplemented with 2.2 g/L of sodium bicarbonate, 5% FBS, and 10 mL of antibiotic-antimycotic solution (Sigma). Cells were maintained in 150-cm^2 culture flasks in an air:carbon dioxide (95:5) atmosphere at 37°. Medium was changed twice per week, and when cells became confluent, they were trypsinized, passed, and reseeded for use in specific studies.

2.3. EROD activity

EROD activity was determined as described [23]. Trypsinized cells were plated into 48-well tissue culture plates (2×10^5 cells/mL), allowed to attain 60% confluency, and treated with 1 nM TCDD, 0.1 to 10 μM resveratrol for 18–24 hr. For kinetic studies, cells were pretreated with 1 nM TCDD for 18 hr, medium was removed, and cells were then treated with DMSO, 10 μM resveratrol, and 0.5 μM MG132, a proteasome inhibitor, and combinations, for 1, 3, and 12 hr. Cells were washed with PBS, 185 μL of PBS was added to each well, and the cells were incubated in a 37° water bath for 2 min. The reaction was started by adding 50 μL ethoxyresorufin (1.25 μg) in a 37° water bath for 13 min. After incubation for 13 min, the reaction was stopped, and EROD activity and protein concentrations were determined on a Cytofluor^{TM} 2350 plate reader as previously described [23]. Each treatment was carried out in triplicate, and results are presented as means ± SEM.

2.4. Microsomal preparation

Cells grown in 150-mm plates were treated with 1 nM TCDD for 24 hr; cells were then trypsinized, resuspended in 10 mL of HEGD buffer (25 mM HEPES, 1.5 mM EDTA, 10% glycerol, 1.0 mM DTT; pH 7.6), and centrifuged. The supernatant was discarded, and 0.5 mL of hypotonic HED buffer (25 mM HEPES, 1.5 mM EDTA, 1.0 mM DTT; pH 7.6) was added to the pellet; the pellet was resuspended and placed on ice for 10–15 min. The samples were centrifuged at 1500 *g* for 10 min at 4° and homogenized using a Teflon pestle/drill apparatus. HEGD buffer (1 mL) was added to

the homogenates and centrifuged at 1500 *g* for 10 min. The resulting supernatant was further centrifuged at 100,000 *g* for 45 min at 4°, and the microsomal pellet was resuspended in 100 μL of Tris–sucrose buffer (38 mM Tris–HCl, 0.2 M sucrose; pH 8.0) and stored at −80° until used. The protein concentration of the microsomal pellet was quantitated by the method of Bradford [24]. Microsomes were diluted with a cofactor solution containing 0.13 mg BSA, 0.01 mg NADPH, 0.01 mg NADH, and 0.06 mg $MgSO_4$ in 0.1 M HEPES in 5-μL aliquots containing 20 μg protein; 5-μL aliquots were added to 96-well Falcon plates at 0°, and treated with DMSO and different concentrations of inhibitors. Fifty microliters of 25 μM ethoxyresorufin was added to each well and incubated at 37° for 10 min, and the reaction was stopped by the addition of 100 μL of MeOH. EROD activity was determined from the fluorescence of resorufin at 530 nm excitation and 585 nm emission wavelength settings on a Cytofluor™ 2350 plate reader as described [23]. Control experiments were carried out using trimethoxyresveratrol and DIM. Each experiment was determined in triplicate, and the results are expressed as means ± SEM.

2.5. Transient transfection assay

The plasmid pRNH11c contains the –1142 to +2434 regulatory region from the human *CYP1A1* gene fused to the bacterial *CAT* reporter gene. Cells were seeded in 100-mm Petri dishes and grown until 70% confluent. Ten micrograms of the test plasmid, 2 μg of the β-galactosidase plasmid, and 62.5 mM calcium chloride were used for transfection. After transfection for 6 hr, cells were shocked using 25% glycerol in PBS and treated with the test chemicals (resveratrol, TCDD, or their combination) for 30–48 hr. Cells were washed with PBS and scraped from the plates. Cell lysates were prepared in 1× lysis buffer (Promega) by a freeze–thaw cycle with liquid nitrogen. Protein concentrations were determined using BSA as a standard. Each cell lysate (20 μg) was incubated with 0.2 mCi *d*-threo-[*dichloroacetyl*-1-^{14}C]chloramphenicol and 4 mM acetyl-CoA as substrates at 37° for 2–5 hr. Following thin-layer chromatography, acetylated metabolites were visualized and quantitated using a Packard Instant Imager. CAT activity was calculated as the percentage of that observed in cells treated with DMSO alone and was normalized relative to β-galactosidase activity.

2.6. Preparation of cytosolic and nuclear extracts

Hepatic cytosol from untreated female Sprague–Dawley rats was prepared essentially as described [22]. Cells grown in 100-mm Petri dishes and treated with DMSO and the test compounds (resveratrol or TCDD) for 30 min to 1 hr were harvested by manual scraping, washed twice in 5 mL of HE buffer (25 mM HEPES, 1.5 mM EDTA; pH 7.6), 0.5 mL of HEGD buffer, incubated on ice, and processed using a Dounce homogenizer. Homogenates were centrifuged at 12,000 *g* for 5 min at 4°; the supernatant was discarded, and the pelleted fraction was resuspended in 0.1 mL of HEGD buffer containing 0.5 M potassium chloride (pH 7.6). After incubation for 30–60 min at 20°, the nuclear extracts were obtained by centrifugation at 12,000 *g* for 10 min at 4°. The supernatants representing nuclear extracts were collected and stored in −80° until used.

2.7. Gel electrophoretic mobility shift assay

Nine picomoles of synthetic human DRE oligonucleotide was labeled at the 5′ end using T4-polynucleotide kinase and [γ-^{32}P]ATP. For the AhR:DRE binding assays, nuclear extracts (2–5 μg) from cells treated with DMSO, 5 nM TCDD, 5 μM resveratrol, or their combination were incubated for 10 min at 20° in 20 mM HEPES–5% (v/v) glycerol, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM EDTA in a final volume of 25 μL with 1 μg poly[d(I-C)] for 10 min at 20° to bind non-specific DNA-binding proteins. A 100-fold excess of unlabeled wild-type and mutant DRE were added for the competition experiments and incubated at 20° for 5 min. Following the addition of [^{32}P]-labeled DNA, the mixture was incubated for an additional 15 min at 20°. Experiments with rat liver cytosol (80 μg/incubation) used different concentrations of various compounds, which were incubated at 20° for 2 hr. The mixtures were treated further with 1 μg of poly[d(I-C)] for 15 min at 20°, and for oligonucleotide competition experiments 100-fold excess of unlabeled wild-type and mutant DRE were added and incubated at 20° for 5 min. Then [^{32}P]-labeled DNA was added to cytosolic extracts and incubated for an additional 15 min at 20°. Reaction mixtures were loaded onto 5% polyacrylamide gel and electrophoresed at 120 V in 0.9 M Tris–borate and 2 mM EDTA, pH 8.0, for 2.5 hr. Gels were dried, and protein–DNA complexes were visualized by autoradiography using a Packard Instant Imager. The gel was also exposed to a phosphoscreen for 12 hr and visualized by autoradiography using a Storm PhosphoImager (Molecular Dynamics).

2.8. RNA preparation and Northern blot analysis

Cells were plated into 100-mm Petri dishes with medium containing 5% charcoal-stripped FBS and, when cells reached 60% confluency, they were treated with 1 nM TCDD, 5 μM resveratrol, or their combinations in serum-free medium for 6 hr. For the kinetic study, cells were pretreated with 1 nM TCDD in serum-free medium for 12 hr, the medium was changed, and DMSO, DHEA (1 μM), and resveratrol (5 μM) with 5 μg/mL of actinomycin D were added to each plate for 2, 6, and 10 hr. After treatment, RNA was extracted from the plates using RNA STAT-60™ purchased from Tel-Test. RNA extracts from different treatment groups were dissolved in nuclease-free water, heated at 55–60° for 15–30 min, vortexed, and quantitated at 260/

280 nm. RNA (30 μg) was mixed with 2× sample buffer [20% formaldehyde, 1.65% 1 M Na_2HPO_4 (pH 6.8), 63.5% formamide, and 15% 6× loading buffer; loading buffer consisted of 0.25% bromophenol blue, 0.25% xylene-cyanol, and 15% Ficoll (Type 400)], electrophoresed on a denaturing 1.2% agarose gel at 60 V for 2.5 hr, and transferred to a Hybond nylon membrane in 1× SPC buffer [20 mM Na_2HPO_4, 2 mM 1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (CDTA), pH 6.8] for 36–48 hr. The membrane was then exposed to UV light for 5 min to cross-link RNA to the membrane and then baked at 80° for 2 hr. Next, the membrane was prehybridized in a solution containing 5× SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 10% dextran sulfate, 0.1% polyvinyl pyrrolidine, 0.1% Ficoll, 0.1% BSA, and 1% SDS for 18–24 hr at 62°. The CYP1A1 or β-tubulin cDNA probes were [^{32}P]-labeled using a Boehringer-Mannheim random primer kit. [^{32}P]-Labeled cDNA (5×10^6 cpm) probes were heated at 100° for 5 min and cooled on ice for 5 min prior to use, and the membrane was hybridized for approximately 24 hr in the prehybridization solution. After hybridization, the membrane was washed at 20° for 15 min (two times) in a solution containing 1× SSPE and 2% SDS, sealed in a plastic bag. Bands were scanned using a Packard Instant Imager, and the membrane was exposed to phosphoscreen for 1–2 days. CYP1A1 signals were visualized by autoradiography and quantitated using a Storm PhosphoImager. The membrane was then stripped with washing solution at 62° for 10 hr, prehybridized for at least 2 hr, and rehybridized with 5×10^6 cpm [^{32}P]-labeled β-tubulin cDNA probe (in 10 μL) for 24 hr. Washing, visualization, and quantitation methods were carried out as described above, and CYP1A1 mRNA was standardized relative to β-tubulin mRNA.

2.9. Statistical analysis

Statistical differences between different treatment groups were determined using Student's *t*-test or ANOVA (Scheffe's), and levels of significance were noted ($P < 0.05$). Results are expressed as means ± SEM for at least three replicate determinations for each treatment group.

3. Results

3.1. Effects of resveratrol on CYP1A1 gene expression and EROD activity

Results summarized in Fig. 1 show that 1 nM TCDD alone induced CYP1A1 mRNA levels (3.5-fold) and CYP1A1-dependent EROD activity in T47D and MCF-7 breast cancer cells, whereas concentrations as high as 10 μM resveratrol were inactive as inducers of CYP1A1 mRNA or EROD activity. Induction of CYP1A1 mRNA levels in T47D cells by 1 nM TCDD was blocked after the co-administration of 10 μM resveratrol. Moreover, in MCF-7 or T47D cells cotreated with 1 nM TCDD plus 0.1 to 10 μM resveratrol, there was a concentration-dependent decrease in EROD activity, and these results were consistent with previous reports on interactions of these compounds with CYP1A1 in various cell lines [16,18,19]. Incubation of resveratrol (0.1 to 10 μM) with TCDD-induced microsomes from MCF-7 and T47D cells only slightly decreased enzyme activity (≤20%) at the 10 μM concentration (Fig. 2), but significantly decreased activity was observed at higher concentrations (data not shown). Incubation with DIM decreased enzyme activity as previously reported [25], and inhibition was also observed for trimethoxyresveratrol. Thus, the inhibitory effect of 10 μM resveratrol was not related to direct interactions of this compound with CYP1A1 protein. Previous studies reported that microsomal EROD activity and benzo[*a*]pyrene (BaP) hydroxylation were inhibited after incubation with 10 μM resveratrol [16,19], whereas in another study the inhibitory effects were similar to those reported in Fig. 2 [17]. The reason for differences between studies may be related to differences in experimental conditions, including the high substrate (ethoxyresorufin) concentrations used in this study.

3.2. Interactions of resveratrol with the AhR

Competitive binding of resveratrol to the AhR was not observed using sucrose density gradients [18], whereas in another report binding was observed in T47D whole cell assays [16]. These differences also were reflected in the results of gel mobility shift assays of nuclear extracts from cells treated with TCDD, resveratrol, and their combination [16,18,19]. Therefore, these latter studies were repeated in both MCF-7 and T47D cells, and the results are summarized in Fig. 3. Nuclear extracts from MCF-7 and T47D cells treated with 5 nM TCDD were incubated with [^{32}P]DRE and analyzed by gel mobility shift assays to give a specifically bound retarded band; the intensity of this band was decreased by competition with excess unlabeled DRE but not by mutant DRE oligonucleotides. Nuclear extracts from cells treated with 5 μM resveratrol alone or in combination with 5 nM TCDD also formed retarded bands; these results were consistent with previous studies in T47D cells [16], but contrasted with results in HepG2 cells where resveratrol did not induce the formation of a nuclear AhR complex and blocked the formation of a TCDD- or BaP-induced nuclear AhR complex [16,19]. The specificity of the effects of resveratrol on transformation of the AhR complex was investigated further using rat liver cytosol (Fig. 3C), which was readily transformed (concentration-dependently) by TCDD to give a retarded band (lanes 9–11), whereas the solvent (DMSO) did not induce transformation (lane 12). The specificity of TCDD-induced transformation was confirmed by the following: competition with unlabeled wild-type DRE (lane 2), but not mutant DRE (lane 1), decreased intensity of the retarded band, and the AhR antagonist 3′-methoxy-4′-nitroflavone blocked TCDD-induced trans-

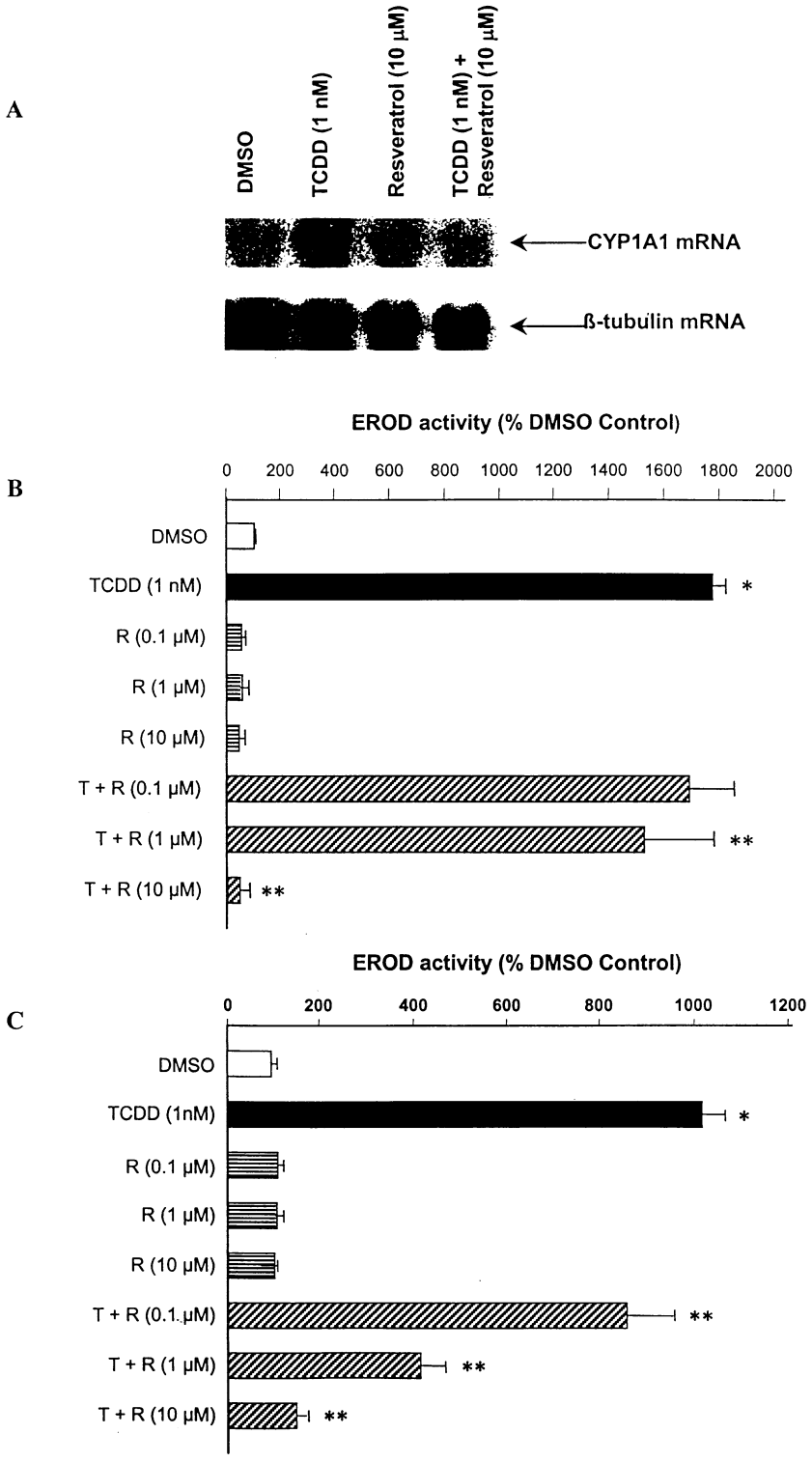

Fig. 1. Effects of resveratrol on *CYP1A1* gene expression in breast cancer cells. (A) CYP1A1 mRNA levels. T47D cells were treated with solvent control (DMSO), 1 nM TCDD, 10 μM resveratrol, and TCDD plus resveratrol (1 nM and 10 μM, respectively), and after 6 hr mRNA was analyzed by northern blot analysis as described in "Materials and methods." Relative CYP1A1 mRNA levels were: DMSO, 1.0 $\pm$ 0.08; TCDD, 3.53 $\pm$ 0.53; reservatrol, 1.1 $\pm$ 0.16; and TCDD plus reservatrol, 0.99 $\pm$ 0.1. TCDD significantly ($P < 0.05$) induced *CYP1A1* gene expression, and this induced response was inhibited significantly ($P < 0.05$) by resveratrol. EROD activity in T47D (B) or MCF-7 (C) cells. Cells were treated with DMSO, 1 nM TCDD, 0.1 to 10 μM resveratrol, and TCDD (1 nM) plus resveratrol (0.1 to 10 μM) for 24 hr, and EROD activity was determined as described in "Materials and methods." TCDD induced EROD activity significantly (*$P < 0.05$) in both cell lines, and the induced response was inhibited significantly (**$P < 0.05$) by 1 and 10 μM resveratrol in T47D and 0.1, 1, and 10 μM resveratrol in MCF-7 cells. Enzyme activities in the control (DMSO) groups were 0.85 and 46.4 pmol/min/mg in T47D and MCF-7 cells, respectively. Results are expressed as means $\pm$ SEM for three separate experiments.

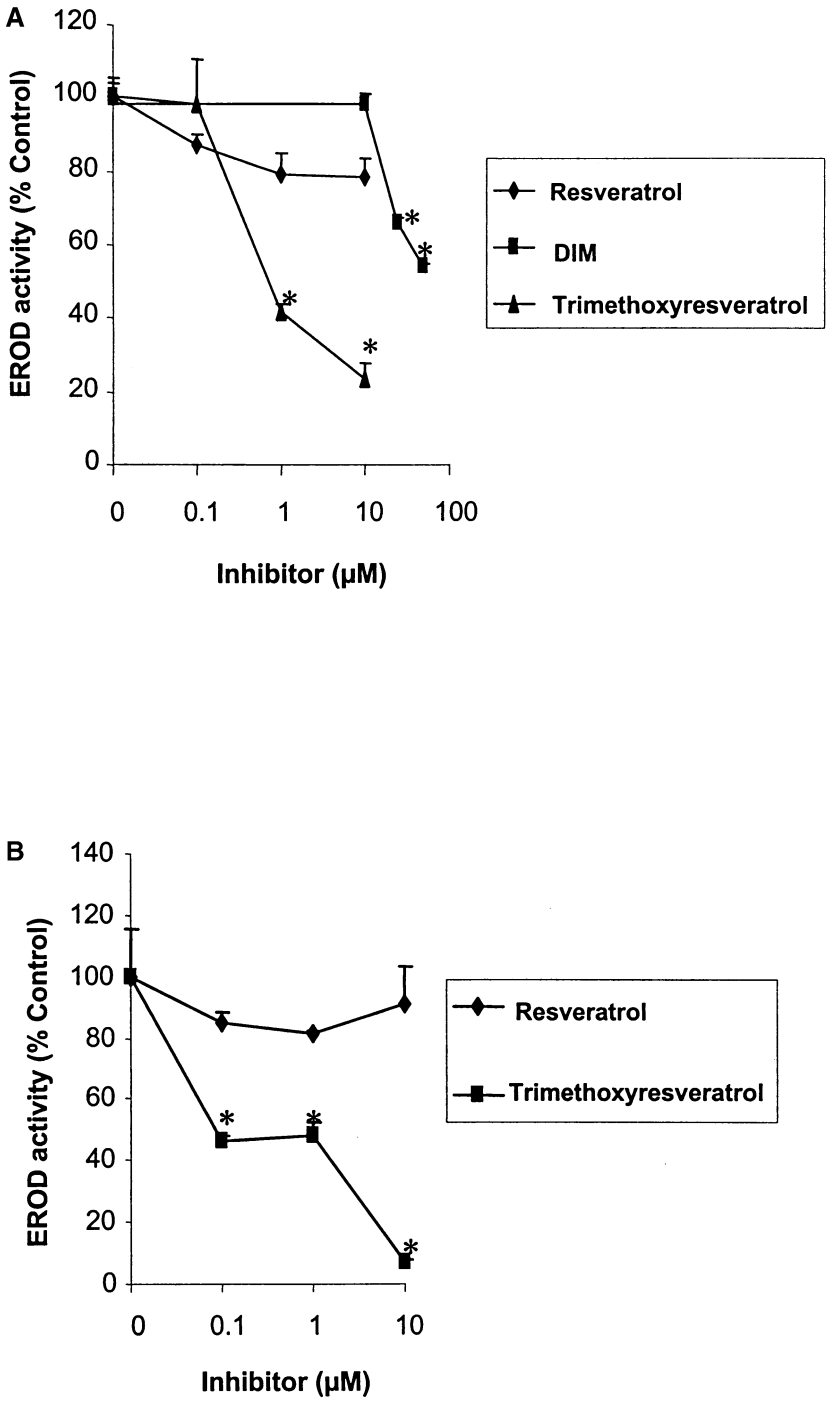

Fig. 2. Interactions of resveratrol with CYP1A1. Microsomes from T47D (A) and MCF-7 (B) cells were incubated with resveratrol (0.1 to 10 μM), trimethoxyresveratrol (0.1 to 10 μM), or DIM (0.1 to 50 μM) for 10 min, and EROD activity was determined as described in "Materials and methods." Resveratrol ($\leq$10 μM) did not inhibit EROD activity, whereas both trimethoxyresveratrol and DIM inhibited EROD activity significantly (*$P < 0.05$). Results are expressed as means $\pm$ SEM for three separate determinations for each treatment group.

formation (lane 3) as previously described [22,26]. In contrast, resveratrol-induced transformation of the cytosolic AhR at concentrations ranging from 0.05 to 5 μM (lanes 6–8) was concentration-independent; co-incubation of resveratrol with TCDD did not markedly affect retarded band intensities (data not shown), whereas 3′-methoxy-4′-nitroflavone blocked transformation of the cytosolic AhR by resveratrol (lane 4). The effects of resveratrol on AhR transformation/nuclear translocation were consistent with the AhR agonist (not antagonist) activity of resveratrol; however, results in Fig. 1 also showed that resveratrol inhibited TCDD-induced *CYP1A1* gene expression.

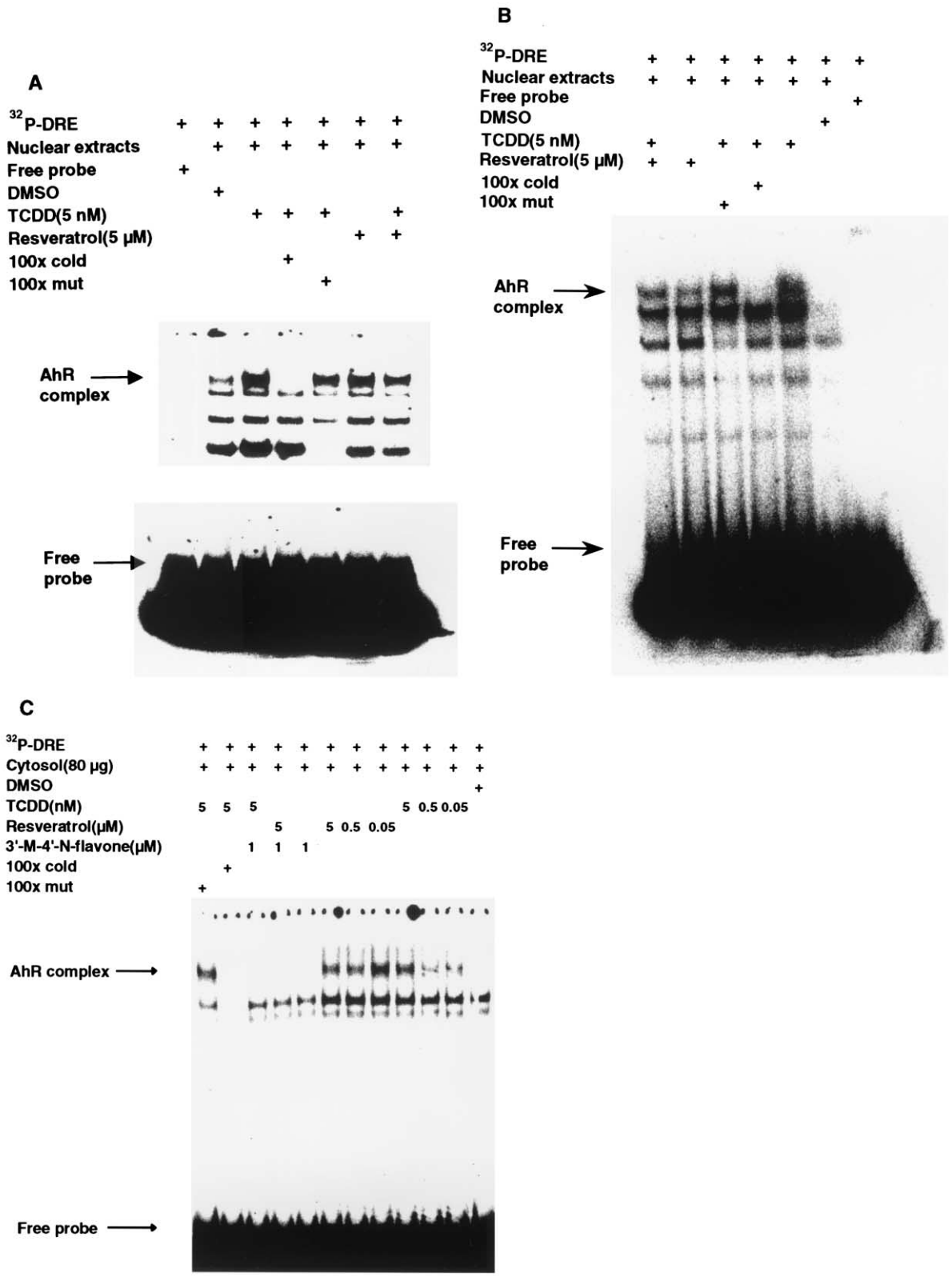

Fig. 3. Resveratrol-induced formation of the nuclear or transformed AhR complex. T47D (A) or MCF-7 (B) cells were treated with 5 nM TCDD, 5 μM resveratrol, or their combination; nuclear extracts were obtained and analyzed by gel mobility shift assays as described in "Materials and methods." A specifically bound AhR–[^{32}P]DRE complex was detected in all treatment groups. (C) Transformation of the rat hepatic AhR. Rat hepatic cytosol was incubated with TCDD, resveratrol, the AhR antagonist 3′-methoxy-4′-nitroflavone, or their combinations, and the transformed complex was analyzed by a gel mobility shift assay as described in "Materials and methods." TCDD (lanes 9–11) and resveratrol (lanes 6–8) induced formation of a specifically bound AhR complex, whereas 3′-methoxy-4′-nitroflavone blocked resveratrol-/TCDD-induced complex formation but this compound alone did not induce transformation (lanes 3–5). This pattern of transformation was observed in duplicate experiments.

The lack of specificity associated with interactions of resveratrol with the AhR in cell culture and in the transformation of rat hepatic cytosol (Fig. 3C) suggested that the inhibitory effects of resveratrol on CYP1A1 mRNA and EROD activity induced by TCDD may be AhR-independent. Previous studies have shown that AhR antagonists such as 3′-methoxy-4′-nitroflavone and other 3′-methoxy-substituted flavones inhibit the formation of the nuclear AhR complex and induction of reporter gene activity by TCDD in cells stably or transiently transfected with constructs containing functional *DRE* promoter inserts [22,26, 27]. Ciolino and Yeh [19] also showed that resveratrol inhibits CAT activity in HepG2 and MCF-7 cells treated with AhR agonists and transiently transfected with a construct containing a rat *CYP1A1* gene promoter insert. In contrast, our results (Fig. 4) show that 1 and 10 μM resveratrol alone did not induce CAT activity in T47D or MCF-7 cells transfected with Ah-responsive pRNH11c; TCDD alone induced CAT activity, and the induced response was not affected by 1 or 10 μM resveratrol, whereas the AhR antagonist 3′-methoxy-4′-nitroflavone blocked TCDD-induced reporter gene activity as previously described [22,26]. Thus, in contrast to 3′-methoxy-4′-nitroflavone, resveratrol did not exhibit AhR antagonist activity for this response. The failure to observe an inhibitory response with resveratrol in the transfection assays cannot be related to metabolism of resveratrol, since previous transfection studies using estrogen-responsive constructs and cell proliferation studies reported anti-estrogenic/estrogenic activities of resveratrol [8–10].

3.3. Effects of resveratrol on CYP1A1 mRNA and protein stability

The apparent conflicting results on the AhR antagonist activity of resveratrol obtained in this and previous studies suggested that other inhibitory mechanisms may also be important. A recent study showed that the steroid hormone DHEA inhibited basal and induced *CYP1A1* gene expression in MCF-7 cells but had no effect on DRE-dependent transcription, using a construct containing the rat *CYP1A1* gene promoter insert [28]. The inhibitory effect of DHEA was associated with post-transcriptional destabilization of CYP1A1 mRNA and, therefore, the time-dependent effects of DHEA and resveratrol were determined in T47D cells pretreated with TCDD to induce CYP1A1 mRNA levels. Then actinomycin D was added to inhibit further transcription, and CYP1A1 mRNA levels (normalized to β-tubulin mRNA) were determined at 0, 2, 6, and 10 hr after the addition of actinomycin D. The results (Fig. 5) show that both DHEA and resveratrol significantly increased the rate of CYP1A1 mRNA degradation, suggesting that, like DHEA, resveratrol also inhibits CYP1A1 by post-transcriptional mechanisms. Moreover, like resveratrol, DHEA also inhibited TCDD-induced EROD activity in T47D cells (data not shown). It is also possible that resveratrol could induce the proteasome-dependent degradation of CYP1A1 protein, and, therefore, the effects of resveratrol, the proteasome inhibitor MG132, and their combination on CYP1A1-dependent EROD activity were determined in T47D cells (Fig. 6). Eighteen hours after treatment with 1 nM TCDD, the medium was changed, and the cells were further incubated with resveratrol or resveratrol plus MG132 for an additional 12 hr. None of the treatments deceased CYP1A1-dependent activity, and there was an increase in activity in cells treated with resveratrol alone. DMSO or resveratrol in combination with MG132 did not affect EROD activity, suggesting that the effects of resveratrol alone or in combination with TCDD on decreased CYP1A1 (Fig. 1) are due to decreased CYP1A1 mRNA stability (Fig. 5).

4. Discussion

Resveratrol exhibits a diverse spectrum of biochemical responses that may be linked to health benefits associated with the moderate consumption of wine [30–32]. Several recent studies have demonstrated that resveratrol decreases CYP1A1 mRNA/protein or related activities in cell culture and *in vivo*, and there is some evidence that this inhibitory response may be related to the AhR antagonist properties of this compound [16–19]. At least two major classes of AhR antagonists have been identified, and these include 3′-methoxy-substituted flavones that block receptor transformation and formation of the nuclear AhR complex [22,26,27] and alternate substituted polychlorinated dibenzofurans (PCDFs). These latter compounds, typified by 6-methyl-1,3,8-trichlorodibenzofuran, compete with TCDD for binding the AhR but form a nuclear AhR complex that only weakly activates *DRE*-dependent responses such as induction of *CYP1A1* gene expression [33,34]. Resveratrol also inhibits *CYP1A1* gene expression and related enzyme activities, and it has been suggested that resveratrol is also an AhR antagonist [16,18,19].

Our results also demonstrated that resveratrol inhibits TCDD-induced CYP1A1 mRNA and related enzyme activity in breast cancer cells; however, interactions of resveratrol with the AhR suggest that these responses may be non-transactivating. Treatment of T47D or MCF-7 cells with resveratrol resulted in the formation of a nuclear AhR complex as determined in gel mobility shift assays, and interactions with TCDD did not affect nuclear AhR complex formation markedly (Fig. 3). These results are consistent with a previous report using breast cancer cells [16] but are in contrast to studies in HepG2 cells [18,19] which showed that treatment with resveratrol alone did not result in the formation of a nuclear AhR complex and, in cotreatment studies (resveratrol + TCDD), resveratrol inhibited TCDD-induced formation of the AhR complex. Differences observed in this assay may be due to cell type (breast vs liver) and, therefore, interactions of resveratrol were investigated further using rat hepatic cytosol which is readily

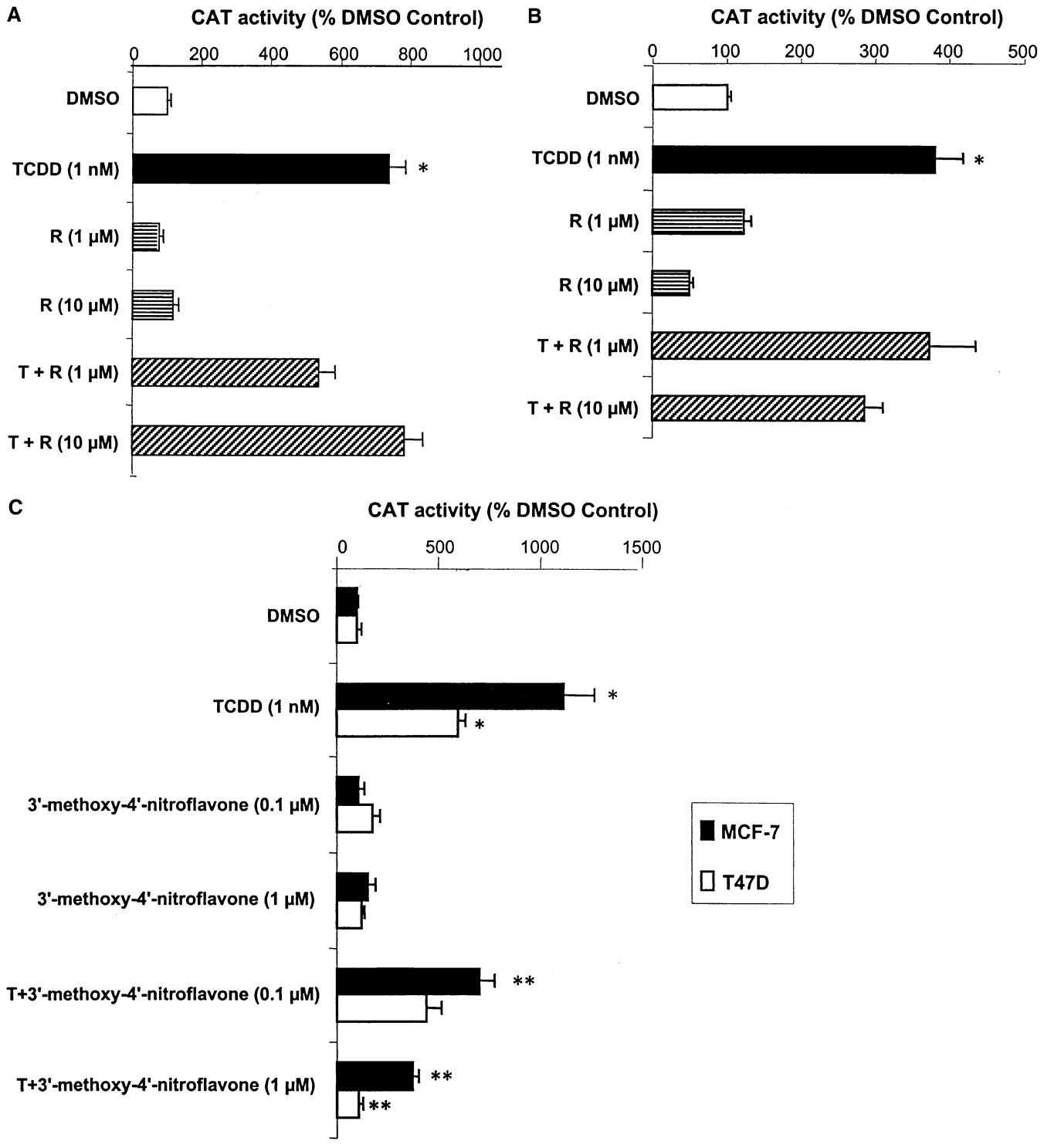

Fig. 4. AhR agonist/antagonist activity of resveratrol in transfection studies. T47D (A) or MCF-7 (B) cells were transfected with pRNH11c, and treated with TCDD (T), resveratrol (R), or their combinations; CAT activity was determined as described in "Materials and methods." In a separate experiment (C), the effects of the AhR antagonist 3′-methoxy-4′-nitroflavone on TCDD-induced CAT activity were also determined. Only TCDD alone induced CAT activity significantly (*$P < 0.05$), and in combination treatments, only 3′-methoxy-4′-nitroflavone decreased the TCDD-induced response significantly (**$P < 0.05$). Results are expressed as means ± SEM for three separate experiments for each treatment group.

transformed by TCDD. Induced transformation is inhibited by pure AhR antagonists such as 3′-methoxy-4′-nitroflavone (Fig. 3C) [22,26,27], whereas resveratrol-induced transformation was concentration-independent and resveratrol plus TCDD gave minimal interactions in the assay (Fig. 5). Seidel and coworkers [35] also have reported that like resveratrol, several structurally different compounds induce the transformation of guinea pig hepatic cytosolic AhR but

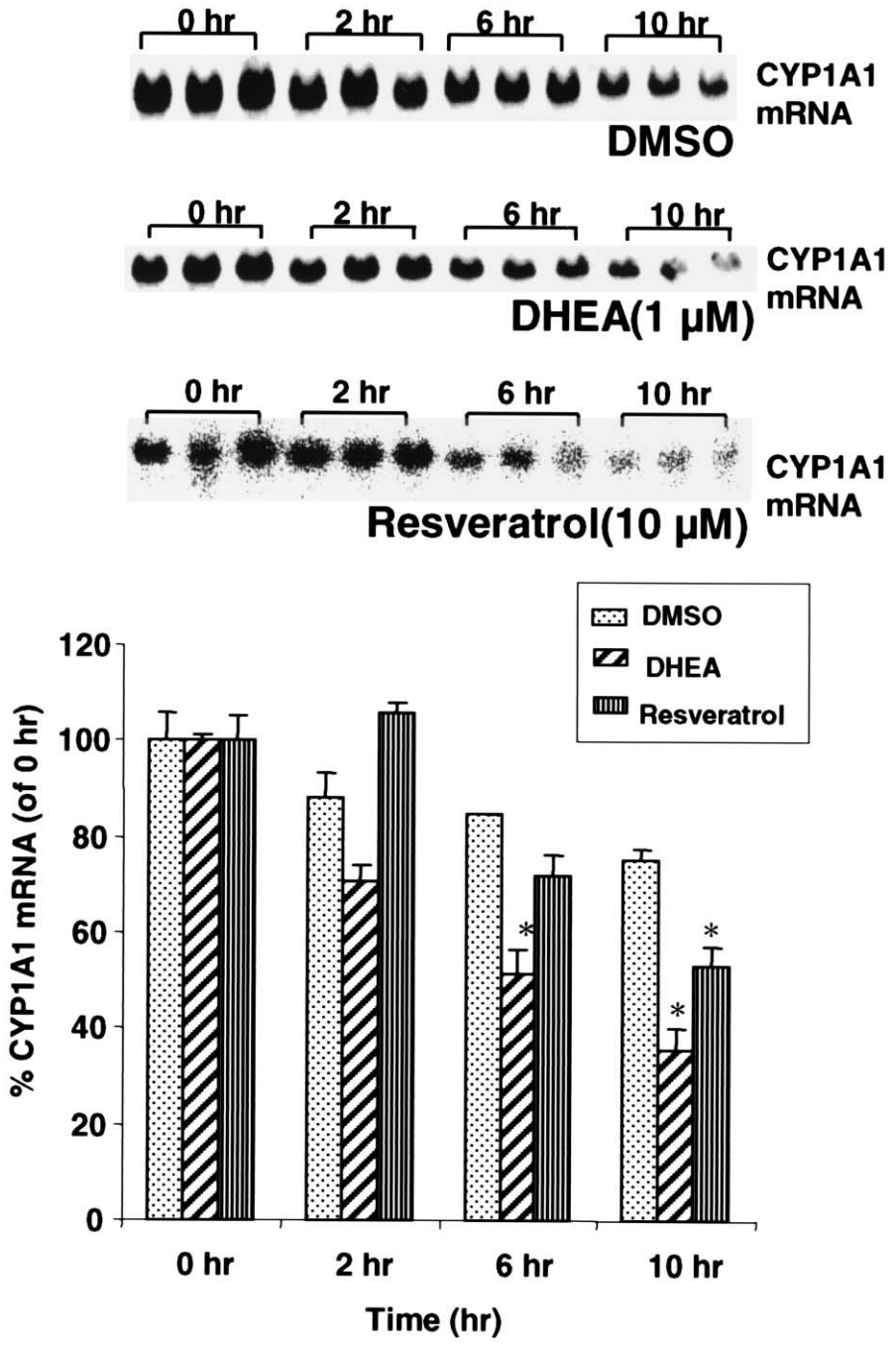

Fig. 5. CYP1A1 mRNA stability in T47D cells treated with resveratrol and DHEA. T47D cells were pretreated with 1 nM TCDD for 12 hr; medium was changed, and cells were treated with 5 μM resveratrol, 1 μM DHEA, or DMSO plus 5 μg/mL of actinomycin D. CYP1A1 mRNA levels were determined 2, 6, and 10 hr after treatment as described in "Materials and methods." CYP1A1 mRNA levels (relative to β-tubulin mRNA) are illustrated for cells treated with DMSO, DHEA, or resveratrol, and both DHEA (6 and 10 hr) and reservatrol (10 hr) decreased CYP1A1 mRNA stability significantly (*$P < 0.05$). Results are expressed as means ± SEM for three separate experiments for each treatment group.

do not exhibit functional AhR agonist activity. These data, coupled with the failure of resveratrol to exhibit AhR agonist activity in functional assays and AhR antagonist activity in cells transfected with Ah-responsive pRNH11c (Fig. 4), suggest that resveratrol–AhR interactions may be nonspecific.

Nevertheless, results of this study and previous reports [16–19] clearly show that resveratrol decreases CYP1A1 mRNA and protein levels, and the latter response is not related to proteasome-dependent degradation of CYP1A1 protein (Fig. 6). Therefore, we investigated the interactions between resveratrol and the down-regulation of *CYP1A1* further by determining the post-transcriptional effects on CYP1A1 mRNA stability. A recent report showed a remarkable similarity between resveratrol and DHEA, both of which inhibited induction of CYP1A1 mRNA and EROD

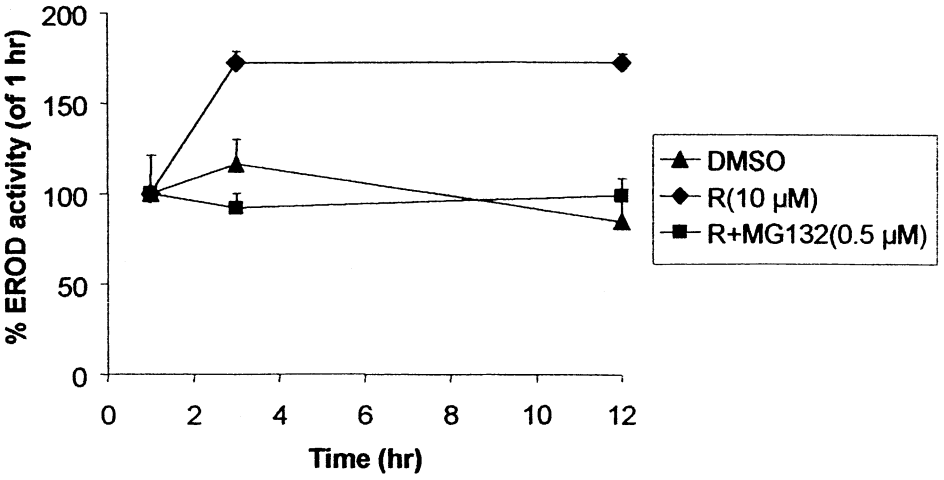

Fig. 6. Effects of resveratrol on CYP1A1-dependent EROD activity. T47D cells were treated with 1 nM TCDD for 18 hr; medium was changed, and cells were then treated with DMSO (control), 10 μM resveratrol, and 10 μM resveratrol plus 0.5 μM MG132 (a proteasome inhibitor). EROD activity was determined after further incubation for 1, 3, and 12 hr as described in "Materials and methods." The concentration of MG132 was sufficient for inhibition of TCDD-induced proteasome-dependent degradation of the AhR in T47D cells [29]. Resveratrol did not affect protein stability as determined by CYP1A1-dependent EROD activity. Results are expressed as means ± SEM for three separate experiments for each treatment group.

activity in breast cancer cells [28]. We also observed these responses for resveratrol (Fig. 1) and DHEA (data not shown). Moreover, like resveratrol (Fig. 4), DHEA did not inhibit induction in cells transfected with an Ah-responsive construct [28]. Our results (Fig. 5) also showed that both DHEA and resveratrol increased the rate of CYP1A1 mRNA degradation in T47D cells, suggesting that resveratrol (like DHEA) also inhibits *CYP1A1* expression by post-transcriptional mechanisms. The importance and specificity of AhR-independent actions of resveratrol are unknown; however, a recent study reported that resveratrol also decreases androgen receptor mRNA levels in LNCaP prostate cancer cells [36]. It is possible that these effects may also be linked to a post-transcriptional AhR-independent mechanism, as reported in this study, and the role of this pathway in mediating resveratrol action, particularly in hormone-responsive cancer cells, is currently being investigated.

Acknowledgments

The financial assistance of the National Institutes of Health (ES09106 and ES04917) and the Texas Agricultural Experiment Station is gratefully acknowledged. S. Safe is a Sid Kyle Professor of Toxicology.

References

[1] Lamuela-Raventos RM, Romero-Perez AI, Waterhouse AL, de la Torre-Boronat MC. Direct HPLC analysis of *cis*- and *trans*-resveratrol and piceid isomers in Spanish red *Vitis vinifera* wines. J Agric Food Chem 1995;43:281–3.

[2] Romero-Perez AI, Ibern-Gomez M, Lamuela-Raventos RM, de la Torre-Boronat MC. Piceid, the major resveratrol derivative in grape juices. J Agric Food Chem 1999;47:1533–6.

[3] Chung MI, Teng CM, Cheng KL, Ko FN, Lin CN. An antiplatelet principle of *Veratrum formosanum.* Planta Med 1992;58:274–6.

[4] Frankel EN, Waterhouse AL, Kinsella JE. Inhibition of human LDL oxidation by resveratrol. Lancet 1993;341:1103–4.

[5] Kimura Y, Okuda H, Kubo M. Effects of stilbenes isolated from medicinal plants on arachidonate metabolism and degranulation in human polymorphonuclear leukocytes. J Ethnopharmacol 1995;45: 131–9.

[6] Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G, Goldberg DM. The red wine phenolics *trans*-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. Clin Chim Acta 1995;235: 207–19.

[7] Ray PS, Maulik G, Cordis GA, Bertelli AA, Bertelli A, Das DK. The red wine antioxidant resveratrol protects isolated rat hearts from ischemia reperfusion injury. Free Radic Biol Med 1999;27:160–9.

[8] Gehm BD, McAndrews JM, Chien P-Y, Jameson JL. Resveratrol, a polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. Proc Natl Acad Sci USA 1997;94:14138–43.

[9] Lu RQ, Serrero G. Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. J Cell Physiol 1999;179:297–304.

[10] Mgbonyebi OP, Russo J, Russo IH. Antiproliferative effect of synthetic resveratrol on human breast epithelial cells. Int J Oncol 1998; 12:865–9.

[11] Turner RT, Evans GL, Zhang M, Maran A, Sibonga JC. Is resveratrol an estrogen agonist in growing rats? Endocrinology 1999;140:50–4.

[12] Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 1997;275:218–20.

[13] Chanvitayapongs S, Draczynska-Lusiak B, Sun AY. Amelioration of oxidative stress by antioxidants and resveratrol in PC12 cells. Neuroreport 1997;8:1499–502.

[14] Day AP, Kemp HJ, Bolton C, Hartog M, Stansbie D. Effect of concentrated red grape juice consumption on serum antioxidant capacity and low-density lipoprotein oxidation. Ann Nutr Metab 1997; 41:353–7.

[15] Subbaramaiah K, Chung WJ, Michaluart P, Telang N, Tanabe T, Inoue H, Jang M, Pezzuto JM, Dannenberg AJ. Resveratrol inhibits

cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. J Biol Chem 1998;273:21875–82.
[16] Casper RF, Quesne M, Rogers IM, Shirota T, Jolivet A, Milgrom E, Savouret JF. Resveratrol has antagonist activity on the aryl hydrocarbon receptor: implications for prevention of dioxin toxicity. Mol Pharmacol 1999;56:784–90.
[17] Chun YJ, Kim MY, Guengerich FP. Resveratrol is a selective human cytochrome P450 1A1 inhibitor. Biochem Biophys Res Commun 1999;262:20–4.
[18] Ciolino HP, Daschner PJ, Yeh GC. Resveratrol inhibits transcription of *CYP1A1 in vitro* by preventing activation of the aryl hydrocarbon receptor. Cancer Res 1998;58:5707–12.
[19] Ciolino HP, Yeh GC. Inhibition of aryl hydrocarbon-induced cytochrome P-450 1A1 enzyme activity and *CYP1A1* expression by resveratrol. Mol Pharmacol 1999;56:760–7.
[20] Ciolino HP, Daschner PJ, Yeh GC. Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. Biochem J 1999;340:715–22.
[21] Ciolino HP, Wang TT, Yeh GC. Diosmin and diosmetin are agonists of the aryl hydrocarbon receptor that differentially affect cytochrome P450 1A1 activity. Cancer Res 1998;58:2754–60.
[22] Lu Y-F, Santostefano M, Cunningham DM, Threadgill MD, Safe S. Identification of 3′-methoxy-4′-nitroflavone as a pure aryl hydrocarbon receptor antagonist and evidence for more than one form of the Ah receptor in MCF-7 human breast cancer cells. Arch Biochem Biophys 1995;316:470–7.
[23] Willett KL, Gardinali PR, Serican JL, Wade TL, Safe S. Characterization of the rat H4IIE bioassay for evaluation of environmental samples containing polynuclear aromatic hydrocarbons (PAHs). Arch Environ Contam Toxicol 1997;32:442–8.
[24] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[25] Chen I, Safe S, Bjeldanes L. Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells. Biochem Pharmacol 1996;51:1069–76.
[26] Henry EC, Kende AS, Rucci G, Totleben MJ, Willey JJ, Dertinger SD, Pollenz RS, Jones JP, Gasiewicz TA. Flavone antagonists bind competitively with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to the aryl hydrocarbon receptor but inhibit nuclear uptake and transformation. Mol Pharmacol 1999;55:716–25.
[27] Gasiewicz TA, Kende AS, Rucci G, Whitney B, Willey JJ. Analysis of structural requirements for Ah receptor antagonist activity: ellipticines, flavones, and related compounds. Biochem Pharmacol 1996; 52:1787–803.
[28] Ciolino HP, Yeh GC. The steroid hormone dehydroepiandrosterone inhibits *CYP1A1* expression *in vitro* by a post-transcriptional mechanism. J Biol Chem 1999;274:35186–90.
[29] Wormke M, Stoner M, Saville B, Safe S. Crosstalk between estrogen receptor α and the aryl hydrocarbon receptor in breast cancer cells involves unidirectional activation of proteasomes. FEBS Lett 2000; 478:109–12.
[30] Gronbaek M, Deis A, Sorensen TIA, Becker U, Schnohr P, Jensen G. Mortality associated with moderate intakes of wine, beer, or spirits. BMJ 1995;310:1165–9.
[31] Hertog MGL, Feskens EJM, Hollman PCH, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 1993;342:1007–11.
[32] Rimm EB, Klatsky A, Grobbee D, Stampfer MJ. Review of moderate alcohol consumption and reduced risk of coronary heart disease: is the effect due to beer, wine, or spirits? BMJ 1996;312: 731–6.
[33] Safe S, Qin C, McDougal A. Development of selective aryl hydrocarbon receptor modulators (SARMs) for treatment of breast cancer. Expert Opin Invest Drugs 1999;8:1385–96.
[34] Sun G, Safe S. Antiestrogenic activities of alternate substituted polychlorinated dibenzofurans in MCF-7 human breast cancer cells. Cancer Chemother Pharmacol 1997;40:239–44.
[35] Seidel SD, Li V, Winter GM, Rogers WJ, Martinez EI, Denison MS. Ah receptor-based chemical screening bioassays: application and limitations for the detection of Ah receptor agonists. Toxicol Sci 2000;55:107–15.
[36] Mitchell SH, Zhu W, Young CY. Resveratrol inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells. Cancer Res 1999;59:5892–5.