Resveratrol Inhibits Transcription of CYPIA1 in Vitro by Preventing Activation of the Aryl Hydrocarbon Receptor

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ABSTRACT

Resveratrol, a compound present in a variety of plants, was recently shown to have potent chemopreventive activity against aryl hydrocarbon-induced tumorigenesis in mice. Therefore, in the present study, we examined the effect of resveratrol on the function of the aryl hydrocarbon receptor (AHR) and the transcription of CYPIA1 in human HepG2 hepatoma cells. Resveratrol inhibited the increase in cytochrome P450 (CYP) 1A1 mRNA caused by the AHR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in a concentration-dependent manner. The induction of transcription of an aryl hydrocarbon-responsive reporter vector containing the CYPIA1 promoter by TCDD was likewise inhibited by resveratrol. Resveratrol also inhibited the constitutive level of CYPIA1 mRNA and reporter vector transcription in HepG2 cells. The increase in CYPIA1 enzyme activity induced by TCDD was inhibited by resveratrol. Resveratrol prevented the TCDD-induced transformation of the cytosolic AHR to its nuclear DNA-binding form. However, resveratrol had no effect on the binding of TCDD to the cytosolic AHR. These data demonstrate that resveratrol inhibits CYPIA1 expression in vitro, and that it does this by preventing the transformation of the AHR to promoter sequences that regulate CYPIA1 transcription. This activity may be an important part of the chemopreventive activity of resveratrol.

INTRODUCTION

Resveratrol (trans-3,4',5-trihydroxystilbene; see Fig. 1 for structure) is a phytoalexin present in a wide variety of plant species, including mulberries, peanuts, and grapes (1), and thus is a constituent of the human diet. Vitis vinifera, or grapes, synthesizes resveratrol in response to fungal infections; thus, it is found in high concentrations in wine, particularly in red wine (2). The protective effect of wine consumption toward coronary heart disease has been attributed to the antioxidant and anticoagulation properties of resveratrol (3, 4). Resveratrol is present in wine, particularly in red wine (2). The protective effect of wine consumption toward coronary heart disease has been attributed to the antioxidant and anticoagulation properties of resveratrol (3, 4). Resveratrol has also been identified in a number of plants used in traditional Asian medicine (5).

Recently, resveratrol has been shown to inhibit cellular events associated with tumor development caused by the aryl hydrocarbon DMBA (6). Aryl hydrocarbons require metabolic activation to be genotoxic. The carcinogenic activation pathway is regulated by the AHR, a cytosolic protein present in a number of human tissues (7). Aryl hydrocarbons and their halogenated derivatives such as TCDD bind the AHR, causing it to translocate to the nucleus, where it heterodimerizes with another protein, aryl hydrocarbon nuclear translocator. Together, these proteins form a transcription factor that initiates the transcription of a number of genes (8). The best characterized response to ligand-activated AHR is the transcriptional induction of the CYPIA1 gene. CYPIA1 encodes the enzyme CYPIA1, which metabolizes aryl hydrocarbons to genotoxic metabolites that bind DNA and cause mutations (9, 10). Inhibition of carcinogen activation, either through the direct inhibition of activating enzymes or through the inhibition of the signal transduction leading to their transcriptional activation, is a characteristic of the so-called “blocking” type of chemopreventive agent (11).

In the present study, we examined the effects of resveratrol on AHR function and CYPIA1 transcription in human HepG2 hepatoma cells. Resveratrol potently inhibited the basal and TCDD-induced expression of CYPIA1, as assessed at the mRNA, transcription, and enzymatic levels. Resveratrol prevented the activation of the cytosolic AHR to its nuclear form capable of binding the XRE of CYPIA1, but it had no effect on TCDD binding to the receptor. These results indicate that resveratrol blocks CYPIA1 transcription in vitro by preventing the receptor from binding to the enhancer sequences of the CYPIA1 promoter that regulate the transcription of the gene. Such activity may be an important part of its chemopreventive ability.

MATERIALS AND METHODS

Materials. HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD), RPMI 1640, glutamine, fetal bovine serum, trypsin/EDTA, and PBS were from BioFluids (Rockville, MD). Resveratrol, HEPES, EDTA, DTT, glyceral, poly(deoxyinosinic-deoxyctydilic acid), sodium molybdate, ETRF, resorufin, Tris-HCl, and protease inhibitors were from Sigma (St. Louis, MO). TCDD was from Midwest Research Institute (Kansas City, MO). [3H]TCDD (specific activity, 28.4 Ci/mmol) was from ChemSyn (Lenexa, KS). [3H]HeP and [3H]ATP were from DuPont New England Nuclear (Boston, MA). RT-PCR was performed with a kit from Stratagene (La Jolla, CA). TBE gels, TBE running buffer, and high-density sample buffer were from Novex (San Diego, CA). LipofectAMINE and TRIzol reagent were from Life Technologies, Inc. (Rockville, MD). The CAT ELISA kit was from Boehringer Mannheim (Indianapolis, IN). Primers for GPDH PCR and pCMZ vector containing β-GAL were from Clontech (Palo Alto, CA). AquaSource and rotube were from Beckman (Palo Alto, CA). The Bradford protein assay kit was from Bio-Rad (Hercules, CA).

Cell Culture. HepG2 cells were grown in RPMI 1640 supplemented with 2 mm glutamine and 10% fetal bovine serum (growth medium). Cells were subcultured weekly using 0.25% trypsin and 0.05% EDTA. All experiments were carried out at 37°C and 5% CO2 on confluent cells in growth medium, except where indicated.

RT-PCR. Resveratrol and TCDD were dissolved in DMSO and stored at −70°C. Confluent HepG2 cells were treated with DMSO (control), resveratrol, or a combination of resveratrol and 1 μM TCDD for 6 h. The cells were washed twice with PBS, and total RNA was isolated using TRIzol reagent. Semiquantitative RT-PCR for CYPIA1 and GPDH was performed in the presence of 1.5 μCi of [32P]dATP (DuPont New England Nuclear, Wilmington, DE) using the primer sequences and conditions described by Dohr et al. (12). cDNA was synthesized from 10 μg of total RNA using a Stratagene RT-PCR kit as instructed. The optimum cycle number that fell within the exponential range of response for CYPIA1 (23 cycles) or GPDH (19 cycles) was used. After PCR, 5 μl of high-density sample buffer were added to the samples, and they were subjected to electrophoresis on a 10% TBE gel in 1× TBE running buffer. The gel was dried, and the results were visualized and quantified on a Bio-Rad

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3 The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; AHR, aryl hydrocarbon receptor; β-GAL, β-galactosidase; CAT, chloramphenicol acetyltransferase; CYP, cytochrome P450; EMSA, electrophoretic mobility shift assay; ETRF, ethoxyresorufin; EROD, ETRF-0-deethylase; GPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; TBE, Tris-borate EDTA; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XRE, xenobiotic responsive element.
complexes were visualized on a Bio-Rad GS-363 Molecular Imaging System. Graphs of the resulting data were generated by normalizing CYP1A1 to GPDH.

CAT/β-GAL Assays. HepG2 cells were plated at 60,000 cells/well in 24-well plates. After 24 h, the cells were transiently transfected with 12.0 μg of a CAT reporter vector containing the full-length rat CYP1A1 promoter (pMC6.3K; Ref. 13) using LipofectAMINE as directed. To control for transfection efficiency, the cells were cotransfected with 1.0 μg of PCMV vector containing β-GAL. After an additional 24 h, the cells were treated with 1 nm TCDD in the presence of DMSO (control), resveratrol, or a combination of resveratrol and 1 nm TCDD for 6 h. The amount of CAT transcription was determined by measuring enzyme activity by the method of Rosenthal (14). The amount of CAT transcription was normalized to β-GAL transcription.

CYP1A1 Activity in Intact HepG2 Cells. HepG2 cells in 24-well plates were treated with 1 ml of growth medium containing 1 nm TCDD for 9 h in the presence of DMSO or the indicated concentrations of resveratrol. At the end of the incubation, the medium was removed, and the wells were washed two times with fresh growth medium. EROD activity, which is a specific assay of the bioactivation capacity of CYP1A1/CYP1A2, was determined in intact cells as described by Kennedy and Jones (15) using 5 μM ETRF in growth medium as a substrate in the presence of 1.5 mM salicylamide to inhibit conjugating enzymes. The assay was carried out at 37°C. The fluorescence of resorufin generated by the conversion of ETRF by CYP1A1 was measured every 10 min for 60 min in a CytoFluor II multiwell fluorescence plate reader (PerSeptive Biosystems, Framingham, MA) with an excitation wavelength of 530 nm and an emission of 590 nm.

EMSA for AHR XRE Binding. Confluent cultures of HepG2 cells were treated with DMSO, 10 nm TCDD, 10 μM resveratrol, or 10 nm TCDD in the presence of 2.5 or 10 μM resveratrol in growth media for 3 h. Nuclear protein was isolated, and EMSA was performed by the method of Denison et al. (16). Synthetic oligonucleotides containing the AHR-binding site of the XRE of CYP1A1 (17) were labeled with [32P]dCTP. The binding reactions were carried out for 30 min and contained 5 μg of nuclear protein, 1 μg of poly(deoxyinosinic-deoxycytidylic acid), and ~50,000 cpm of labeled probe in a final volume of 20 μl of binding buffer [25 mM Tris (pH 7.9), 50 mM KCl, 1 mM MgCl2, 1.5 mM EDTA, 0.5 mM DTT, and 5% glycerol]. To determine the specificity of binding to the oligonucleotide, a 200-fold excess of unlabeled specific probe was added to the extract from TCDD-treated cells. DNA-protein complexes were separated under nondenaturing conditions on a 4% polyacrylamide gel using 0.5X TBE [45 mM Tris (pH 7.5), 45 mM boric acid, and 2 mM EDTA] as a running buffer. The gels were dried, and the DNA-protein complexes were visualized on a Bio-Rad GS-363 Molecular Imaging System.

AHR Ligand Binding Assay. HepG2 cells were grown to confluence in 175-cm² flasks. The cells were washed once in PBS, harvested by trypsinization, and pelleted by centrifugation at 800 × g for 10 min at 4°C. The pellet was washed once in cold PBS, resuspended as described above, and resuspended in cold buffer [25 mM HEPES, 1 mM EDTA, 1 mM DTT, 20 mM sodium molybdate, and 10% glycerol (pH 7.4)] with protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 300 μg/ml EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, and 0.7 μg/ml pepstatin A). The cells were homogenized by 30 strokes with a Dounce glass homogenizer on ice, and the homogenate was centrifuged at 100,000 × g for 60 min at 4°C. The supernatant (cytosol) was removed, and protein was assayed by the Bradford method (18). The cytosol was aliquoted and stored at −70°C. Specific binding to the AHR was measured by scintillation counting centrifugation as described by Raha et al. (19). Cytosolic protein (1 mg) was incubated with 10 nm [3H]TCDD in the presence of DMSO (control), 10 μM unlabeled TCDD (positive control), or 50 μM resveratrol in a total volume of 500 μl of the above buffer for 2 h at 4°C. Samples were applied to 5-30% (w/v) linear sucrose density gradients in 12-nl Beckman Quick-Seal rotor tubes. The gradients were centrifuged for 2 h at 63,000 rpm (372,000 × g) in a Beckman VTI-65-1 rotor. Twenty-five fractions of seven drops each (~500 μl) were collected from the bottom of the tubes and assayed for radioactivity using Aquasure scintillation fluid.

RESULTS

Resveratrol Inhibits CYP1A1 mRNA Levels. Confluent cultures of HepG2 human hepatoma cells were treated with DMSO (control) or the prototypical AHR ligand TCDD for 6 h. The relative levels of CYP1A1 and GPDH mRNA were determined by a semiquantitative RT-PCR. Treatment of HepG2 cells with 1 nm TCDD caused an approximately 9-fold increase in CYP1A1 mRNA compared to DMSO-treated cells (Fig. 2). In cells cotreated with resveratrol, this increase in CYP1A1 mRNA was inhibited in a concentration-dependent manner. In cultures treated with TCDD and the highest concentrations of resveratrol tested (10 or 20 μM), CYP1A1 mRNA was inhibited to a similar degree as with 10 nm TCDD alone.
Resveratrol Inhibits CYP1A1 Transcription.

Resveratrol Inhibits CYP1A1 Promoter-driven Transcription. HepG2 cells were transiently transfected with an aryl hydrocarbon-responsive CAT reporter vector that contains the full-length CYP1A1 promoter. Transcription of this vector is increased in response to known AHR ligands in their rank order of affinity for the receptor (TCDD > 3-methylcholanthrene > benzo(a)pyrene > DMBA; data not shown). The treatment of transfected cells with 1 nM TCDD for 6 h resulted in a 10-fold increase in CAT transcription. Cotreatment with resveratrol inhibited TCDD-induced CAT transcription in a concentration-dependent manner (Fig. 4). At 20 \( \mu \text{M} \) resveratrol, CAT transcription was only 27% of the control levels.

There was also a low but detectable level of CAT transcription in cells transfected with the aryl hydrocarbon-responsive reporter vector in the absence of any treatment. The treatment of transfected cells with resveratrol in the absence of any other treatment resulted in a concentration-dependent decrease in the basal level of CAT transcription of the CYP1A1 promoter vector (Fig. 5).

Resveratrol Inhibits CYP1A1 Enzyme Activity in Intact Cells. CYP1A1 enzyme activity in intact HepG2 cells was measured by the EROD assay, which is specific for CYP1A1/CYP1A2 activity. The treatment of these cells with AHR ligands such as TCDD results in a concentration-dependent increase in cellular EROD activity (data not shown). Treatment of the cells with 1 nM TCDD for 9 h resulted in an EROD activity of 18.04 ± 0.28 pmol/min/well. This increase was inhibited in a concentration-dependent manner by cotreatment with resveratrol, with an IC\(_{50}\) of less than 1 \( \mu \text{M} \) (Fig. 6). Complete inhibition of TCDD-induced EROD activity was seen in cultures treated with ≥5 \( \mu \text{M} \) resveratrol.

Effect of Resveratrol on the Transformation of the AHR. Upon ligand binding, the cytosolic AHR is transformed to its nuclear form, which gains the ability to bind the XRE. Using EMSA with a labeled oligonucleotide representing the XRE sequence of CYP1A1, we examined the effect of resveratrol on the transformation of the AHR by TCDD and its translocation to the nucleus. As seen in Fig. 7, there was a detectable amount of AHR XRE-binding activity in nuclear extracts from untreated cells (Lane 1), as has been reported in other cell lines (20–22). This agrees with the data presented above, which demonstrates that there is a low level of CYP1A1 expression in untreated cells. The treatment of cells with 20 nM TCDD caused an increase in the intensity of the band shift (Lane 2). Cells treated with 10 \( \mu \text{M} \) resveratrol in the absence of any other treatment had no detectable XRE-binding capacity (Lane 3). Nuclear extracts from cells cotreated with TCDD and 2.5 \( \mu \text{M} \) resveratrol showed a partial inhibition of the band shift (Lane 4); in extract from cells treated with TCDD plus 10

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Fig. 3. Inhibition of constitutive CYP1A1 mRNA levels by resveratrol. Cells were treated with DMSO (control) or the indicated concentrations of resveratrol for 6 h, and RT-PCR for CYP1A1 and GPDH mRNA was performed as described previously, except that the cycle number was increased from 23 to 25 to facilitate detection. In the bar graph, the amount of CYP1A1 mRNA was normalized to the amount of GPDH mRNA. \( n = 3 \); bars, SE.

Fig. 4. Resveratrol inhibition of TCDD-induced CAT transcription. HepG2 cells were transfected with an aryl hydrocarbon-responsive reporter vector containing the full-length CYP1A1 promoter and treated with DMSO (control) or 1 nM TCDD in the presence of the indicated concentrations of resveratrol for 6 h. The amount of CAT transcription was normalized to the transcription of \( \beta \)-GAL. \( n = 4 \); bars, SE.

Fig. 5. Resveratrol inhibition of basal CAT transcription. Cells transfected with the aryl hydrocarbon-responsive reporter vector were incubated with DMSO (control) or resveratrol for 6 h, and the relative CAT transcription was determined as described. \( n = 4 \); bars, SE.

Fig. 6. Resveratrol inhibition of TCDD-induced EROD activity. HepG2 cells were treated with DMSO (control) or resveratrol for 9 h, and EROD activity was measured. \( n = 3 \); bars, SE.
μM resveratrol, there was a complete inhibition of AHR binding activity (Lane 5). Specificity is shown by the lack of the band shift in Lane 6, in which the nuclear extract of TCDD-treated cells was incubated with an excess of unlabelled XRE in addition to the labeled probe. This band shift was also inhibited by preincubation of the nuclear extract with a polyclonal antibody to the AHR (data not shown). Two other cell preparations were made under identical conditions; Fig. 7 is a typical EMSA.

**Effect of Resveratrol on TCDD Binding to the Cytosolic AHR.**

The ability of resveratrol to prevent the transformation of the AHR to its DNA-binding form led us to examine the effect of resveratrol on the binding of TCDD to the cytosolic receptor. Cytosol of untreated HepG2 cells was incubated for 2 h with [3H]TCDD in the presence of a 1000-fold excess of unlabelled TCDD as a positive control or a 5000-fold excess of resveratrol. Specific binding of [3H]TCDD to the AHR was then analyzed by sucrose density gradient centrifugation. As shown in Fig. 8, unlabeled TCDD substantially inhibited [3H]TCDD binding, but resveratrol had no effect on binding. This experiment was performed three times; results of these other experiments were essentially identical to those shown in Fig. 8. The effect of resveratrol on specific [3H]TCDD binding was also analyzed by hydroxyapatite chromatography (23) with similar results.

**DISCUSSION**

Strong epidemiological evidence indicates that the consumption of diets rich in plant materials is associated with a reduced risk of cancer (24, 25). A central tenet of current chemoprevention theory is that minor dietary constituents, particularly from plant sources, may exert profound biological effects that thereby inhibit carcinogenesis (26–28). Chemoprevention may occur at any of the three stages of carcinogenesis (initiation, promotion, and progression), and putative chemopreventive agents have been classified according to the stage(s) that they affect (29). Several phytochemicals that have chemopreventive activity in animal models of carcinogenesis have been identified, including flavonoids (30–32), curcumin (23), indole-3-carbinol (33), and retinoids (34, 35). Although these compounds have a multiplicity of biochemical activities that may affect carcinogenesis, they have also been shown to affect carcinogen activation through interactions with the AHR or by directly inhibiting CYP1A enzyme activity (36–39). Because the enzymatic activity of the CYP1A family, particularly CYP1A1, catalyzes the metabolic activation of aryl hydrocarbons to genotoxic forms, inhibition of CYP1A1 expression or activity may be chemopreventive. Such blocking activity inhibits carcinogenesis at the initiation stage by preventing the formation of genotoxic metabolites or by increasing detoxification mechanisms. Recently, the polyphenolic dietary compound resveratrol was shown to prevent the initiation of tumorigenesis induced in mice by the aryl hydrocarbon DMBA (6). Because the tumorigenic properties of aryl hydrocarbons are a direct result of their metabolism by CYP1A enzymes, the transcription of which is induced by the AHR, we examined the effects of resveratrol on AHR function and CYP1A expression. This study was carried out in human HepG2 hepatoma cells, which have been extensively used as a model system to investigate the regulation of CYP1A expression (40–43). We used TCDD in these experiments because it is the most potent AHR ligand known, and its effects on CYP1A transcription and AHR function have been well studied (8, 9, 12, 17). To our knowledge, there has been no study on the effect of resveratrol on the signal transduction pathway leading to carcinogen activation.

Resveratrol inhibited the expression of CYP1A1 induced by TCDD. This is indicated by a concentration-dependent decrease in the amount of CYP1A1 mRNA (Fig. 2), CYP1A1 promoter-controlled transcrip-
The existence of a second AHR binding site would also be consistent with the results of experiments showing that resveratrol inhibits AHR function without altering ligand binding by affecting one or more of the AHR-interacting protein (53), all of which have been shown to affect the DNA-binding capacity of the AHR. Furthermore, in addition to inhibit AHR function without altering ligand binding by affecting one or more of the AHR-interacting protein (53), all of which have been shown to affect the DNA-binding capacity of the AHR. Furthermore, in addition to inhibit AHR function without altering ligand binding by affecting one or more of these proteins.

Further study will be required to determine how resveratrol inhibits the DNA-binding capacity of the AHR. Furthermore, in addition to CYP1A1, the AHR also mediates the transcription of a number of genes (the so-called “AH-battery”) that are involved in carcinogen detoxification (54). The effect of resveratrol on their expression is unknown. The results of the current work demonstrate that resveratrol potently inhibits CYP1A1 expression in vitro, and that it exerts this effect by preventing AHR activation and XRE binding. This activity may be responsible for the chemopreventive effect of resveratrol (6), in that resveratrol may inhibit the activation and DNA-adduct formation in vivo of those carcinogens that require CYP1A1 enzyme activity for their mutagenic or genotoxic effect. Thus far, only one study demonstrating the inhibitory activity of resveratrol on carcinogen-induced tumorigenesis has been published (6). Further study is needed to determine the full extent of the inhibitory activity of resveratrol on AHR-mediated CYP1A1 transcription.
chemopreventive effect of resveratrol in animal models of tumorigenesis.

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