Inhibition of aryl hydrocarbon receptor-dependent transcription by resveratrol or kaempferol is independent of estrogen receptor α expression in human breast cancer cells

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Abstract

Resveratrol and kaempferol are natural chemopreventative agents that are also aryl hydrocarbon receptor (AHR) antagonists and estrogen receptor (ER) agonists. In this study we evaluated the role of ER α in resveratrol- and kaempferol-mediated inhibition of AHR-dependent transcription. Kaempferol or resveratrol inhibited dioxin-induced cytochrome P450 1A1 (CYP1A1) and CYP1B1 expression levels and recruitment of AHR, ER α and co-activators to *CYP1A1* and *CYP1B1*. Both phytochemicals induced the expression and recruitment of ER α to *gene amplified in breast cancer 1 (GREB1)*. RNAi-mediated knockdown of ER α in T-47D cells did not affect the inhibitory action of either phytochemical on AHR activity. Both compounds also inhibited AHR-dependent transcription in ER α -negative MDA-MB-231 and BT-549 breast cancer cells. These data show that ER α does not contribute to the AHR-inhibitory activities of resveratrol and kaempferol.

Keywords

Resveratrol; Kaempferol; Dioxin; Aryl hydrocarbon receptor; Estrogen receptor

1. Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor and a member of the basic-helix-loop-helix Per (Period) ARNT (aryl hydrocarbon receptor nuclear translocator) SIM (single-minded) (bHLH/PAS) family. The AHR mediates the toxic effects of polycyclic chlorinated and aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and benzo[*a*]-pyrene (B[*a*]P) [1]. AHR exhibits profound ligand binding diversity, binding dioxins, polycyclic aromatic hydrocarbons (PAHs), coplanar polychlorinated biphenyl congeners and numerous natural compounds [2]. Certain phytochemicals act as AHR agonists [2], whereas polyphenols, such as resveratrol and the

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Conflict of interest

The authors have no conflict of interest to declare.

flavonoid, kaempferol are AHR antagonists [3,4]. Ligand-dependent activation of AHR causes its translocation to the nucleus where it dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT). AHR/ARNT complexes bind to its cognate DNA recognition sequence termed AHR response elements (AHREs) located in regulatory regions of phase I (cytochrome P4501A1 (CYP1A1), CYP1A2, CYP1B1) and phase II (NQO1, UGT1A6) drug metabolizing enzymes [1].

Crosstalk has been observed between AHR and many different signalling pathways [5,6], including acting as an inhibitor of estrogen-action [7]. Estrogen signalling is mediated by estrogen receptor α (ER α) and ER β , which are ligand-activated transcription factors and members of the nuclear receptor super-family of transcription factors [8]. ERs regulate gene expression in two ways, via direct DNA-binding to estrogen responsive elements (EREs) in target promoters or via protein–protein interactions with other transcription factors [8]. AHR and ER α physically interact, and activated AHR inhibits many ER-dependent responses through several different mechanisms [7,9]. ER-dependent modulation of AHR-mediated transcription is less clear as it varies from activation, inhibition to having no effect [7,9]. Some of these differences in AHR-ER α interactions may be due, in part, to cell context and the specific response. More recently, it has been shown that a number of AHR agonists directly activate ER α [10]. The ability of some compounds to modulate the activities of more than one receptor system may also contribute to crosstalk between these pathways.

Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenol present in grapes, peanuts and berries [11]. Kaempferol (3,4',5,7-tetrahydroxyflavone) is found in tea, broccoli, grapefruit and other plant sources, and accounts for approximately 25–33% of the mean dietary flavonol intake in the USA [12]. Resveratrol and kaempferol have received a lot of attention due to their anti-carcinogenic, cardioprotective and anti-aging properties [11,13]. These properties have been attributed to their ability to act as anti-oxidants, anti-inflammatory agents, and pro-apoptotic agents. Resveratrol and kaempferol have also been reported to inhibit the enzymatic activities of some phases I and II drug metabolizing enzymes including CYP1A1, CYP1B1, CYP1A2, CYP3A4, and sulfotransferase 1E1 (SULT1E1) and SULT1A2 [14,15].

Resveratrol and kaempferol also inhibit AHR-dependent transcription by preventing AHR/ARNT binding to the AHRE [16]. This is mediated, in part, by their ability to competitively displace TCDD from the AHR [4,16]. A single dose of 10 μ M resveratrol has been reported to inhibit the AHR-dependent activation of a saturating (100 nM) concentration of TCDD even after 48 h [17]. Interestingly, resveratrol was rapidly metabolized in HepG2 human hepatoma cells to several metabolic products within the first 8 h of treatment, including one of its the major phase I metabolites, piceatannol (3,4,3',5'-tetrahydroxy-*trans*-stilbene) [18]. Piceatannol is a tyrosine-kinase inhibitor with known anti-cancer and anti-inflammatory properties [19], but its ability to inhibit AHR-dependent transcription has not been determined. In addition to their ability to modulate AHR signalling, resveratrol and kaempferol also activate ER α and ER β and have been classified as phytoestrogens [20,21]. It remains unclear if the ability of resveratrol and kaempferol to activate ER α influences their ability to inhibit AHR-dependent transcription.

The aims of the present study were to investigate the role of ER α in the inhibitory effects of resveratrol and kaempferol on AHR-dependent transcription and determine the ability of piceatannol to inhibit AHR activity. Our data show that the estrogenic properties of resveratrol and kaempferol and ER α expression levels are independent of their ability to inhibit AHR-dependent transcription.

2. Materials and methods

2.1. Chemicals

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Wellington Laboratories (Guelph, ON, Canada). Resveratrol, kaempferol, piceatannol, 17 β -estradiol (E2), ICI 182,780 (ICI) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Cell culture media, fetal bovine serum (FBS), and trypsin were all purchased from Wisent (St. Bruno, QC). All other chemicals and biochemicals were of the highest quality available from commercial vendors.

2.2. Cell culture

T-47D, BT-549 and MDA-MB-231 human breast carcinoma cells were purchased from ATCC (Manassas, VA). T-47D cells were cultured in 1:1 mixture of DMEM (Dulbecco's modified Eagle's medium) and F12 Ham's nutrient mixture medium and supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. BT-549 cells were cultured in low glucose DMEM supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. MDA-MB-231 cells were cultured in low glucose DMEM supplemented with 10% (v/v) FBS, 1% penicillin/streptomycin and 1X non-essential amino acids. All cell lines were maintained at 37 °C and 5% CO₂ and were sub-cultured every 2–3 days or when they reached 80% confluency.

2.3. Chromatin immunoprecipitation (ChIP) assays

T-47D cells were plated in 10 cm dishes in 1:1 mixture of DMEM/F12 supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. After 48 h, cells were treated with either 1 nM TCDD, 1 nM TCDD + 10 μ M resveratrol, 1 nM TCDD + 10 μ M kaempferol, 1 nM TCDD + 10 μ M piceatannol or DMSO (0.1%) for 1 h. For estrogenic ChIP studies, T-47D cells were plated in 10 cm dishes in phenol red-free DMEM/F12 media supplemented with 5% dextran-coated charcoal (DCC)-treated FBS and 1% penicillin/streptomycin for 72 h. Cells were then treated with 10 nM E2, 10 μ M resveratrol, 10 μ M kaempferol, 10 μ M piceatannol or DMSO (0.1%) for 1 h and ChIP assays were performed as previously described [22] using 1 μ g of anti-AHR; H-211, anti-ARNT; H-172, anti-ER α ; HC-20, anti-CBP; A-22, anti-NCoA3; M-397 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-dimethyl-Histone H3 (Lys4); 07-303, anti-acetyl-Histone H3 (Lys9); 07-352 (Upstate, Temecula, CA), anti-trimethyl Histone H3 (Lys4); 39159 (Active Motif, Carlsbad, CA) or anti-Histone H3; ab1791-100 (Abcam, Cambridge, MA). Isolated DNA was quantified by quantitative real-time PCR (qPCR) using Kapa SYBR FAST qPCR Master Mix (KapaBiosystems, Woburn, MA). Primers used to amplify *CYP1A1* promoter and enhancer and *CYP1B1* enhancer regions have all been previously described [22]. *CYP1B1* promoter region primers were designed based on the *CYP1B1* gene sequence previously described

[23]. Data are reported as percentages relative to 5% total input chromatin. All histone H3 modifications were normalized to total H3 to ensure that any differences in immunoprecipitated DNA were not due to reduced levels of total H3.

2.4. RNA Isolation and qPCR

T-47D, BT-549 and MDA-MB-231 cells were seeded in six-well plates and grown their respective completed media. Cells were treated with DMSO (0.1%), 1 nM TCDD, 10 nM TCDD, 10 μ M resveratrol, 10 μ M kaempferol or 10 μ M piceatannol, alone or each of the phytochemicals were also treated in combination with TCDD for 1.5 h, 3 h, 6 h and 24 h. RNA was isolated using RNeasy spin columns (Qiagen). For estrogenic studies, T-47D cells were seeded in six-well plates in phenol red-free DMEM/F12 media supplemented with 5% DCC-treated FBS and 1% penicillin/streptomycin for 72 h. Cells were treated with 10 nM E2, 10 μ M resveratrol, 10 μ M kaempferol, 10 μ M piceatannol, 10 nM E2 + 100 μ M ICI, 10 μ M resveratrol + 100 μ M ICI, 10 μ M kaempferol + 100 μ M ICI, 10 μ M piceatannol + 100 μ M ICI or DMSO (0.1%) for 24 h. For cDNA synthesis, 500 ng of extracted RNA was reversed transcribed using SuperScriptII (Invitrogen) and random hexamer primers. qPCR was performed with 1 μ l of the cDNA synthesis reaction using Kapa SYBR FAST qPCR Master Mix (KapaBiosystems). Primers used to amplify CYP1A1, CYP1B1 and gene amplified in breast cancer 1 (GREB1) mRNAs have been previously described [24]. All target transcripts were normalized to the 18S rRNA and analyzed using the comparative C_T ($-C_T$) method. RNA data are presented as mean and standard error of the mean of at least two independent replicates.

2.5. Transient transfection and RNAi

ER α (siER α -11; J-003401-11-0020 and siER α -14; J-003401-14-0020) ON-TARGETplus siRNAs and Dharma-FECT1 transfection reagent were purchased from Dharmacon (Lafayette, CO). Briefly, T-47D cells were seeded 300,000 per well in six-well plates containing 2 ml of medium. After 24 h, 100 nM of each siRNA against ER α or non-targeting siRNA #2 (D-001810-02-20) (Dharmacon) were transfected using 4 μ l of DharmaFECT1. After 48 h, cells were treated with 1 nM TCDD, 1 nM TCDD + 10 μ M resveratrol, 1 nM TCDD + 10 μ M kaempferol or DMSO (0.1%) for 24 h for mRNA analysis as described above.

2.6. Western blot

T-47D cells were transfected with siER α or NT as described above. Whole cell extracts were prepared 48 h after transfection and proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked in 2% (w/v) ECL-Advanced blocking agent for 1 h at room temperature and then incubated with anti-ER α (HC-20), anti-AHR (SA-210, Biomol) or anti- β -actin antibody (Sigma) overnight at 4 °C. The membrane was then washed and incubated with appropriate secondary antibody for 1 h at room temperature. After washing, the bands were visualized using ECL-Advanced chemiluminescent substrate (GE Healthcare) according to the manufacturer's instructions.

2.7. Statistical analysis

All data are presented as means and standard error of the mean. ChIP and siRNA experiments were performed in triplicate and statistically analyzed using the Student's two-tailed *t*-test to assess statistical significance ($P < 0.05$). RNA data represent at least two independent replicates and were statistically analyzed using the Student's two-tailed *t*-test.

3. Results

3.1. Resveratrol and kaempferol inhibit TCDD-induced CYP1A1 and CYP1B1 mRNA expression

We first performed a series of dose-response experiments to evaluate the ability of resveratrol and kaempferol to inhibit the TCDD-induced CYP1A1 and CYP1B1 mRNA expression in T-47D human breast carcinoma cells. We observed that 10 μ M resveratrol inhibited induction of CYP1A1 and CYP1B1 mRNA levels by 1 nM TCDD but not 10 nM TCDD (Fig. 1B). This was in contrast to a recent study demonstrating that 10 μ M resveratrol inhibited 100 nM TCDD-induced CYP1A1 and CYP1B1 mRNA expression levels in MCF-7 human breast adenocarcinoma cells and HepG2 human hepatoma cells [17]. The reason for the discrepancy between the two studies is unclear; however, our data are consistent with other reports [4]. Kaempferol at a dose of 10 μ M, but not 1 μ M, inhibited the induction of CYP1A1 and CYP1B1 mRNA levels by 1, 10 and 100 nM TCDD, revealing it to be a more potent inhibitor of AHR-dependent transcription (Fig. 1C). The ability of kaempferol to inhibit AHR-dependent transcription following treatment with 10 nM TCDD was not observed after 48 h incubation (data not shown). Similar findings were observed in MCF-7 breast cells (data not shown).

We then performed a time course study to determine the kinetics of inhibition of AHR-dependent transcription by both phytochemicals. T-47D cells were treated with 1 nM TCDD alone or in combination with 10 μ M resveratrol or 10 μ M kaempferol from 1.5 to 24 h. The effects on TCDD-induced CYP1A1 and CYP1B1 mRNA expression were determined using qPCR. We observed inhibition of TCDD-induced CYP1A1 and CYP1B1 mRNA expression by resveratrol and kaempferol as early as 1.5 h of treatment (Fig. 1D and E).

To determine if resveratrol or kaempferol could inhibit TCDD-activated AHR or if TCDD could induce AHR-dependent gene expression in cells that were already exposed to phytochemicals, we performed a series of experiments in T-47D cells which were pre-treated with TCDD or phytochemicals. For these experiments, T-47D cells were pre-treated with either TCDD or phytochemicals for 2 h and then co-treated for 6 h with phytochemicals or TCDD, respectively, prior to determining the effects on CYP1A1 and CYP1B1 mRNA expression levels. Cells pre-treated with TCDD followed by treatment with resveratrol or kaempferol demonstrated significantly lower induction of CYP1A1 and CYP1B1 mRNA compared to TCDD treatment alone (Fig. 2A and B). Similarly, cells pre-treated with resveratrol or kaempferol also displayed significantly lower TCDD-induced CYP1A1 and CYP1B1 mRNA expression levels compared to TCDD treated cells (Fig. 2C and D). These findings show that resveratrol and kaempferol can inhibit activated AHR as well as prevent the induction of AHR-dependent transcription.

3.2. Resveratrol and kaempferol prevented TCDD-induced AHR/ARNT and co-activator recruitment and epigenetic changes at CYP1A1 and CYP1B1

Chromatin immunoprecipitation (ChIP) assays were used to investigate the mechanisms by which resveratrol and kaempferol inhibit TCDD-induced CYP1A1 and CYP1B1 mRNA levels. As expected TCDD-induced recruitment of AHR and ARNT to *CYP1A1* and *CYP1B1* enhancer and promoter regions was observed following 1 h treatment (Fig. 3). Co-treatment of TCDD with resveratrol or kaempferol resulted in significantly lower recruitment of AHR and ARNT to both regions compared to TCDD alone. Co-treatment of TCDD with either phytochemical also prevented the recruitment of co-activators, Creb binding protein (CBP) and nuclear receptor co-activator 3 (NCoA3/AIB1/SRC-3), and the TCDD-dependent increases in acetylation of histone H3K9 at both *CYP1A1* and *CYP1B1* (Fig. 4). These data indicate that both phytochemicals interrupt the assembly of an activated AHR/ARNT complex which also prevents subsequent downstream steps that are required for AHR-mediated transcription. Co-treatment with phytochemicals prevented TCDD-dependent increases in histone H3 lysine 9 acetylation (H3K9Ac) and decreases in histone H3 lysine 9 dimethylation (H3K4Me2) at *CYP1A1* and increases in H3K9Ac at *CYP1B1*. No changes in H3K4Me2 status following treatment with TCDD alone or in combination with phytochemicals was observed at *CYP1B1*.

3.3. Piceatannol inhibits TCDD-induced CYP1A1 and CYP1B1 expression and recruitment of AHR and ARNT to CYP1A1 and CYP1B1

A single treatment of 10 μ M resveratrol has been reported to inhibit TCDD-induced gene expression even after 48 h [17] and (MacPherson and Matthews unpublished data). However, it has also been shown that greater than 90% of resveratrol is metabolized after 8 h treatment in HepG2 cells [18]. Piceatannol is generated during phase I metabolism of resveratrol by CYP1A1, CYP1A2 or CYP1B1 [19], and represents one of its main hydroxylated metabolites. Therefore we were interested in determining the ability of piceatannol to inhibit TCDD-dependent induction of CYP1A1 and CYP1B1 expression levels. We observed that 10 μ M piceatannol inhibited 1 nM TCDD-induced CYP1A1 and CYP1B1 mRNA levels, but not those induced by 10 nM TCDD after 24 h of co-treatment (Fig. 5A and B). Moreover, piceatannol also inhibited TCDD-induced recruitment of AHR and ARNT to *CYP1A1* and *CYP1B1* enhancer regions (Fig. 5C and D). These data show that piceatannol is an inhibitor of AHR-dependent transcription and suggest that piceatannol may contribute to the prolonged inhibition of AHR-dependent gene expression following resveratrol treatment.

3.4. Estrogenic activity of resveratrol and kaempferol

We and others have previously demonstrated the AHR ligand-dependent recruitment of ER α to *CYP1A1* and *CYP1B1* [10,22] and we have suggested that ER α may influence AHR/ARNT activity through this mechanism [22]. Resveratrol and kaempferol have been described as multifunctional ligands activating multiple signalling pathways, including estrogen signalling pathway [9]. As such, we were interested to determine whether the estrogenic properties of these phytochemicals influenced their ability to inhibit AHR-dependent transcription. Resveratrol and kaempferol significantly induced the expression of the estrogen-responsive, GREB1 mRNA levels in T-47D cells; however, this induction was

lower than that induced by E2 (Fig. 6A). Resveratrol- and kaempferol-dependent increases in GREB1 mRNA expression levels were inhibited by co-treatment with the ER antagonist ICI 182,780. Piceatannol did not induce significant increases in GREB1 mRNA expression levels.

To determine the ability of both phytochemicals to induce recruitment of ER α and its associated co-activator NCoA3 to the upstream regulatory region of *GREB1* (referred to as *GREB1* enhancer 3) we performed ChIP assays. The *GREB1* enhancer 3 region is an estrogen-responsive genomic region identified by others and important in the ER α -dependent regulation of *GREB1* [25]. ChIP assays revealed that 1 h treatment of T-47D cells with resveratrol and kaempferol induced a similar level of recruitment of ER α to the *GREB1* enhancer 3 compared to 10 nM E2, whereas piceatannol induced only a modest increase in recruitment of ER α above untreated controls (Fig. 6B). Both phytochemicals also induced recruitment of the co-activator NCoA3 to the *GREB1* enhancer 3 region, but the level of recruitment was lower than that induced by E2 (Fig. 6C). Piceatannol induced a slight, but significant recruitment of NCoA3 to *GREB1* enhancer 3 region.

3.5. Inhibition of TCDD-induced CYP1A1 and CYP1B1 mRNA expression by resveratrol and kaempferol is independent of ER α

Since resveratrol and kaempferol activate ERs, we were interested to determine the role of ER α in the resveratrol- or the kaempferol-mediated inhibition of AHR-dependent transcription. In agreement with our previous work, we detected ER α at the *CYP1A1* and *CYP1B1* enhancer regions following treatment with TCDD (Fig. 7) [22]. ChIP assays showed that resveratrol and kaempferol inhibited the TCDD-induced recruitment of ER α to *CYP1A1* and *CYP1B1*, suggesting that ER α may not be directly involved in the inhibition of AHR-transcription mediated by these two phytochemicals. We then used RNAi-mediated knockdown of ER α in T-47D cells and determined the ability of resveratrol and kaempferol to inhibit the TCDD-induced CYP1A1 and CYP1B1 mRNA expression levels. Knockdown of ER α using two independent siRNA sequences did not affect inhibition of TCDD-induced CYP1A1 and CYP1B1 mRNA expression by resveratrol and kaempferol compared to non-targeting control (Fig. 8). Both siRNA sequences resulted in similar knockdown of ER α protein levels but had no effect on AHR protein expression. Moreover, TCDD-dependent gene expression in two ER α -negative breast cancer cell lines; MDA-MB-231 and BT-549, was also significantly inhibited by resveratrol and kaempferol (Fig. 9).

4. Discussion

Resveratrol and kaempferol are phytochemicals that have received considerable attention due to their *in vitro* and *in vivo* anti-cancer properties. Both compounds are known to modulate the activities of a number of signalling pathways including AHR- and ER-dependent transcription. Previous studies have suggested that ER α expression levels influence the ability of resveratrol to inhibit AHR-dependent transcription [4]; however, this has not been experimentally determined. Although some studies have reported that knockdown of ER α does not affect AHR-responsiveness in human breast cancer cells, we have shown that knockdown of ER α reduces AHR-responsiveness in a gene-specific manner

[24]. Moreover, overexpression of ER α in ER α -negative breast cancer cells restores AHR-responsiveness [26]. In this study we evaluated the role of ER α in resveratrol- and kaempferol-mediated inhibition of AHR-dependent transcription. Our data reveal that the ER α expression levels are independent of the ability of resveratrol and kaempferol to inhibit AHR-dependent transcription.

We observed that kaempferol (10 μ M) was more effective than resveratrol (10 μ M) at inhibiting TCDD (1 nM)-induced AHR-mediated transcription and recruitment of AHR and ARNT to *CYP1A1* and *CYP1B1* enhancer and proximal promoter regions and at reducing TCDD-dependent increases in *CYP1A1* and *CYP1B1* mRNA levels. Similarly, kaempferol was found to be the most effective flavonoid at inhibiting the agonistic effect of TCDD and cigarette smoke condensate on AHR-dependent activity [27]. We observed that the TCDD-dependent occupancy of CBP and NCoA3, and H3K9Ac levels at *CYP1A1* and *CYP1B1* was also inhibited by co-treatment with either phytochemical. Recruitment of AHR and ARNT to both the enhancer and proximal promoters regions of *CYP1A1* and *CYP1B1* is in agreement with our previous studies in MCF-7 human breast cancer cells [22], supporting the notion that the regulation of *CYP1A1* and *CYP1B1* occurs through enhancer-promoter looping, whereby factors bound to the enhancer sequence interact with the basal transcriptional machinery present at the proximal promoter. Others have reported that TCDD induces AHR/ARNT recruitment to the upstream AHRE-containing enhancer regions of *CYP1A1* and *CYP1B1* but not to the proximal promoter regions of both genes [28]. This discrepancy is most likely due to cell culture conditions and differences in assay methodologies between laboratories.

Resveratrol is rapidly metabolized following treatment of human hepatoma HepG2 cells, but a single dose of resveratrol inhibits TCDD-induced transcription for at least 48 h in the same cell line [17]. Our analysis of piceatannol, one of several phase I metabolites of resveratrol [18,19] supports the hypothesis that prolonged inhibition of resveratrol might be mediated by its metabolites. In humans and rodents, resveratrol is metabolized to glucuronidated and sulfated conjugates, with resveratrol glucuronide predominantly circulating in human plasma [29]. Similar to resveratrol, kaempferol is predominantly found as a glucuronide conjugate, kaempferol 3-glucuronide, in plasma and urine [30]. The phase II conjugation of resveratrol and kaempferol significantly reduces the bioavailability of both phytochemicals *in vivo*, which might weaken the *in vitro* anti-cancer, anti-inflammatory, and anti-atherogenic activities attributed to them. However, resveratrol administered by subcutaneous injection to female Sprague–Dawley rats completely prevented B[a]P-dependent induction of *CYP1A1* protein expression in several tissues [4]. Moreover, phase II metabolites of the flavones quercetin, quercetin-3-*O*-glucuronide and quercetin-3'-*O*-sulfate retained their anti-oxidant and inhibition of lipoxygenase activity, respectively. These findings demonstrate the effectiveness of resveratrol at inhibiting AHR-mediated activity *in vivo* and show that phase II metabolic products of certain phytochemicals retain biological activity.

Our analysis of the estrogenic activity of resveratrol, kaempferol and piceatannol is similar to previous studies [20,31] but with some notable differences. In agreement with these studies, we observed super-induction of ERE-regulated reporter activity with resveratrol but not with kaempferol at the doses examined. Both compounds induced a lower level of

GREB1 mRNA expression but induced a similar level of recruitment of ER α to *GREB1* enhancer 3 upstream regulatory region. Interestingly, NCoA3 occupancy at *GREB1* enhancer 3 induced by resveratrol or kaempferol was lower than that induced by E2. This suggests that resveratrol- and kaempferol-ER α might induce structurally distinct conformations from that of E2-ER α altering the effectiveness of the ligand-bound receptor to interact or recruit co-activators similar to that reported for genistein-ER α conformation [32]. Piceatannol displayed weak estrogenic activities in T-47D cells, which contrasts a report describing this compound as a strong estrogen receptor agonist [33]. Estrogenic as well as anti-estrogenic activities have been reported for both resveratrol and kaempferol [34,35]. Resveratrol has been shown to inhibit cell proliferation as well as induce apoptosis and cell cycle arrest in several breast cancer cell lines [36]. Resveratrol decreases ROS generation and inhibits breast tumour cell invasiveness and delays the development of spontaneous mammary tumours in HER-2/neu transgenic mice [37]. However, resveratrol has been reported to accelerate N-methyl-N-nitrosourea-induced mammary carcinogenesis in pre-pubertal rats [38] and induce receptor-mediated proliferation in osteoblasts and breast cancer cells [20]. Collectively, these studies suggest that the effects of resveratrol are influenced by cellular conditions, assay parameters and model system used.

Inhibitory ER-AHR crosstalk may contribute to the AHR antagonist activities by certain phytochemicals. Despite the estrogenic action of resveratrol and kaempferol, siRNA-mediated knockdown of ER α did not affect the ability of either compound to inhibit AHR-dependent transcription in T-47D cells. Both compounds also inhibited TCDD-induced CYP1A1 and CYP1B1 mRNA expression levels in ER α -negative MDA-MB-231 and BT-549 human breast cancer cells. Since T-47D cells express low levels of ER β and both BT-549 and MDA-MB-231 express ER β [39], we cannot exclude the possible role of ER β in mediating the inhibitory action of resveratrol or kaempferol on the AHR signalling pathway. However, resveratrol effectively inhibits AHR-mediated transcription in HepG2 human hepatoma cells which do not express ER α or ER β [40]. Pharmacological dissection of the role of ERs in resveratrol- or kaempferol-mediated inhibition of AHR action must be carefully considered since 4-hydroxytamoxifen and ICI 182,780 have recently been reported to activate AHR [41].

Another mechanism by which resveratrol may inhibit AHR-dependent transcription is through regulation and/or activation of NAD-dependent deacetylase sirtuin-1 (SIRT1) [42]. SIRT1 is a class III histone deacetylase that modulates the activity of histones as well as a number of transcription factors, including p53 and forkhead protein O3a (FOXO3a) factors [43]. Recent studies have questioned whether resveratrol is a *bona fide* activator of SIRT1 [44]. We did not observe any recruitment of SIRT1 to AHR-regulated genes in the presence of TCDD alone or in combination with resveratrol (data not shown).

In summary, our findings show that the ability of resveratrol and kaempferol to inhibit AHR-dependent transcription is independent of ER α expression. We also provide evidence that metabolites of phytochemicals may also contribute to the ability of resveratrol to inhibit AHR-mediated action and may also be important in mediating other biological actions and anti-neoplastic properties of these compounds.

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Fig. 1.

(A) Chemical structures of resveratrol, kaempferol and piceatannol. (B and C) Concentration-dependent inhibition of TCDD-induced CYP1A1 mRNA expression levels by resveratrol and kaempferol in T-47D cells. T-47D cells were treated with 1–100 nM TCDD alone or in combination with 10 μ M resveratrol or kaempferol for 24 h. (D and E) Time-dependent increases in TCDD-induced CYP1A1 and CYP1B1 mRNA expression levels and their inhibition by co-treatment with resveratrol and kaempferol in T-47D cells. T-47D cells were treated with 1 nM TCDD, 1 nM TCDD + 10 μ M resveratrol or 1 nM TCDD + 10 μ M kaempferol for 1.5–24 h. Results shown are means $\pm$ S.E.M for three independent experiments. Expression levels significantly ($P < 0.05$) different compared to time-matched TCDD treatments are denoted with an asterisk. T; TCDD, Resv; resveratrol, Kae; kaempferol.

Fig. 2.

CYP1A1 and CYP1B1 mRNA expression of T-47D cells pre-treated with 1 nM TCDD, 10 μ M resveratrol or 10 μ M kaempferol. (A and B) T-47D cells were pre-treated with control (DMSO) or 1 nM TCDD for 2 h and then treated with 10 μ M resveratrol or 10 μ M kaempferol for 6 h. RNA was isolated and reverse transcribed as described in Section 2. Results are shown as means $\pm$ S.E.M for three independent experiments. mRNA expression levels of TCDD + resveratrol or kaempferol co-treatments significantly ($P < 0.05$) lower than TCDD + DMSO are denoted with an asterisk. (C and D) T-47D cells were pre-treated with either 10 μ M resveratrol or 10 μ M kaempferol for 2 h and then treated with 1 nM TCDD for 6 h. RNA was isolated and reverse transcribed as described in Section 2. Results shown are as means $\pm$ S.E.M for three independent experiments. mRNA expression levels of resveratrol or kaempferol + TCDD co-treatments significantly ($P < 0.05$) different than DMSO + TCDD are denoted with an asterisk.

Fig. 3.

Resveratrol and kaempferol inhibit TCDD-induced recruitment of AHR and ARNT to *CYP1A1* (A) enhancer and (B) promoter and *CYP1B1* (C) enhancer and (D) promoter. T-47D cells were treated with 1 nM TCDD or co-treated with 1 nM TCDD + 10 μ M resveratrol or 1 nM TCDD + 10 μ M kaempferol for 1 h and cells were harvested for ChIP assays as described in Section 2. Results shown are means $\pm$ S.E.M for three independent experiments. Percent recruitment levels significantly ($P < 0.05$) different than control are denoted with an asterisk, percent recruitment levels significantly ($P < 0.05$) different than TCDD treatment are denoted with a pound sign. Resv; resveratrol, Kae; kaempferol.

Fig. 4.

Resveratrol and kaempferol inhibit TCDD-induced recruitment of CBP, NCoA3 and H3K4Me2 and H3K9Ac at (A) *CYP1A1* and (B) *CYP1B1* enhancer regions. T-47D cells were treated with 1 nM TCDD or co-treated with 1 nM TCDD + 10 μ M resveratrol or 1 nM TCDD + 10 μ M kaempferol for 1 h and were harvested for ChIP assays as described in Section 2. Results shown are means $\pm$ S.E.M for three independent experiments. Percent recruitment levels significantly ($P < 0.05$) different than control are denoted with an asterisk, percent recruitment levels significantly ($P < 0.05$) different than TCDD treatment are denoted with a pound sign. Resv; resveratrol, Kae; kaempferol.

Fig. 5.

Piceatannol inhibits TCDD-induced expression of CYP1A1 and CYP1B1 and TCDD-induced recruitment of AHR and ARNT to *CYP1A1* and *CYP1B1* enhancer regions in T-47D cells. (A and B) Concentration-dependent inhibition on TCDD-induced CYP1A1 and CYP1B1 mRNA expression levels by piceatannol. T-47D cells were treated with 1 nM or 10 nM TCDD alone or in combination of 10 μM piceatannol for 24 h and mRNA was prepared as described in Section 2. (C–F) AHR and ARNT recruitment to *CYP1A1* and *CYP1B1* enhancer regions was inhibited by piceatannol co-treatment. T-47D cells were treated with 1 nM TCDD alone or in combination with 10 μM piceatannol for 1 h and cells were harvested for ChIP assays. Results shown are means ± S.E.M for three independent experiments. Changes in mRNA expression and percent recruitment levels significantly ($P < 0.05$) different than TCDD are denoted with an asterisk, percent recruitment levels significantly ($P < 0.05$) different than control are denoted with a pound sign. Pic; piceatannol.

Fig. 6.

(A) GREB1 mRNA induction by AHR inhibitors. T-47D cells were plated in media devoid of estrogens and treated with 10 nM E2, 10 μM resveratrol, 10 μM kaempferol or 10 μM piceatannol or in co-treatment with 100 nM ICI for 24 h. mRNA expression levels significantly ($P < 0.05$) different than control are denoted with an asterisk, mRNA expression levels significantly ($P < 0.05$) different than E2 treatment are denoted with a pound sign, mRNA expression levels of cells co-treated with ICI significantly ($P < 0.05$) lower than treatment alone are denoted with a double dagger. (B & C) Recruitment of ERα and NCoA3 to the *GREB1* enhancer 3 region by the three phytochemicals. T-47D cells plated in media devoid of estrogens were treated with 10 nM E2, 10 μM resveratrol, 10 μM kaempferol or 10 μM piceatannol for 1 h and ChIP assays were performed as described in Section 2. Percent recruitment levels significantly ($P < 0.05$) different than control are denoted with an asterisk, percent recruitment significantly ($P < 0.05$) different than E2 treatment are denoted with a pound sign. Results shown are means $\pm$ S.E.M for three

independent experiments. Resv; resveratrol, Kae; kaempferol, Pic; piceatannol, ICI; ICI 182,780.

Fig. 7.

Resveratrol and kaempferol inhibit TCDD-induced recruitment of ERα to (A) *CYP1A1* and (B) *CYP1B1* enhancer regions. T-47D cells were treated with 1 nM TCDD alone or in combination with 10 μM resveratrol or 10 μM kaempferol for 1 h and ChIP assays were performed as described in Section 2. Percent recruitment levels significantly ($P < 0.05$) different than control are denoted with an asterisk, percent recruitment levels significantly ($P < 0.05$) different than TCDD treatment alone are denoted with a pound sign. Results shown are means $\pm$ S.E.M for three independent experiments. Resv; resveratrol, Kae; kaempferol.

Fig. 8.

RNAi-mediated ER α knockdown in T-47D cells. (A and B) CYP1A1 and CYP1B1 mRNA expression. T-47D cells were transfected with specific siRNA against ER α for 48 h. Cells were then treated with 1 nMTCDD alone or in combination with 10 μ M resveratrol or 10 μ M kaempferol for 24 h and RNA was isolated and reverse transcribed. Changes in mRNA expression was then determined using qPCR. Data were normalized to non-targeting (NT) DMSO (control) and to ribosomal 18s levels. Results shown are means $\pm$ S.E.M for three independent experiments. Changes in mRNA expression levels within similarly transfected cells significantly ($P < 0.05$) lower than TCDD treated cells are denoted with an asterisk. (C) Western blot analysis of RNAi-mediated ER α knockdown in T-47D cells. Cell extracts were probed with anti-ER α or anti-AHR or anti- β -actin antibodies as described in Section 2. This figure is representative of three independent experiments. NT; non-targeting, Resv; resveratrol, Kae; kaempferol.

Fig. 9.

Inhibition of TCDD-induced CYP1A1 and CYP1B1 mRNA in ER α -negative MDA-MB-231 (A and B) and BT-549 (C and D) cells by resveratrol and kaempferol. MDA-MB-231 and BT-549 cells were treated with 1 nM TCDD alone or in combination with 10 μ M resveratrol or 10 μ M kaempferol for 24 h and RNA was isolated and reverse transcribed. Changes in mRNA expression levels were determined using qPCR. Data were normalized against DMSO (control) to ribosomal 18s levels. Results shown are means $\pm$ S.E.M for three independent experiments. Significant differences ($P < 0.05$) compared to TCDD treated cells are denoted with an asterisk. Resv; resveratrol, Kae; kaempferol.