Estrogen receptor expression is required for low-dose resveratrol mediated repression of aryl hydrocarbon receptor activity

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ABSTRACT

The putative cardio-protective and chemo-preventive properties of the red wine phenolic resveratrol (RES) have made it the subject of a growing body of clinical and basic research. We have begun investigations focusing on the effects of RES on the activity of the aryl hydrocarbon receptor complex. Our evidence suggests that RES is a potent repressor of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) inducible gene transcription in estrogen receptor-positive human breast, lung and colon cancer cell lines. RES activates the transcription of the estrogen receptor target genes to the same degree as estradiol (E2) in human MCF-7 breast cancer cells. Unlike E2, which can only diminish TCDD-inducible CYP1A1 gene transcription by approximately 50%, RES can completely abrogate this response. Furthermore, 50% repression of TCDD-inducible transcription can be achieved with 100 nM RES, approximately 2.5 orders of magnitude lower than concentrations required for maximal inhibition, suggesting that multiple mechanisms are responsible for this effect. RES (100 nM) does not prevent ligand binding of a TCDD analogue nor does it prevent AHR from binding to its response element in the 5’ regulatory region of the CYP1A1 gene. Small inhibitory RNAs directed to ERα have demonstrated that RES-mediated repression of CYP1A1 is dependent on ERα. While CYP1A1 protein levels in MCF-7 cells are refractory to the low dose transcriptional effects of RES, a concomitant decrease in CYP1A1 protein levels are observed in Caco-2 cells. These results highlight a low-dose RES effect that could occur at nutritionally relevant exposures and are distinct from the high dose effects often characterized.
Introduction

Resveratrol (3,4’-5-trihydroxystilbene, RES) is found in abundance in many foodstuffs, including grapes and berries, and is available as an over-the-counter dietary supplement. Its virtues as a cardio-protective agent are thought to account for the beneficial effects of red wine; the so-called French Paradox (Ulrich et al., 2005). RES inhibits proliferation in many tumor cell lines including breast, colon, and cervical carcinomas. RES can repress the activity of several transcription factors, including NF-κB, AP-1 and the androgen receptor (Mitchell et al., 1999; Manna et al., 2000; Yu et al., 2001). As such, the putative cardio-protective and chemopreventive properties of RES have made it the focus of intense research over the past decade. However, most of the documented pharmacological effects of RES, including inhibition of MAP kinases, activation of sirtuins, inhibition of cell cycle progression, and as a pharmacological antagonist of the aryl hydrocarbon receptor (AHR), occur at concentrations in the mid to high micromolar range (Casper et al., 1999; Ciolino and Yeh, 1999; Ulrich et al., 2005). In addition, the estrogentic activity of RES is well documented, acting as an agonist at micromolar concentrations for both the estrogen receptor α and β [ERα/β] (Gehm et al., 1997; Bowers et al., 2000). Concentrations of RES in red wines range from 1.5-3 mg/L (Goldberg et al., 1995). Due to these factors and the low bioavailability of RES after oral ingestion (Marier et al., 2002), micro-molar plasma concentrations cannot be achieved by normal food intake or moderate consumption of red wine [for review see (Cottart et al.)]. Thus, the mechanisms by which the chemo-protective effects of RES, associated with the so-called French Paradox, are manifested remain to be determined.

One potential explanation stems from the observation that nanomolar concentrations of RES can activate membrane-associated ER, leading to MAPK activation (Klinge et al., 2005),
and ultimately, ER target gene expression. This intrigued us as we observed in MCF-7 cells that ERα was greatly enriched at the CYP1A1 enhancer in response to estrogen during TCDD-activated transcription (Beischlag and Perdew, 2005). Likewise, we were able to monitor AHR on the PS2 promoter under similar conditions, consistent with the observations of other investigators (Ohtake et al., 2003; Matthews et al., 2005).

AHR and ARNT form a heterodimeric transcription factor that binds a variety of environmental pollutants, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and mediate an organism’s response to these contaminants. The AHR activates the transcription of several genes, including those encoding several xenobiotic-metabolizing enzymes (e.g. CYP1A1/1A2/1B1). Unliganded AHR exists in the cytoplasm as part of a multimeric complex. Upon ligand binding, AHR translocates to the nucleus where it associates with ARNT to form a functional transcription factor, the aryl hydrocarbon receptor complex [AHRC] (Beischlag et al., 2008). It does so by the recruitment of transcriptional co-activators and co-repressors that serve to modify chromatin structure, stabilize core transcriptional machinery, and mediate RNA chain elongation. AHR is capable of recruiting the p160/bHLH-PAS co-activators SRC-1, NCoA2/GRIP1 and p/CIP (Kumar and Perdew, 1999; Beischlag et al., 2002), NcoA4 (Kollara and Brown, 2006), CoCoA (Kim and Stallcup, 2004), GAC63 (Chen et al., 2006), CBP (Kobayashi et al., 1997) and TRIP230 (Beischlag et al., 2004). In addition to these classical transcriptional co-activators, AHR appears to recruit other transcription factors during transcription including ERα (Ohtake et al., 2003; Beischlag and Perdew, 2005; Matthews et al., 2005) and NF-κB (Tian et al., 1999). The possibility that environmental contaminants and naturally occurring dietary compounds can differentially regulate the AHRC and its recruitment of other transcription factors is intriguing. An understanding of the mechanisms by which this
regulation occurs could aid in our understanding of the AHRC and its role in development, homeostasis and the complex pathologies responsible for a wide spectrum of human diseases, including chemical carcinogenesis, solid tumor growth and atherosclerosis.

We wanted to test the hypothesis that RES mediates some of its effects on AHR-activated transcription through binding to the ER. To address this issue, the effect of a range of concentrations of RES on AHR-mediated gene expression in several human cell lines was examined. Both low concentrations similar to those readily achievable through regular dietary intake as well as high concentrations, commonly used to activate sirtuins, were tested. RES at nanomolar concentrations is able to repress AHR-mediated induction of CYP1A1 in an ERα dependent manner and without reducing the level of AHRC at the CYP1A1 promoter. In contrast, 10 μM RES markedly reduced AHRC on the CYP1A1 promoter and resulted in near complete inhibition of CYP1A1 expression and metabolic activity.
Methods

Cell Lines and Reagents

Polyclonal anti-ERα and CYP1A1 antibodies were purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-AHR polyclonal antibody (SA-210) was purchased from Biomol. Anti-XAP2(ARA9) monoclonal antibody was purchased from Novus Biological and anti-HOP mouse monoclonal antibody F5 was kindly provided by David Toft (Mayo Clinic, Rochester, MN). Human lung cancer BEAS-2B, human colon cancer Caco-2 and human breast cancer MCF-7 cells were purchased from ATCC. BEAS-2B cells were maintained in serum-free LHC-9 media (Invitrogen). MCF-7 cells were maintained in serum free Dulbecco’s modified Eagle medium (D-MEM) lacking phenol red with high glucose, for at least 48 h prior to any treatment or experimental manipulation to ensure that unnecessary activation/down-regulation of ER or other signal transduction pathways would not confound the experimental parameters examined. Similarly, Caco-2 cells were maintained in D-MEM and 2% dextran-treated charcoal-stripped fetal bovine serum for 48 h prior to any treatment. TCDD was kindly provided by Steve Safe (Texas A&M University).

Reverse Transcription and Real-Time PCR

Reverse transcription and real-time PCR were performed as described previously (Beischlag and Perdew, 2005). In brief, prior to treatment with ligand, MCF-7 cells were exposed to cycloheximide (10 μg/ml) for 1 h to halt protein translation. Subsequently, cells were treated either with vehicle (Me₂SO), TCDD, RES, or a combination of TCDD and increasing concentrations of RES, for 6 h. Cells were harvested in TriZol and total RNA was isolated and
subjected to reverse transcription using a High Capacity cDNA Archive kit (Applied Biosystems). Complimentary DNA was amplified by real-time PCR using a Power SYBR Green PCR kit (Applied Biosystems) according to manufacturer’s protocols. Oligonucleotide pairs used to amplify human cDNA sequences were described previously (Beischlag and Perdew, 2005). DNA was amplified for 40 cycles in a StepOne Plus Real-Time PCR System (Applied Biosystems). Oligonucleotides for amplification of CYP1A1 enhancer chromatin isolated using the ChIP assay were identical to those reported by Hestermann and Brown (Hestermann and Brown, 2003).

**Western Blot Analysis**

In order to determine the effects of RES on TCDD-inducible CYP1A1 protein levels, MCF-7 cells were treated either with vehicle (Me2SO), TCDD, RES, or a combination of TCDD and increasing concentrations of RES for 24 h. Whole cell extracts of MCF-7 cells were resolved by SDS-PAGE and protein blot analysis was performed as described previously (Beischlag et al., 2002), with minor modifications. Western blots were probed with affinity purified rabbit anti-CYP1A1 (Santa Cruz), and anti-XAP2 monoclonal antibody, or anti-HOP F5 monoclonal antibody (as loading control in MCF-7 and Caco-2 cells, respectively). After incubation with primary antibodies, blots were incubated with a biotin labeled goat anti-rabbit IgG. Blots were washed and incubated with [$^{125}$I]-labeled streptavidin (Amersham) and exposed to film overnight. The level of radioactivity was quantified using a Cyclone phosphor imaging system and OptiQuant software (Packard).
Cytochrome P450-GLO Assays

We assessed cytochrome P450-1A1 (CYP1A1) enzymatic activity using the p450GLO (Promega) indirect luminescence assay according to manufacturer’s protocols. This assay is dependent on the formulation of luciferin from a synthetic CYP1A1 substrate, luciferin-chloroethyl ether (L-CEE). In brief, cells were maintained as described above in 24 well plates. After 24 h of serum deprivation in Phenol-Red-free media, cells were treated with vehicle, or TCDD and various concentrations of RES for a further 24 h. Media was replaced with fresh media containing 50 μM L-CEE and incubated overnight. Eighteen h later 100 μl of media was mixed with 100 μl of luciferase detection reagent and luminescence was measured in a GLOMAX 20/20 luminometer (Promega).

Competition Ligand Binding Assays

MCF-7 cytosol was prepared by manual cell disruption of cells in MENG, 20 mM Na₂MoO₄, and protease inhibitor cocktail (Sigma) using a steel Dounce homogenizer. The crude homogenate was centrifuged at 100,000 x g for 60 min at 4° C and the supernatant was collected as cytosol. Protein content was determined by BCA assay (Pierce), and cytosol samples were stored at -80° C until use. Ligand binding assays were performed using the previously described AHR photoaffinity ligand 2-azido-3-[¹²⁵I]iodo-7,8-dibromodibenzo-p-dioxin (Poland et al., 1986). Varying concentrations of benzo[a]pyrene (B[a]P) or RES were added to 150 μl aliquots of cytosol and incubated at room temperature for 10 min followed by the addition of a saturating amount (700,000 cpm, 1.25 nM) of photoaffinity ligand. After a 30 min incubation at room temperature to achieve steady-state receptor binding levels, samples were placed on ice for 5 min to prevent further association/dissociation of receptor-ligand complexes. Next, 75 μl of a
charcoal (1%)/dextran (0.1%) solution (made in MENG) was added to each tube and incubated for 10 min on ice, after which samples were centrifuged at 3,000 x g for 10 min at 4° C. Ligand-receptor complexes were covalently cross-linked by exposure to ultraviolet light at a distance of 8 cm. Aliquots were resolved by 8% SDS-PAGE, and gels were transferred to PVDF membrane. Cross-linked bands were visualized by autoradiography, and quantified using a Cyclone PhosphorImager (Perkin Elmer). Data was plotted and statistical analysis was performed using GraphPad Prism 4.00 (GraphPad Software).

**Chromatin Immunoprecipitation (ChIP) Assays**

ChIP assays from fixed MCF-7 cell lysates were performed as previously described (Beischlag et al., 2004). In brief, cells were treated with either 0.1% vehicle as control, 10 nM E2, 2 nM TCDD or a combination of E2 and TCDD for 1 h. Cells were fixed in 1% formaldehyde for 10 min at room temperature and subjected to ChIP analysis using agarose gel electrophoreses and Real-Time PCR.

**Sub-cellular Localization Assay**

MCF-7 cells were transfected with 500 ng of human AHR-eGFP expression vector using SuperFect transfection reagent. (Qiagen). Five hours after transfection, media was replaced and cells were cultured in Phenol-Red-free DMEM without sera for 24 h prior to treatment with ligand. Cells were then treated with either vehicle (DMSO), RES (50 µM), TCDD (2 nM), or a combination of TCDD and increasing concentrations of RES (1-10 µM). To identify nuclei, fifty minutes after treatment with ligand tissue culture media was supplemented with Hoechst 33342 (bis-benzimide H) to a final concentration of 5 µg/ml and allowed cells were allowed to incubate
for a further 10 min at 37 °C in a humidified CO2 incubator. One hour after treatment cells were fixed in 4% paraformaldehyde at room temperature for 15 min. Fluorescence micrographs of cells 24 h after transfection were obtained with an Olympus Fluoview FV10i laser scanning confocal microscope and a 60X objective.

**ERα Fluorescence Polarization Competition Assay**

ERα competition assay was performed using the Estrogen Receptor-α Competitor Assay, Green from PanVera #P2614, P2698, Invitrogen Corp. Essentially, compounds and vehicle were diluted to twice their final assay concentrations in PanVera supplied ES2 screening buffer and added to 6 x 50 mm borosilicate glass cell culture tubes. A master mix was made of screening buffer with human recombinant ERα added for a final concentration of 6 pmol/μl, and ES2 fluoromone added for a final concentration of 400 nM. The master mix was added to diluted test compounds in a 1:1 volume, mixed gently, and incubated in the dark at room temperature for 2 h. Samples were then measured for fluorescence polarization using the PanVera Beacon 2000 polarization reader with 485 nm excitation and 530 nm emission filters at 25° C.

**RNA Interference**

“Knock-down” of ER was performed essentially as described previously (Hollingshead et al., 2008). In brief, MCF-7 and Caco-2 cells were maintained in D-MEM supplemented with Na+-pyruvate and 7% FBS, and cells grown to approximately 50% confluence, at which time cells were washed with PBS and maintained in D-MEM with 1% dextran/charcoal stripped FBS, without antibiotics, 24 h prior to transfection. BEAS-2B cells were maintained in LHC media. Cells were transfected with either ERα, ERβ or siGFP Shortcut siRNA (New England Biolabs).
using Dharmafect I (Dharmacon) according to manufacturers’ protocols. Twenty-four h after transfection, cells were supplemented with 5 mg/ml bovine serum albumin and treated with cycloheximide (10 μg/ml). One h later, cells were treated as described above and CYP1A1 mRNA levels were determined.

**Statistical Analysis**

For multiple comparisons, statistical analyses were performed using a 1-way ANOVA and Tukey’s Multiple Comparison test. For multiple comparisons using more than one variable (i.e. siRNA experiments), statistical significance was determined using a 2-way ANOVA with Tukey’s Multiple Comparison test. Values are presented as means ± standard error of the mean. A P value < 0.05 was considered to be significant.
Results

Effects of resveratrol on TCDD-mediated accumulation of \textit{CYP1A1} mRNA. Various reports have identified RES as a pharmacological antagonist of the AHR (Ciolino et al., 1998; Casper et al., 1999), and a partial or super-agonist of the ER (Ciolino et al., 1998). We were interested in exploring the outcome of RES on the cross-talk that exists between the AHR and ER. Phenol-red dye found in regular D-MEM displays ER agonist activity (Berthois et al., 1986) and like endogenous estrogens found in regular fetal bovine sera can lead to extreme down-regulation of ER abating the pharmacological effects of any modulatory agent. Therefore, in order to ensure an appropriate and robust ER-dependent response all cell lines were either deprived of fetal bovine sera or maintained in low levels (≤ 2%) dextran-treated, charcoal-stripped sera in phenol red-free media for at least 24 h prior to any experimental manipulation. The TCDD-dependent accumulation of \textit{CYP1A1} mRNA in various human cancer cell lines was first examined after exposure to increasing amounts of RES. In order to eliminate secondary transcriptional effects of RES, cells were pre-treated for 1h with cyclohexamide (10 μg/ml). Initial studies with RES have shown that it is capable of repressing TCDD-responsive \textit{CYP1A1} transcription in MCF-7, Caco-2, and BEAS-2B human cell lines (Figures 1A, B and C). TCDD-dependent \textit{CYP1A1} mRNA accumulation was reduced by approximately 50% by 100 nM RES in each of the three cell lines tested, which is well below the EC$_{50}$ reported for RES to displace [1,6-\textsuperscript{3}H]TCDD from the receptor (Casper et al., 1999). Approximately 90-95% repression of \textit{CYP1A1} expression was achieved with 50 μM RES in MCF-7 and Caco-2 cells, which were treated with a wide range of doses. Furthermore, the response at the mRNA level appeared to be biphasic, especially in Caco-2 cells. The lower range of RES repression (below 100 nM) was not explored in all cell lines. Superinduction of \textit{CYP1A1} transcription by cycloheximide is common in many cancer
cell lines (Joiakim et al., 2004). Superinduction in response to cycloheximide was negligible in
the MCF7 cells (Beischlag and Perdew, 2005), and modest in CaCo-2 and BEAS-2B. We did
observe an increase in basal CYP1A1 expression that resulted in a proportional decrease in the
*fold* expression by TCDD which we have reported previously (Beischlag and Perdew, 2005).
Treatment with cycloheximide had no effect on the profile of TCDD activation or RES
repression in either of these cells as evidenced below in Figure 7 where cycloheximide was not
used.

**Effects of resveratrol on TCDD-inducible CYP1A1 protein levels.** Next, we examined the
functional consequences of decreased *CYP1A1* mRNA accumulation after RES treatment on
protein levels in MCF-7 and Caco-2 cells. Surprisingly, no decrease in CYP1A1 protein levels
in MCF-7 cells was observed in response to nanomolar concentrations of RES (Figure 2A).
Only concentrations in excess of the reported EC$_{50}$ for RES to displace [1,6-$^3$H]TCDD from the
receptor led to a concomitant decrease in CYP1A1 protein (Casper et al., 1999). In order to
examine changes in protein levels, a longer exposure time frame of 16 h was used, as opposed to
6 h treatment used in figure 1. This longer time frame of exposure may lead to significant RES
metabolism in MCF-7 cells reducing its effect on CYP1A1 expression. However, Western blots
of Caco-2 lysates reveal that 100 nM RES efficiently blocks CYP1A1 protein expression
(approximately 50%, Figure 2B) and exposure to increasing concentrations of RES led to a dose-
dependent reduction in TCDD-dependent protein levels. These data suggest multiple
mechanisms are responsible for the repressive effect of RES on AHR activity and the degree of
their respective effects may be cell-type specific. Furthermore, the more physiologically relevant
mode of action would be at concentrations below those that act as a pharmacological antagonist of AHR.

**Effects of resveratrol on TCDD-inducible CYP1A1 activity.** To confirm these findings we performed correlative studies to determine CYP1A1 enzymatic activity using the P450-GLO Assay. Due to the assay’s reliance on CYP1A1 activity to convert a synthetic inert precursor of luciferin to a usable substrate, results would be expected to coincide with protein expression. The effect of RES in both the MCF-7 and Caco-2 cell lines (Figure 3A and B) indeed mirrored the observed changes in CYP1A1 protein levels. For these studies, we employed a larger range of doses for RES; from 1 nM to 50 μM. In MCF-7 cells, using doses ranging from 1-500 nM RES, the trend was towards a mild enhancement of TCDD inducible CYP1A1 activity. A sharp decrease in this activity was observed at doses of RES above 500 nM. Likewise, Caco-2 CYP1A1 oxidative activity mirrored changes observed at the protein level. Doses of RES above 10 nM resulted in a significant decrease in TCDD inducible activity. A distinct biphasic response was observed in both Caco-2 and BEAS-2B cells, with a dose-dependent decrease in CYP1A1 activity to approximately 50% of maximal observed with 100 and 500 nM RES, respectively (Figure 3B and C). Non-linear regression analysis using an F-test to determine the best-fit to a one- or two-site model confirmed this (p<0.001). In addition concentrations above 500 nM further decrease CYP1A1 activity, with complete abrogation of activity observed at 10 μM.

**Pharmacological effect of RES on AHR-ligand binding.** Our preliminary results indicate that RES has the ability to repress TCDD signaling at concentrations lower than what could be
attributed to the pharmacologically relevant antagonist properties reported in other studies (Ciolino et al., 1998; Casper et al., 1999; Ciolino and Yeh, 1999). Furthermore, we observed a biphasic response to RES in all cell lines exposed to TCDD at the level of mRNA. Therefore, we wished to confirm observations reported previously that RES is an AHR antagonist only in the micromolar range, to eliminate the possibility of experimental error on our part and to resolve micromolar and sub-micromolar actions of RES. MCF-7 cells were chosen for these studies as they express primarily ERα and not ERβ in estrogen-deprived cells (Shaw et al., 2006), and thus the likelihood of observing effects attributable to a mixed population of receptors was reduced. A pilot displacement study using cytosolic lysates derived from MCF-7 cells and the TCDD analog, 2-azido-3-[125]iodo-7,8-dibromodibenzo-p-dioxin, suggests that the IC50 for RES is approximately 5 μM (Figure 4A). This is in agreement with other investigators’ previously determined values. Furthermore, the apparent RES-dependent displacement of radioactive ligand displayed simple first-order kinetics with little or no displacement at 500 nM. This result also indicates that the effects observed in this report at high nanomolar concentrations of RES are not due to its pharmacological antagonist properties with the AHR, and thus the concentrations used had been titrated appropriately.

Effects of RES on AHR DNA binding and nuclear translocation. In order to better understand this phenomena, we asked whether nanomolar RES had any effect on the translocation profile of the AHR or its ability to associate with dioxin response elements (DRE’s) in the upstream activating sequences (UAS) of the CYP1A1 gene. Therefore, we performed chromatin immunoprecipitation (ChIP) experiments followed by Real-Time PCR in MCF-7 cells to determine if nanomolar RES concentrations blocked the association of AHR with its cognate
DNA response element, the DRE. Enrichment of the CYP1A1 UAS is seen in both panels in a TCDD dependent fashion. Co-treatment of MCF-7 cells with TCDD and 100 nM RES did not diminish this enrichment (Figure 4B, bottom left panel). However, co-treatment of cells with 10 μM RES is effective in preventing TCDD-dependent AHR association with DREs (Figure 4B, bottom right panel), consistent with recent observations made in Oliver Hankinson’s laboratory (Beedanagari et al., 2009). In addition, the amount of CYP1A1 enhancer precipitated with an antibody directed towards ERα was increased by 100 nM RES and 2 nM TCDD demonstrating that ERα is present at the CYP1A1 regulatory regions under these conditions (Figure 4C). Likewise, concentrations of RES that can partially displace 2-azido-3-[125]iodo-7,8-dibromodibenzo-p-dioxin from the receptor (i.e. 1 and 5 μM) did not alter the pattern of fluorescence of an AHR-enhanced green fluorescence protein (eGFP) moiety upon treatment with TCDD (Figure 5). In the absence of dioxin, green fluorescence appears to be homogenous (Figure 5A) and treatment with RES has no effect on this pattern (Figure 5B). Upon treatment with 2 nM TCDD, a profound and rapid translocation of the AHR-eGFP moiety to the nucleus is observed within 45 min which can be confirmed by the nuclear fluorescence of the Hoechst 33342 stain in the middle panel (Figure 5C). After co-treatment of MCF-7 cells with TCDD and either 100 nM or 1 μM RES, the majority of fluorescence in the cell appears to be nuclear (Figure 5, panels D and E). Only co-treatment with 10 μM RES was sufficient to block the accumulation of nuclear fluorescence in MCF-7 cells (Figure 5F). These data strongly suggest that nanomolar concentrations of RES, are sufficient to repress the accumulation of TCDD-dependent CYP1A1 mRNA and protein expression, but do not affect the ability of the AHR to translocate to the nucleus upon exposure to TCDD, nor do they interfere with the ability of the AHR to associate with its cognate DNA response element.
**ERα Fluorescence Polarization Competition Assay.** The data presented to this point suggest that RES is capable of repressing AHR activity at a 100 nM concentration. In order to test whether RES can significantly interact with the ERα in the nanomolar range a competition assay that measures the ability of a compound to displace a fluorescent ERα ligand, ES2 was utilized. ES2 fluoromone bound to the ERα tumbles slowly in solution, giving a higher polarization reading (mP). Compounds that compete the fluoromone away from the ERα result in free fluoromone that tumbles faster and provides a lower mP value. Non-specific binding effects can be seen by the shift of mP from ERα and fluoromone alone to the addition of 2% DMSO in the reaction (Figure 6). Addition of estradiol (E2) at higher concentrations displaces the fluoromone. Resveratrol shows minimal ERα competition at a dose of 10 nM, is at parity with E2 at a dose of 100 nM, and stronger competition at higher doses. This data suggests that resveratrol at 100 nM would be capable of significantly binding to the ER. The ability of 500 nM RES to almost totally inhibit in this assay relative to E2 was surprising and may suggest that the manner in which RES interacts with ERα in vitro differs.

**Role of estrogen receptors in RES-mediated repression of AHR signaling.** There have been few reports documenting significant perturbations in cellular physiology mediated by RES at sub-micromolar concentrations. One report, described the activation of the MAP kinase signaling cascade by 100 nM RES, mimicking the effect of estradiol (Klinge et al., 2005). We have previously shown that estradiol can repress TCDD-dependent CYP1A1 expression (Beischlag and Perdew, 2005). Therefore, we were interested in determining whether estrogen receptors were essential for the repressive effects of RES on AHR activity. To this end, we used...
small inhibitory RNA directed against either ERα or ERβ to investigate the possible contribution of these proteins to this phenomenon. All cell lines transfected with a non-specific, scrambled GFP siRNA displayed a repression profile for RES similar to that observed in Figure 2 (Figures 7A, B and C). In MCF7 cells, which express predominantly ERα and little ERβ, only siRNA directed to ERα was used. This completely ablated the repressive effect of RES on TCDD-inducible CYP1A1 mRNA accumulation (Figure 7A). A representative Western blot analysis of ERα protein levels in MCF-7 cells after transfection with either GFP or siRNA directed toward ERα is displayed in Figure 7D. A similar profile for the ERα siRNA was observed in BEAS-2B cells, while siRNA directed to ERβ was ineffective (Figure 7B). Knockdown of either ERα or ERβ failed to prevent repression of TCDD signaling in Caco-2 cells, though they did enhance the accumulation of TCDD-dependent CYP1A1 mRNA levels (Figure 7C). This may reflect transfection efficiency and hence, the ability of these siRNAs to block target protein expression in this cell line as transfection with a combination of both siRNAs blocked the ability of RES to repress TCDD-mediated accumulation of CYP1A1 mRNA. These results suggest that both ERα and ERβ are capable of repressing CYP1A1 induction in the presence of RES, depending on the cell line examined.
Discussion

The putative intrinsic, anti-aging, cardio and chemo-protective effects of resveratrol, a red wine phenolic, have made RES an attractive focus for the development of new pharmacological strategies for the treatment of a myriad of disease states. At high concentrations (micromolar), RES acts as a pharmacological antagonist of the aryl hydrocarbon receptor, a weak agonist for estrogen receptors, and binds sirtuins. However, these levels are not attainable through regular diet and are thus not likely to account for the protective effects attributed to RES. We have observed that low, nanomolar doses of RES are capable of significantly repressing dioxin inducible CYP1A1 transcription in human MCF-7 breast tumor, Caco-2 colonic tumor, and BEAS-2B lung epithelial cell lines. These effects are dose-dependent and lead to a concomitant decrease in CYP1A1 protein and activity levels. While 100 nM RES repressed dioxin inducible CYP1A1 transcription by approximately 50%, it did not prevent nuclear translocation of the activated receptor or binding of the receptor to the regulatory regions of the CYP1A1 gene, nor did it displace 2-azido-3-\[^{125}\text{I}]\text{oio}-7,8-dibromodibenzo-p-dioxin from the aryl hydrocarbon receptor. These data strongly suggest a role for nanomolar concentrations of RES in the assembly and disassembly of transcription complexes at the CYP1A1 promoter and ultimately in the physiological manifestation of AHR activation by environmental pollutants. Therefore, this mechanism may account for some of the putative chemo-protective effects of dietary RES.

RES is marketed commercially as a nutritional supplement and as a preventive medicine (Jang et al., 1997; Kundu and Surh, 2004). However, the effects of RES most often studied, namely those mediated by NF-κB, AP-1 and sirtuins and other targets are realized at concentrations (30-100 μM) well in excess of those reasonably achievable even by the over
consumption of concentrated dietary supplements. Thus, the mechanisms responsible for the chemo-protective properties of resveratrol observed in dietary studies remain to be determined. Mean peak plasma concentrations in humans range from the pico-molar to mid-nanomolar after oral ingestion of quantities associated with normal dietary consumption and reach the low micromolar range only after consumption of gram quantities [for review see (Cottart et al.)]. Certainly, local concentrations could be higher in the G.I. mucosa where there is significant first-pass metabolism.

We have found that concentrations of RES that are more likely achievable through dietary sources have a profound effect on CYP1A1 mRNA accumulation, protein levels and activity. With the exception of MCF-7 cells, the effects of RES on CYP1A1 mRNA accumulation lead to a concomitant decrease in CYP1A1 protein levels and/or CYP1A1 enzymatic activity. The rapid and extensive biotransformation of RES has been noted and metabolism is likely to occur in cell culture. Despite this, the acute effects of RES on CYP1A1 mRNA accumulation can be observed as perturbations in CYP1A1 protein levels and associated enzymatic activity (in BEAS-2B and Caco-2 cells) for as much as 24 h following treatment. The unexpected increase in CYP1A1 protein levels observed in MCF-7 cells after exposure to nanomolar concentrations of RES is not consistent with observed decreases in TCDD-dependent CYP1A1 mRNA levels. However, the modest increases in protein levels are mirrored in the P450-GLO assay, an indirect indicator of enzymatic activity. This data would suggest that potential carcinogen (e.g. benzo(a)pyrene) metabolism would be decreased by low-dose resveratrol exposure. Since concentrations lower than 5 nM RES were not tested in the P450-GLO assay in Caco-2 and BEAS-2B cells, it is difficult to ascertain if the sub-micromolar effects demonstrate the kinetics of a one site model. Therefore, RES may be eliciting its effects via more than one pathway.
However, ablation of ERα expression appears to indicate that ERα is the primary target of RES at nanomolar concentrations in these studies. In addition, these findings indicate that RES concentrations as low as 5 nM may have significant chemo-protective properties.

Several findings presented in this report support a distinct mechanism(s) or target that is sensitive to low, nanomolar concentrations of RES that differ mechanistically from the effects of RES most widely reported in the literature. First, interrogation of the CYP1A1 TCDD-responsive upstream enhancer region by chromatin immunoprecipitation with an affinity-purified antibody directed against AHR revealed that 100 nM RES could not prevent TCDD-inducible association of AHR with its cognate response elements (Figure 4B). However, 10 μM RES efficiently blocked TCDD-mediated binding of AHR to this region of chromatin. Second, these effects are elicited at concentrations which do not displace 2-azido-3-[125I]iodo-7,8-dibromodibenzo-p-dioxin from the receptor and are below the EC50 reported for RES to displace [1,6-3H]TCDD from the receptor (Casper et al., 1999). Finally, RES concentrations below 10 μM are not effective at blocking the TCDD-dependent translocation of AHR to the nucleus. These data strongly suggest that the intrinsic properties of RES lead to action via multiple mechanisms. Therefore, the low dose effect of RES on TCDD-responsive gene transcription is distinct from the high dose effects associated with its properties as a pharmacological antagonist of AHR. Furthermore, any nutritional benefits derived from RES ingestion are likely to involve mechanisms separate from those seen with high doses. For example, the physiological effects on sirtuins and other cellular factors are likely unattainable by RES administered orally.

It is well established that treatment of cells with TCDD or other activators of AHR alone is sufficient to recruit ER to the dioxin response element-containing regulatory regions of the CYP1A1 gene (Beischlag and Perdew, 2005; Matthews et al., 2005; Abdelrahim et al., 2006).
Additionally, we have demonstrated previously by sequential ChIP using affinity purified antibodies to AHR and ER that activated ER associates with the CYP1A1 enhancer in an AHR-dependent fashion (Beischlag and Perdew, 2005). Therefore, it may not be necessary for RES to direct estrogen receptors to AHR target genes, simply that RES bind to ERα while at the enhancer region. Additionally, RES has been reported to activate ERα via an estrogen-sensitive site in the cell membrane that activates the mitogen-activated protein kinase (MAPK) signaling cascade (Klinge et al., 2005). This finding, along with the fact that we have previously implicated ER in estrogen-mediated repression of AHR transcriptional activity (Beischlag and Perdew, 2005), led us to investigate the role of ER in this phenomena. Immunoprecipitated chromatin fractions from MCF7 cells using an antibody directed to ERα were enriched with CYP1A1 enhancer sequences in both a RES- (100 nM) and TCDD-dependent fashion. Reduction of estrogen receptor expression with siRNAs abrogated the repressive effect of resveratrol on dioxin inducible gene transcription. In addition, direct measurement of RES binding to ERα by time resolved fluorescence indicates that RES is capable of interacting with ERα at nanomolar concentrations. This may indicate its potential as a selective modulator of ER in addition to its Type I ER ligand activity (Levenson et al., 2003). Indeed, the ability to selectively modulate ERα activity with a synthetic compound, WAY-169916, to yield ER-mediated repression of NF-κB induced gene expression has been established (Chadwick et al., 2005). Interestingly this compound is both a selective ER and AHR ligand (Murray et al., 2009). Clearly, further studies are needed to ascertain whether RES is a selective ER ligand.

In order to facilitate the transcription of TCDD responsive genes in the appropriate spatial and temporal manner, the AHR must assemble a large array of ancillary co-activators, co-repressors and chromatin remodeling enzymes, such as Brahma/brg-1 (Wang and Hankinson,
Hence, the RES-mediated repression of TCDD-inducible gene induction appears to be mediated by the presence of ER in an AHR transcription complex at the CYP1A1 promoter and may not directly mediate signals received from a RES-activated MAPK signaling cascade. However, there are conflicting reports concerning activation of the MAPK signaling cascade and AHR activity (Andrieux et al., 2004). For example, U0126, an inhibitor of the MAP kinase kinase MEK1, leads to increased activation of the receptor even in the absence of ligand (Andrieux et al., 2004). This suggests that MAPK plays a role in suppressing the basal function of AHR. We did not examine the MAPK signaling cascade pharmacologically in this study, but further investigation into the role of this signaling cascade seems warranted.

In conclusion, we have documented the repression of ligand inducible AHR signaling by concentrations of RES well below those attributed to either the pharmacological antagonism of the receptor or the widely reported activation of other cellular constituents such as sirtuins. Furthermore, we have established that ERα is a target for low dose exposure to RES. The data presented here provides a plausible explanation for some of the chemo-preventive effects of dietary consumption of RES-containing foodstuffs.

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References


Footnotes

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Legends for Figures

1. Dose-dependent repression of CYP1A1 transcription by resveratrol. Human MCF-7 cells (A) and human Caco-2 cells (B) were maintained in phenol red-free, FBS free media for at least 24 h prior to treatment, while human BEAS-2B (C) cells were maintained in FHC-9 media. Cells were pre-incubated with 10 μg/ml cycloheximide 1 h before the addition of ligand. Cells were treated for 6 h with either DMSO, RES alone, (10 μM), TCDD alone (2 nM) or TCDD in combination with varying doses of RES (0.05-50 μM). Measurement of GAPDH mRNA was used to standardize CYP1A1 mRNA accumulation. Data was analyzed using a 1-way ANOVA and Bonferroni’s Multiple Comparison Test (**p<0.01, ***p<0.001).

Figure 2. Effect of resveratrol on CYP1A1 protein expression in MCF-7 and Caco-2 cells.
Western blots of (A) MCF-7 cell, and (B) Caco-2 cell lysates. Cells were treated either with vehicle, RES, or 2 nM TCDD alone, or TCDD with various concentrations of RES, cells were harvested 16 h later and lysates were fractionated by SDS-PAGE. In Figure B analysis of lysates from two separate experiments are shown.

Figure 3. Alterations in CYP1A1 activity in response to RES in MCF-7, Caco-2 and BEAS-2B cells as assessed by P450-GLO assay. MCF-7 cells (A), Caco-2 cells (B) and BEAS-2B cells (C) were maintained as described above prior to treatment with ligand. Cells were treated for 24 h with either DMSO, resveratrol alone, (100nM and 10 μM), TCDD alone (2 nM) or TCDD in combination with varying doses of resveratrol (0.005 -50 μM). CYP1A1
activity was measured by using the P450-GLO assay (Promega) according to the manufacturer’s protocol. After 24 h, LCEE was added to each well and cells were incubated overnight. Following incubation, recombinant luciferase was added to each well and luminescence was measured. Data is presented as mean (n=3) ± s.e.m. (**p<0.01). Right hand panels depict the data presented in a log-dose response relationship. Curves were fitted using GraphPad Prism 4.0 and non-linear regression analysis (F-test, for best fit comparisons) in order to predict a one- or two-site model.

Figure 4. Low dose resveratrol (100 nM) does not displace TCDD from the receptor or prevent DNA binding. (A) An AHR ligand displacement study using 2-azido-3-[125]iodo-7,8-dibromodibenzo-p-dioxin was performed. The image below the displacement plot depicts an autoradiograph of SDS-PAGE fractionated receptor coupled to the photo-affinity ligand after pre-incubation with increasing concentrations of RES. (B) Representative micrographs of ChIP analysis of AHR at the CYP1A1 enhancer during repression by low (100 nM) and high (10 μM) doses of RES. Below each panel appears Real-Time PCR analysis of immunoprecipitated CYP1A1 enhancer chromatin. Data was obtained from at least 2 separate experiments. (C) ChIP analysis of ERα at the CYP1A1 enhancer in response to combinations of 100 nM RES and 2 nM TCDD.

Figure 5. RES does not prevent nuclear translocation of AHR in MCF-7 cells. MCF-7 cells were transfected with AHR-eGFP using SuperFect transfection reagent (Qiagen). Cells were maintained in phenol-red-free, FBS-free media for at least 24 h after transfection and prior to treatment with ligand. Cells were treated with either vehicle (DMSO), RES (50 μM), TCDD (2
nM) or TCDD in combination with various concentrations of RES (0.1-10 μM) for 1 h. Cells were fixed and receptor status was assessed by immunofluorescence. Nuclei were identified using Hoechst 33342.

Figure 6. Fluorescence polarization ERα competition assay. ERα competition assay was performed using the Estrogen Receptor-α Competitor Assay, Green from PanVera #P2614, P2698, Invitrogen Corp. Vehicle (DMSO), varying concentrations of (10-200 nM), or E2 (1 nM to 1 μM) were diluted in ES2 screening buffer and added to 60 x 150 mm borosilicate glass cell culture tubes. Recombinant ERα was added for a final concentration of 6 pmol/μl, and ES2 fluoromone added for a final concentration of 400 nM. Samples were incubated in the dark at room temperature for 2 h and were measured for fluorescence polarization using a PanVera Beacon 2000 polarization reader with 485 nm excitation and 530 nm emission filters at 25° C. Results shown are the mean of samples done in triplicate.

Figure 7. Role of estrogen receptors in RES-mediated repression of AHR-driven transcription. Small inhibitory RNAs directed against ERα, ERβ and GFP were used to test their effects on AHR-mediated gene transcription in MCF-7 (A), Caco-2 (B) and BEAS-2B cells (C). Cells were transfected with either siGFP as control or ERα and/or ERβ RNAs 24 h prior to ligand treatment, otherwise cells were subsequently treated as described in Figure 1. CYP1A1 mRNA levels were determined as described above. Data is presented as mean (n=3) ± standard deviation (*p<0.05; **p<0.01). (D) Western blot of ERα protein knock-down of target proteins in MCF-7 cells, AhR and XAP2 are shown to control for protein loading.
Figure 2

A. MCF-7 Cells

CYP1A1

XAP2

TCDD 2 nM

RES (μM)

10 0.001 0.01 1.0 10 50 100

B. CaCo2 Cells

CYP1A1

HOP

DMSO

TCDD 5 nM

RES (μM)

10 0.1 0.5 1.0 5.0