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Resveratrol Has Antagonist Activity on the Aryl Hydrocarbon Receptor: Implications for Prevention of Dioxin Toxicity

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ABSTRACT

Aryl hydrocarbon receptor (AhR) ligands such as dioxin and benzo[*a*]pyrene are environmental contaminants with many adverse health effects, including immunosuppression, carcinogenesis, and endothelial cell damage. We show here that a wine component, resveratrol (3,5,4′-trihydroxystilbene), is a competitive antagonist of dioxin and other AhR ligands. Resveratrol promotes AhR translocation to the nucleus and binding to DNA at dioxin-responsive elements but subsequent transactivation does not take place. Resveratrol inhibits the transactivation of several dioxin-inducible genes including cytochrome P-450 1A1 and interleukin-1β, both ex vivo and in vivo. Resveratrol has adequate potency and nontoxicity to warrant clinical testing as a prophylactic agent against aryl hydrocarbon-induced pathology.

Dioxins and other aryl hydrocarbon receptor (AhR) ligands, such as polycyclic aromatic hydrocarbons (PAHs), are environmental toxicants generated by the chemical industry. They are present in air pollution from industrial furnace gas and cigarette smoke. Many AhR ligands, especially the halogenated ones, have a long biologic half-life in the human body (up to 4 to 12 years in body fat for dioxins and dibenzofurans), resulting in cumulative increases in body burden. The PAHs in cigarette smoke, such as benzo[*a*]pyrene (BaP) and anthracene derivatives, and dioxins have been shown to cause immunosuppression (Kerkvliet, 1995) and/or endocrine disruption (Safe et al., 1998). They have been linked to cancer and ischemic heart disease in several epidemiological studies (Flesch-Janys et al., 1995; Boyle, 1997; Bertazzi et al., 1998).

In the process of screening for dioxin antagonists that have potential uses in human medicine, we noticed that red wine contains a variety of phenolic compounds, several of which had close structural homology to flavonoid ligands of the AhR with antagonistic abilities. We thus considered the possibility that a polyphenolic component of red wine might have antagonistic activity on the AhR. In the present study, we demonstrate that the trihydroxystilbene resveratrol (3,5,4′-trihydroxystilbene), found in red wine, is a pure AhR competitive antagonist and has the requisite properties of potency and nontoxicity to warrant clinical testing as a possible prophylactic agent against aryl hydrocarbon-induced pathology.

Materials and Methods

Chemicals. TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) was a generous gift from Dr. S. Safe, Texas A&M University, College Station, TX). 2,3,7,8-tetrachloro[1,6-^{3}H]dibenzo-*p*-dioxin (TC[1,6-^{3}H]DD; specific activity, 28 Ci/mmol) was purchased from Terrachem (Lenexa, KS). Dioxin stock solutions were initially dissolved in dimethyl sulfoxide and handled under a fume hood. TCDD stock was subsequently diluted in ethanol for use in experiments described below. All TCDD containing waste was treated according to French regulations. All other chemicals were purchased from Sigma Chemicals (St. Quentin, France).

Transfection and Reporter Assay. T-47D cells were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and 0.6 U/ml insulin. They were stably transfected with a construct bearing a single dioxin response element linked to the thymidine kinase promoter and the chloramphenicol acetyltransferase reporter gene (DRE-TK-CAT) with Superfect (Quiagen, Courtaboeuf, France) as the DNA carrier. Selected clonal colonies were grown in the

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; PAH, polycyclic aromatic hydrocarbons; BaP, benzo[*a*]pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TC[1,6-^{3}H]DD, 2,3,7,8-tetrachloro[1,6-^{3}H]dibenzo-*p*-dioxin; DRE, dioxin responsive element; CAT, chloramphenicol acetyl transferase; TK, thymidine kinase promoter; α-NF, α-naphthoflavone; NQOR, NAD(P)H quinone oxidoreductase; Il-1β, interleukin-1β; GFP, green fluorescent protein; DMBA, 7,12-dimethylbenzanthracene; I3C, indole-3-carbinol; LTR, long terminal repeat.

presence of 0.2 mg/ml geneticin (G418). Assays for TCDD inducibility of the integrated DRE-TK-CAT construct were performed after a 48-h treatment with dioxin, plus or minus red wine components as described in the text. All chemicals applied to the cells were diluted in ethanol; control cells received ethanol alone. CAT expression was assayed on whole cell extracts (100 μg of protein) with a CAT enzyme-linked immunosorbent assay (Roche, Meylan, France) according to supplier's specifications. Experiments were done in quadruplicate. For transient experiments, CAT vector DNA was transfected with Superfect according to supplier's recommendations.

NAD(P)H Quinone Oxidoreductase Assay. T-47D cells were incubated for 72 h in the presence of drugs [control, ethanol alone; TCDD, dioxin (10^{-9} M); Res, resveratrol (5×10^{-6} M); α-NF, α-naphthoflavone (10^{-6} M)], and NQOR activity was measured as described previously (Montano and Katzenellenbogen, 1997). Enzyme activity was calculated as nanomoles of cytochrome *c* reduced per minute. Specific quinone reductase activity is expressed as units (nanomoles of cytochrome *c* reduced per minute) per mg of protein (U/mg).

Western Blotting Experiments. Cells were treated with drugs as described in the figure legends. They were then collected and washed in cold PBS buffer and lysed by repeated freeze-thawing in isotonic conditions [20 mM HEPES, pH 7.6, 100 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, with Complete protease cocktail (Roche, Meylan, France)]. Total extract proteins (75 μg) were submitted to denaturing polyacrylamide gel electrophoresis. The gel was electrically blotted on a polyvinylidene difluoride membrane (Dupont, Paris, France). The membrane was saturated with 5% nonfat dry milk and incubated with various antibodies, including: a rabbit polyclonal antibody against cytochrome P-450 1A1 (CYP1A1; Daiichi Pure Chemicals Co., Tokyo, Japan), a goat polyclonal antibody against interleukin-1β (Il-1β; Santa Cruz Biotechnologies, Santa Cruz, CA) ,or a mouse monoclonal antibody against the AhR (Affinity Bioreagents Inc., Golden, CO) all at 1 μg/ml. Immune complexes were detected by chemiluminescence with the Enhanced Chemiluminescence kit (Amersham France, Les Ulis, France) or Ultra Super Signal (Pierce, Paris, France) as suggested by manufacturers.

Receptor binding competition assay was performed with rabbit liver cytosol as receptor source. Rabbit liver cytosol was prepared at 4°C in 20 mM HEPES, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, with Complete protease cocktail (Roche, Meylan, France) (cytosol buffer) by homogenization in a Ultra-Turax homogenizer (Bioblock Scientific, Illkirch, France) followed with 20 strokes in a Dounce Homogenizer. The homogenate was centrifuged 15 min at 15,000*g*. The supernatant was then centrifuged at 105,000*g* for 65 min. The cytosol was aliquoted and kept at −80°C. Cytosol was thawed and adjusted at 0.6 mg/ml in cytosol buffer for binding assays. Diluted cytosol (1 ml) was incubated with 2 nM TC[1,6-^{3}H]DD in presence of the desired amounts of competitors for 4 h at 4°C. Nondisplaceable binding was then assessed by incubating aliquots with 100 μl of a 2% activated charcoal suspension in cytosol buffer for 90 min at 4°C, followed by centrifugation at 15,000*g* for 10 min. The supernatants (500 μl) were counted in 5 ml of Ultima Gold cocktail (Packard, Meriden, CT) in a Beckman liquid scintillation counter (45% counting efficiency). Binding competition assays were repeated at least twice for each competitor and each point was performed in duplicate.

Whole-Cell Binding Assay. Cells were plated in 6-well culture dishes in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and 0.6 U/ml insulin. At 70% confluency, cells were rinsed and established in 2 ml of fresh medium. Binding was performed in the CO_2 incubator at 37°C in two steps. Competitors alone were added for 1 h, then 5 nM TC[1,6-^{3}H]DD was added and incubation was continued for 3 h. Cells were then taken at 4°C and washed 4 times for 10 min with 2 ml of PBS containing 0.5 mg/ml BSA at 4°C. Cells were lysed in 800 μl of cytosol buffer containing 1% Nonidet P-40. Protein contents was measured and aliquots were counted for radioactivity as described above.

In Vitro DNA Binding. Gel retardation experiments were performed with T-47D cell extracts. Cells were treated with drugs as indicated in the figure legend (Fig. 3) for 60 min in the CO_2 incubator. Cells were then collected, washed with PBS, and lysed by three freeze-thaw cycles in the cytosol buffer described above. After centrifugation at 15,000*g* for 20 min at 4°C, the supernatant was saved. The crude nuclear pellet was extracted 10 min on ice by three volumes of cytosol buffer plus 0.6 M KCl. After centrifugation, both supernatants were mixed. The mix was adjusted to 60 mM final concentration, and used in the gel retardation assay as described by Cuthill et al. (1991). The probe used for gel retardation was a 35-base-pair oligonucleotide bearing a single DRE: 5′-AGCTTAGCTAGGCG**TTGCGTGAGA**AGGACCG-3′

Nuclear Translocation. In situ visualization of green fluorescent protein (GFP)-tagged AhR transfected in T-47D cells grown on coverslips was performed as described previously (Chang and Puga, 1998). Cells were treated for 90 min with the indicated drugs.

In Vivo Antagonism Experiments. Female Sprague-Dawley rats (3 to 6 months old) were assembled in groups of four rats and treated with a s.c. injection [group 1, olive oil (control); group 2, 5 mg/kg BaP/7,12-dimethylbenz[*a*]anthracene (DMBA); group 3, 1 mg/kg BaP/DMBA; group 4, 0.5 mg/kg BaP/DMBA] to determine optimal conditions of CYP1A1 induction (not shown). For in vivo competition, female Sprague-Dawley rats (3 to 6 months old) were assembled in groups of three and treated by s.c. injection with the following: 1 mg/kg BaP/DMBA; 1 mg/kg BaP/DMBA and 1 mg/kg resveratrol; 1 mg/kg BaP/DMBA and 5 mg/kg resveratrol; 5 mg/kg resveratrol alone; and vehicle (olive oil) alone for the control animals. Injections were performed on days 1 and 7. Rats were sacrificed by carbon dioxide exposure on day 11. Lungs and kidneys were removed and snap frozen in liquid nitrogen. Tissue samples were homogenized in isotonic conditions as described above. Aliquots (30–60 μg of protein per lane) were submitted to polyacrylamide denaturing gel electrophoresis. β-Actin was added to monitor loading variability. Western blotting was done as described above.

Results

Resveratrol Antagonizes AhR-Mediated Transactivation. The model system used in our initial experiments consisted of an AhR-positive human breast cancer cell line (T-47D) stably transfected with a DRE linked to the TK and the CAT. Representative wine compounds such as flavonoids (epicatechin, quercetin), phenolic acids (*p*-coumaric, caffeic, and vanillic acids), and the trihydroxystilbene resveratrol were added to the cells alone or in the presence of TCDD, the prototypical AhR ligand.

Resveratrol, in the micromolar range, elicited a dose response inhibition of dioxin-mediated transactivation (Fig. 1, A and B) without any apparent agonistic activity (data not shown). In contrast, none of the other compounds tested had any AhR antagonistic activity. Quercetin displayed moderate agonistic effects at 10^{-6} M (data not shown). Challenging the cells with the various drugs used in all described experiments did not modify the level of expression of AhR in either wild-type T-47D cells or in the stably transfected cell line, as observed by Western blot (data not shown). The effect of resveratrol on AhR-mediated transactivation seems quite specific: resveratrol modified neither the transactivation of a retinoic-responsive CAT construct by all-*trans*-retinoic acid, nor that of a progesterone-responsive CAT construct by synthetic progestins (data not shown). Resveratrol displayed very limited estrogenic ability on a CAT construct bearing the vitellogenin A2 estrogen responsive element: 10 μM res-

veratrol elicited a response equivalent to that of 2×10^{-11} M estradiol (data not shown).

The observed effect of resveratrol in this gene transfection model system suggested that it may exert antagonistic activity on the induction in vivo of genes known to be regulated by AhR ligands such as dioxins. We therefore performed further experiments to analyze the mechanism of action of resveratrol.

Resveratrol Binds and Translocates AhR to the Nucleus. Whole-cell binding competition experiments were performed on T-47D cells and HepG2 cells. Resveratrol displaced TC[1,6-^{3}H]DD from the human AhR with an EC_{50} of 10^{-7} M in both cell lines (Fig. 2A). We extended this result by performing in vitro binding competition experiments with rabbit liver cytosol as the source of AhR and comparing various dioxin competitors (Fig. 2B): In this case, Resveratrol displaced TC[1,6-^{3}H]DD from the rabbit AhR with an EC_{50} of 6×10^{-6} M. The competition efficiency of resveratrol in this latter model was lower than α-NF ($EC_{50} = 5 \times 10^{-9}$ M) but higher than that of indole-3-carbinol (I3C) ($EC_{50} = 6 \times 10^{-5}$ M), an established natural AhR ligand (Bjeldanes et al., 1991).

Gel retardation experiments showed that TCDD, resveratrol, and α-NF all induced to a similar degree the binding of AhR to DNA (Fig. 3). Binding was specific because it was suppressed by a 100-fold excess of unlabeled oligonucleotide or the addition of an anti-AhR antibody during preincubation. Neither resveratrol nor α-NF inhibited TCDD-mediated AhR DNA binding. The inhibitory activity of resveratrol therefore takes place during the interaction between AhR and the transcriptional complex. We confirmed this result by visualizing in situ the resveratrol-AhR complex. To this effect, we used a GFP-tagged AhR vector (Chang and Puga, 1998) transiently expressed in wild-type T-47D cells. As shown in Fig. 4, resveratrol induced AhR nuclear translocation in a manner similar to that of dioxin and α-NF.

Resveratrol Blocks TCDD-Mediated Induction of a Variety of Genes. TCDD-sensitive genes comprise Phase I and Phase II genes, as well as an array of various other genes. Phase I genes are the cytochromes P-450 1A1, 1A2, and 1B1. CYP1A1 is the major inducible enzyme responsible for the oxidative metabolism of PAHs and other xenobiotics. The products of this metabolism include reactive oxygen species (Park et al., 1996; Shertzer et al., 1998) and, in the case of PAHs, proximate carcinogenic metabolites (Okey et al., 1994) and DNA adducts (Arif et al., 1999; Lagueux et al.,

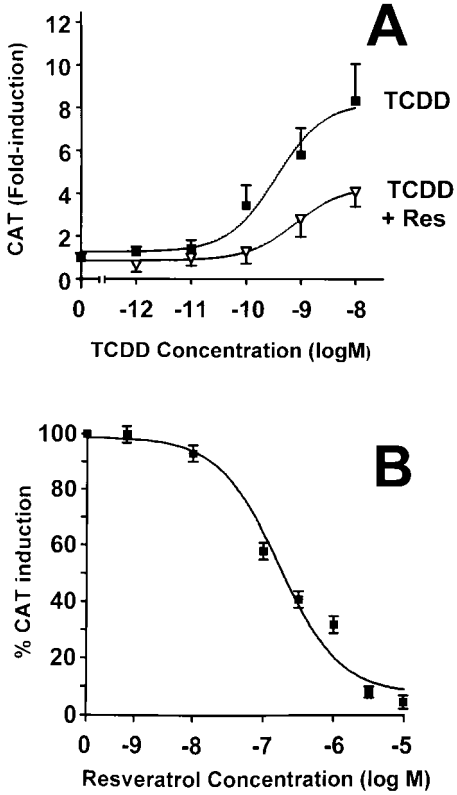

Fig. 1. Resveratrol inhibits TCDD-mediated transactivation in a dose-dependent manner. A, T-47D cells stably transformed with the DRE-TK-CAT construct (clone DRE82) were treated for 48 h with dioxin at concentrations from 10^{-12} to 10^{-8} M, alone (TCDD, ■) or in presence of 10^{-6} M resveratrol (TCDD + Res, △). CAT assays are represented as mean ± S.E. of four experiments. B, Cells were treated with 10^{-9} M TCDD alone or in the presence of resveratrol at concentrations ranging from 10^{-9} to 10^{-5} M. 100% TCDD was obtained by comparison of cells treated with 10^{-9} M TCDD versus control cells receiving 0.1% ethanol.

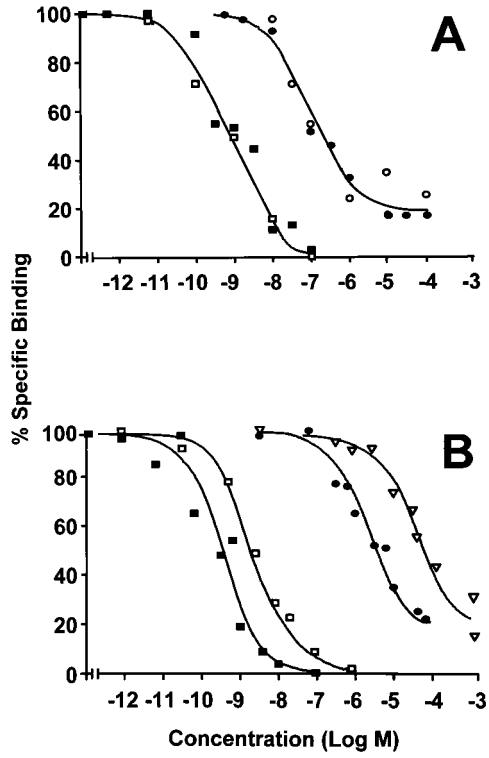

Fig. 2. Binding competition assays for the AhR. Whole-cell and in vitro cytosol binding competition assays with labeled dioxin are described in *Materials and Methods*. A, whole-cell binding competition assay performed with labeled dioxin (5 nM) versus unlabeled dioxin (■, □) or resveratrol (●, ○) on T-47D cells (■, ●) or HepG2 (□, ○). In both cell lines, resveratrol displaces labeled dioxin with an EC_{50} value of 10^{-7} M. Total TCDD binding in T-47D averaged 3150 dpm/mg whole-cell protein, of which 43% remained bound in the presence of 200 nM unlabeled TCDD and was considered nonspecific binding. With a specific activity of 4937 dpm per picomole of TC[1,6-^{3}H]DD, specific binding is 360 fm/mg protein in T-47D cells (150 fm/mg protein in HepG2 cells). B, in vitro binding competition assay for rabbit liver cytosol AhR of TC[1,6-^{3}H]DD (2 nM) versus unlabeled dioxin (TCDD), resveratrol (Res), α-NF, and I3C. Resveratrol displaces TC[1,6-^{3}H]DD with a EC_{50} value of 6×10^{-6} M, lower than α-NF ($EC_{50} = 5 \times 10^{-9}$ M) but higher than I3C ($EC_{50} = 6 \times 10^{-5}$ M). Total binding in 950 μl of cytosol was 1800 dpm (3160 dpm/mg protein), of which 27% was nonspecific binding. With a specific activity of 4937 dpm per picomole, the specific binding corresponds to 467 fmol of AhR per mg cytosolic protein from rabbit liver.

1999). We tested the ability of resveratrol to block TCDD-mediated CYP1A1 protein production in T-47D cells by Western blot analysis. A 10^{-6} molar concentration of Resveratrol blocked TCDD (10^{-10} M) induction of the CYP1A1 protein (Fig. 5). The inhibitory activity of resveratrol was slightly lower than that of α-NF (Santostefano et al., 1993; Wilhelmsson et al., 1994).

Phase II genes comprise a collection of conjugating enzymes, such as glutathione-*S*-transferase, UDP-glucuronyl transferase and NQOR. These enzymes are principally involved in the metabolization of xenobiotics (including AhR ligands) to more readily excreted water-soluble metabolites (Montano and Katzenellenbogen, 1997). Neither resveratrol nor α-NF inhibited dioxin-mediated induction of NQOR activity in T-47D cells (Fig. 6).

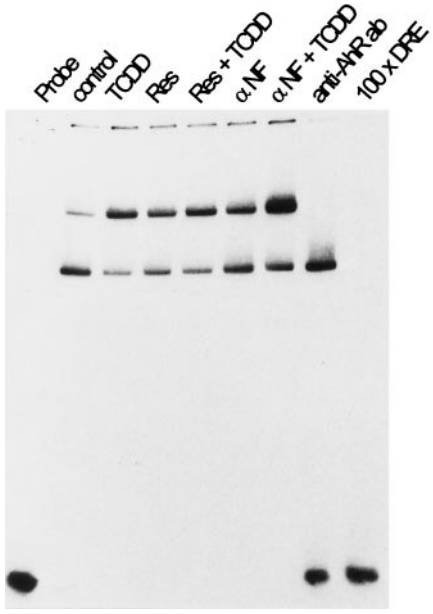

Fig. 3. Resveratrol elicits binding of AhR to specific DNA and does not inhibit dioxin-mediated DNA binding. T-47D cells were treated for 1 h with dioxin (TCDD) at 10^{-9} M, resveratrol (Res) at 5×10^{-6} M, α-NF at 10^{-6} M alone or in combinations as indicated. Cell extracts were processed for gel retardation electrophoresis in nondenaturing conditions. The arrow points to the specific retardation band. The lower band is a nonspecific species consistently observed in the literature (Cuthill et al., 1991; Wilhelmsson et al., 1994).

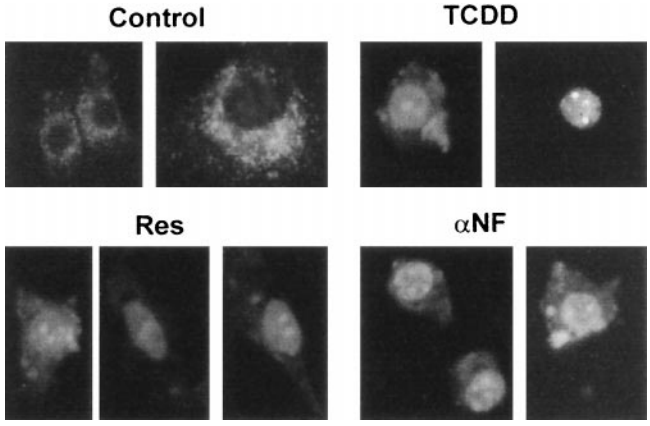

Fig. 4. Resveratrol elicits nuclear translocation of the dioxin receptor similar to dioxin and α-NF. T-47D cells grown on coverslips were transiently transfected by the AhR-GFP plasmid (Chang and Puga, 1998). After 48 h, they were treated for 90 min with TCDD at 10^{-9} M (TCDD), resveratrol at 5×10^{-6} M (Res) or α-NF at 10^{-6} M as indicated on the figure. Two representative cells are shown for each experimental condition. Two hundred cells were examined in each condition. Nuclear fluorescence exceeded cytoplasmic fluorescence in 4% of control cells, 70% of dioxin-treated cells, 50% of α-NF -treated cells, and 71% of resveratrol-treated cells.

Besides phase I and phase II genes, several other genes have been shown to be TCDD-sensitive (Lai et al., 1996). We examined the effects of TCDD, alone or in the presence of resveratrol on two of these genes: the promoter in the HIV-1 long terminal repeat (LTR) and Il-1β. The promoter in the LTR of HIV-1 contains a consensus DRE. TCDD-inducibility of HIV expression, and TCDD activation of latent HIV-1 expression and replication has been reported (Gollapudi et al., 1996) We investigated the antagonist effect of resveratrol by using a construct bearing the promoter of the HIV-1 LTR linked to the CAT gene (Israel et al., 1989). When T-47D cells transiently transfected with this construct were challenged with 10^{-9} M TCDD, a 3-fold induction of CAT activity was observed (Fig. 7). The addition of 5.10^{-6} M resveratrol completely abolished the transactivation of the HIV LTR by TCDD.

TCDD is known to increase the expression of Il-1β in rat liver keratinocytes (Sutter et al., 1991) and the endometrial cell line RL95–2 (Charles and Shiverick, 1997). The ability of TCDD to induce both the reactive oxygen species-generating CYP1A1 as well as the proinflammatory Il-1β prompted us to hypothesize that AhR might mediate the inflammatory effects displayed by its ligands on the cardiovascular system (Toborek et al., 1995). We therefore analyzed the ability of resveratrol to inhibit TCDD-mediated Il-1β overexpression. The choice of the protein rather than the mRNA for pre–Il-1β as the experimental endpoint of AhR activation, was dictated by the known dissociation between Il-1β transcription and translation. Induction of pre–Il-1β overexpression was less sensitive to dioxin than CYP1A1 with 5×10^{-9} M TCDD required to give a clear-cut signal. As can be seen in Fig. 5, resveratrol (5×10^{-6} M) efficiently repressed the stimulatory effects of 5.10^{-9} M TCDD on pre–Il-1β (31-kDa precursor) expression in the endometrial adenocarcinoma cell line RL95–2. Surprisingly, α-NF was not an efficient TCDD antagonist in RL95–2 cells.

Resveratrol Antagonizes AhR Ligands In Vivo. Finally, we extended the results obtained ex vivo by assessing the antagonist effects of resveratrol toward tobacco-related AhR ligands in vivo in various tissues of the rat. Female

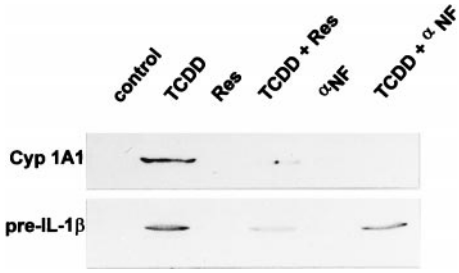

Fig. 5. Resveratrol inhibits TCDD-mediated accumulation of CYP1A1 in T-47D cells as well as the 31-kDa pre–Il-1β in RL95–2 cells. Both cell lines were treated 48 h as shown on the figure either by ethanol (control), 10^{-10} M TCDD for CYP1A1 induction or 5×10^{-9} M TCDD for pre–Il-1β induction (TCDD), 5×10^{-6} M resveratrol (Res), a combination of TCDD and resveratrol (TCDD + Res), 10^{-6} M α-NF or a combination of TCDD and α-NF (TCDD + α-NF). Cell extracts were submitted to Western blotting analysis as described.

Sprague-Dawley rats were treated as described in *Materials and Methods* with a combination of BaP and DMBA, two AhR ligands found in high concentrations in cigarette smoke (40 to 100 ng in mainstream smoke per cigarette). Preliminary dose-response experiments showed that CYP1A1 induction was detectable after treatment with 1 mg/kg of BaP plus DMBA (data not shown). This treatment was then repeated with or without concomitant treatment with resveratrol (1 and 5 mg/kg). After treatment, the animals were sacrificed and CYP1A1 protein production was assayed by Western blot in whole-cell extracts of several organs. As can be seen in Fig. 8, BaP/DMBA elicited CYP1A1 expression in lung and kidney. This induction was totally suppressed by administration of an equal dose of resveratrol. Similar results were demonstrated in liver and spleen but not in ovary, where Western blotting revealed constitutive expression, which was not induced by BaP/DMBA (data not shown). Immunocytochemical analysis of rat bone and testis revealed a similar induction of CYP1A1 by BaP/DMBA that was efficiently counteracted by resveratrol (data not shown).

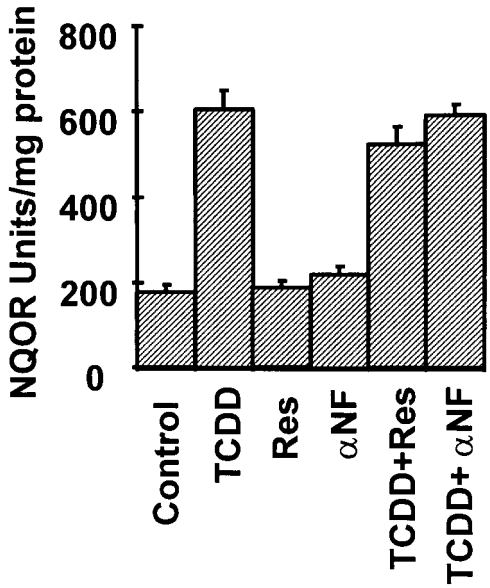

Fig. 6. Neither resveratrol nor α-NF inhibits induction of NQOR by TCDD oxin. T-47D cells were incubated 3 days with drugs as indicated (control, ethanol alone; TCDD, dioxin 10^{-9} M; Res, resveratrol 5×10^{-6} M; NF, α-NF 10^{-6} M) and NQOR activity was measured as described previously (Montano and Katzenellenbogen, 1997). Enzyme activity was calculated as nanomoles of cytochrome *c* reduced per minute and the specific quinone reductase activity is expressed as units (nanomoles of cytochrome *c* reduced per minute) per mg of protein (U/mg). TCDD significantly increased NQOR activity and this induction was inhibited by neither resveratrol (Res) nor α-NF. Res or α-NF alone were ineffective in inducing NQOR activity. $^*P < .001$ compared with control as determined by one-way ANOVA and Bonferroni's method as a post hoc test.

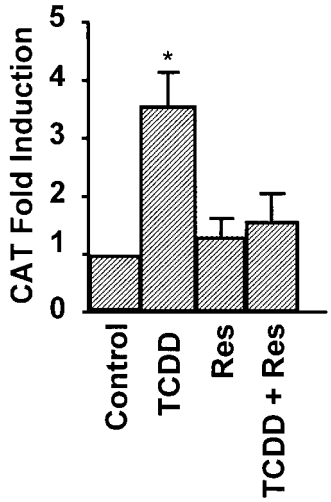

Fig. 7. Resveratrol inhibits transactivation of HIV-LTR-CAT by TCDD. T47-D cells were transiently transfected with a CAT construct bearing the promoter from the HIV LTR (−453/+82). Cells were then treated with 10^{-9} M dioxin (TCDD), 5×10^{-6} M resveratrol (Res) alone or in combination. Transfections and CAT assays were performed twice with triplicate transfections. $^*P = .01$ by one-way ANOVA on ranks followed by Dunnett's test for multiple comparisons with Sigmastat software (SPSS, Chicago, IL).

Discussion

In contrast to animals, plants are able to metabolize phenylalanine into cinnamic acid. Cinnamic acid is then polymerized into stilbenes such as resveratrol by resveratrol synthase or into flavonoids by chalcone synthase and chalcone isomerase (Soleas et al., 1997b). Several natural flavonoids are known to bind the AhR and either activate or repress its *trans*-activating ability (Gasiewicz and Rucci, 1991; Santostefano et al., 1993; Wilhelmsson et al., 1994). In addition, previous studies of synthetic derivatives of flavonoids have demonstrated binding to the AhR and the ability to antagonize, either totally or partially, dioxin-induced CYP1A1 (Lu et al., 1995). Generally, good antagonists are planar phenolic compounds with lateral electron-rich substitutions such as $-NO_2$ or $-NCS$ (Henry et al., 1999). When hydroxyls are present instead, the compounds become either moderate affinity antagonists or partial agonists such as quercetin (R.F.C. and J.-F.S., unpublished observations) or α-NF (Santostefano et al., 1993; Wilhelmsson et al., 1994). Flavonoids have not been examined extensively for potential therapeutic antidioxin activity because they often display adverse effects. These effects may be mediated by AhR agonistic effects at high concentration (α-NF) (Santostefano et al., 1993; Wilhelmsson et al., 1994) or toxic side-effects as demonstrated for α-NF (Collman et al., 1986) or quercetin (Sahu and Washington, 1991). We also observed in the course of the present study that quercetin has AhR-agonistic activities (R.F.C. and J.-F.S., unpublished observations).

Resveratrol is a phytoalexin, existing in *cis* and *trans* configurations, in a variety of spermatophyte plants including eucalyptus, peanuts, and grapes (Soleas et al., 1997b). Resveratrol is present in some red wines in concentrations between 1 and 8 mg/liter (Siemann and Creasy, 1992; Goldberg, 1995) but absent in nonalcoholic beverages because it is not

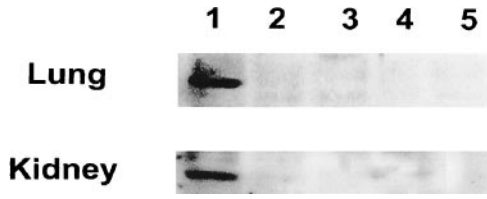

Fig. 8. A, Effect of resveratrol on the induction of CYP1A1 in rat lung and kidney by AhR ligand treatment in vivo. Lane 1, 1 mg/kg BaP/DMBA; lane 2, 1 mg/kg BaP/DMBA plus 1 mg/kg resveratrol; lane 3, 1 mg/kg BaP/DMBA plus 5 mg/kg resveratrol; lane 4, 5 mg/kg resveratrol; lane 5, vehicle only (olive oil). Each lane contains 30 μg (lung) or 60 μg (kidney) of whole tissue extract proteins. Western blot was performed as in Fig. 5.

water-soluble. Resveratrol has previously been proposed to be responsible for the cardioprotective effects of red wine (the so-called 'French Paradox') (Renaud and de Lorgeril, 1992; Pace-Asciak et al., 1995). However, it was later dismissed as the primary protective agent because it had no additive beneficial effect on platelet aggregation and lipid metabolism compared with alcohol alone (Soleas et al., 1997a).

We deonstrate here that resveratrol is able to compete with TCDD for AhR binding and efficiently block the induction of CYP1A1 and pre–Il-1β expression by AhR ligands, both ex vivo and in vivo. In contrast, resveratrol did not counteract the induction of NQOR activity by TCDD. The effect of AhRs on NQOR expression has been reported to occur through binding to an antioxidant responsive element and not a DRE (Montano and Katzenellenbogen, 1997). These authors also report inducibility of NQOR by the antiestrogen hydroxytamoxifen, whereas estradiol represses NQOR. It is possible that NQOR induction by TCDD is related to the antiestrogenic effects of dioxins (Safe et al., 1998).

The inhibition by resveratrol of the induction of CYP1A1 expression by TCDD in HepG2 cells was reported while this article was being written (Ciolino et al., 1998). However, these authors report the inability of resveratrol to bind AhR, which is discordant with our data. Essentially, Ciolino et al. report that resveratrol did not displace TCDD from its receptor but nevertheless precluded AhR binding to DNA (Ciolino et al., 1998). We have clearly demonstrated resveratrol binding to the AhR and its ability to displace TCDD in three different systems, either ex vivo or in vitro. However, in the case for ex vivo binding competition in intact human cells (T-47D and HepG2), we must state that fully efficient competition was only achieved when resveratrol was preincubated with the cells before addition of TC[1,6-^{3}H]DD. This was *not* necessary for in vitro binding competition assays with rabbit liver cytosol. This difference in methodology may explain the discrepancy between our results and those of Ciolino et al. (1998) when using human cells.

Concerning the influence of resveratrol on AhR transformation and subsequent binding to its cognate site, it is well known for most DNA binding proteins that technical differences in the execution of the gel retardation assay can lead to divergent results, especially when stringent incubation and/or electrophoretic conditions are used. Indeed, in the case of α-NF and AhR, it has been successively reported that α-NF inhibits AhR binding to DNA (Gasiewicz and Rucci, 1991) or elicits binding similar to TCDD (Santostefano et al., 1993; Wilhelmsson et al., 1994). As can be seen in Fig. 3, our results concur with the latter reports, because both α-NF and resveratrol mediate AhR binding to its cognate responsive element. Moreover, Fig. 4 demonstrates the ability of resveratrol to elicit the nuclear shuttling of an AhR-GFP fusion protein.

This ability of resveratrol to induce AhR binding to DNA while precluding transactivation is reminiscent of previous papers on dibenzofuran derivatives such as 6-methyl-1,3,8-trichlorodibenzofuran. The group of Safe has successively reported the inhibition of TCDD effects by dibenzofurans (Merchant et al., 1992a) as well as the inhibition of BaP effects (Merchant et al., 1992b), both in vivo (Astroff et al., 1988) and in vitro (Merchant et al., 1992a). This similarity in the mechanism of action of 6-methyl-1,3,8-trichlorodibenzofuran and resveratrol, coupled with the efficiency of resveratrol in multiple tissues in vivo, supports the possibility that resveratrol may be effective as a protective agent against AhR ligands.

Halogenated aromatics and PAHs, AhR ligands with distinct toxicities, have been implicated in a variety of diseases including endocrine disruption (Safe et al., 1998), cancer (Flesch-Janys et al., 1995; Boyle, 1997), and immunosuppression (Kerkvliet, 1995). Still, epidemiological studies of carcinogenic, inflammatory, and/or cardiovascular effects of dioxins have been hard-pressed to show unequivocal results. It now seems that insufficient duration of follow-up in these studies may in part explain these difficulties: the latest studies on the Seveso population now detect an increase in cancer and cardiovascular mortality (Bertazzi et al., 1998).

Cigarette smoke contains BaP and other TCDD-like compounds at concentrations that induce CYP1A1 activity in the lungs (Bilimoria and Ecobichon, 1980) and in vascular endothelial cells, where they have been shown to cause endothelial cell damage and dysfunction through the generation of reactive oxygen species (Toborek et al., 1995). CYP1A1-mediated oxidative metabolism of BaP results in reactive carcinogenic intermediates (Okey et al., 1994), DNA adducts (Arif et al., 1999; Lagueux et al., 1999), and the production of reactive oxygen species, which can lead to oxidative DNA damage (Park et al., 1996). AhR ligands, through reactive oxygen generation, are also able to cause peroxidation of low-density lipoproteins, leading to the formation of cytotoxic oxysterols (Berliner and Heinecke, 1996; Steinberg, 1997). In addition, we (present study) and others (Sutter et al., 1991; Charles and Shiverick, 1997) have demonstrated that AhR ligands can induce IL-1β expression, which is known to be associated with inflammation. The impact of AhR ligands on inflammatory processes should, therefore, be considered in terms of chronic toxicity. Accordingly, cigarette smoking has been identified as the cause of at least eight different human cancers as well as ischemic heart disease (Boyle, 1997).

We performed all of the in vitro and ex vivo pharmacological and biochemical studies with dioxin (TCDD), to ascertain that resveratrol was a genuine AhR antagonist. Then, when we considered in vivo experiments, and in keeping with all of the above-mentioned data on tobacco-related AhR ligands, we decided to mimic the tobacco-smoking situation by using a mix of DMBA and BaP. We are aware of the differences between these ligands, namely that BaP and DMBA are metabolized by CYP1A1 to proximate carcinogens and DNA adducts and also have other targets in addition to AhR. Still, they do bind the AhR with high affinity and elicit its transactivating ability (Merchant et al., 1992b). Moreover, exposure to tobacco smoke AhR ligands is much more frequent than TCDD intoxication and is of greater concern in terms of its impact on human health. The daily intake of TCDD is expressed in p.p.t. (ng/kg) whereas daily BaP intake of smokers is in the 0.1- to 0.5-mg range. If resveratrol had only partially counteracted the effects of BaP and DMBA, our interest in it as a potential therapeutic agent would have been much weaker. However, we demonstrate complete antagonism by resveratrol of tobacco-related AhR ligand induction of CYP1A1 in vivo. Our data, therefore, suggest that resveratrol may prevent the adverse effects of PAHs in vivo, through a number of mechanisms, including the antagonism of ligand binding to the AhR and down-regulation of genes such as CYP1A1 and Il-1β.

Resveratrol concentrations in human blood have not been measured previously, but it has been reported that rats fed a

single oral dose of red wine (4 ml containing 26 μg of resveratrol) achieved a 10^{-4} M concentration of resveratrol in liver and kidney for more than two h. At 2 h, the plasma concentration of resveratrol was 10^{-5} M and the resveratrol concentration in the cardiac tissue remained at 10^{-5} M for 4 h (Bertelli et al., 1996). If the same relatively slow metabolism of resveratrol occurs in humans, the micromolar concentration necessary to block activation of the AhR should be easily attained. It should be stressed that the literature does not contain any evidence of toxicity for resveratrol, in contrast to other flavonoids as discussed above (Collman et al., 1986; Sahu and Washington, 1991; Wilhelmsson et al., 1994).

In conclusion, we have demonstrated that resveratrol is a competitive antagonist for the AhR with a specificity for this receptor. We believe that the identification of a nontoxic antagonist of AhR without agonistic activity is potentially of great medical interest. Resveratrol is able to block AhR ligand-mediated increased expression of CYP1A1 and Il-1β at micromolar concentrations. Although less efficient against dioxin-mediated *trans*-activation than α-NF, resveratrol has the advantage of being devoid of any known toxicity. Because AhR ligands exert deleterious effects on human health, we believe resveratrol warrants further clinical study for potential prevention of a variety of adverse effects of these environmental toxicants.

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