Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect *CYP1A1* transcription differentially

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Transcriptional activation of the human *CYP1A1* gene (coding for cytochrome P450 1A1) is mediated by the aryl hydrocarbon receptor (AhR). In the present study we have examined the effect of the common dietary polyphenolic compounds quercetin and kaempferol on the transcription of *CYP1A1* and the function of the AhR in MCF-7 human breast cancer cells. Quercetin caused a time- and concentration-dependent increase in the amount of CYP1A1 mRNA and CYP1A1 enzyme activity in MCF-7 cells. The increase in CYP1A1 mRNA caused by quercetin was prevented by the transcription inhibitor actinomycin D. Quercetin also caused an increase in the transcription of a chloramphenicol reporter vector containing the *CYP1A1* promoter. Quercetin failed to induce CYP1A1 enzyme activity in AhR-deficient MCF-7 cells. Gel retardation studies demonstrated that quercetin activated the ability of the AhR to bind to an oligonucleotide containing the xenobiotic-responsive element (XRE) of the *CYP1A1* promoter. These results indicate that quercetin's effect is mediated by the AhR. Kaempferol did not affect *CYP1A1* expression by itself but it inhibited the transcription of *CYP1A1* induced by the prototypical AhR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), as measured by a decrease in TCDD-induced *CYP1A1* promoter-driven reporter vector activity, and CYP1A1 mRNA in cells. Kaempferol also abolished TCDD-induced XRE binding in a gel-shift assay. Both compounds were able to compete with TCDD for binding to a cytosolic extract of MCF-7 cells. Known ligands of the AhR are, for the most part, man-made compounds such as halogenated and polycyclic aromatic hydrocarbons. These results demonstrate that the dietary flavonols quercetin and kaempferol are natural, dietary ligands of the AhR that exert different effects on *CYP1A1* transcription.

Key words: chemoprevention, flavonoid, MCF-7 cells, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, xenobiotic-responsive element.

INTRODUCTION

Exposure to environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) and their halogenated derivatives such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes the induction of the *CYP1A* gene family, which encode cytochromes P450 1A1 and 1A2 [1]. These enzymes catalyse the metabolic activation of PAHs, generating genotoxic metabolites that bind DNA [2] and thus mediate PAH-induced carcinogenesis. Transcriptional activation of *CYP1A1* is regulated by the aryl hydrocarbon receptor (AhR), a cytosolic protein that belongs to the basic helix–loop–helix protein family. The AhR has been detected in several different tissues and cell types [3,4]; it is thought to mediate the broad spectrum of biological responses that PAH or TCDD elicits, including tumorigenesis, teratogenesis, tumour promotion and thymic atrophy [5]. After the binding of PAH or TCDD, the AhR translocates to the nucleus, where it heterodimerizes with a protein partner, the AhR nuclear translocator, forming a transcription factor that binds the xenobiotic-responsive elements (XREs) present in the 5′-promoter of *CYP1A1*, inducing transcription [6]. Several non-PAH compounds have also been shown to be inducers of *CYP1A1* [7–9] but the known ligands of the AhR are mainly man-made compounds. Known natural ligands of the AhR include: indolo[3,2-*b*]carbazole, an acid derivative of a compound found in some vegetables [10–12]; curcumin, a polyphenolic compound found in the spice turmeric [13]; tryptophan metabolites [14]; and bilirubin [15]. Other natural exogenous or endogenous ligands of the AhR have been postulated but not demonstrated.

Flavonoids, a large group of polyphenolic derivatives of benzo-γ-pyrone, are one of the most prevalent class of compounds in edible plants and thus in human diets [16]. Total dietary flavonoid intake has been estimated to be as high as 1 g/day [17] but recent studies have indicated that intake varies widely [18,19]. The most abundant flavonoids are the flavonols quercetin and kaempferol, which exist as a variety of glycosides or in aglycone form. Recent studies have shown that either form of these compounds is absorbed by the human gut [20]. The aglycone forms of quercetin and kaempferol are similar in structure, differing only by one hydroxy group in the B-ring (Figure 1). Quercetin has been extensively studied, particularly with regard to biochemical mechanisms that affect carcinogenesis. In animal models, it has chemopreventive activity against tumorigenesis induced by AhR ligands such as PAHs [21,22]. In cell culture models, it exerts a multiplicity of biochemical effects that are relevant to carcinogenesis, including metal chelation [23], antioxidant properties [24], the inhibition of hepatic enzymes, including CYP1A1, involved in carcinogen activation [25], and the induction of Phase II (conjugating) enzymes [26]. Despite this, there has been to our knowledge no study that has examined the effect of quercetin or kaempferol on the AhR and *CYP1A1* transcription.

We have hypothesized that dietary polyphenolic compounds

Abbreviations used: AhR, aryl hydrocarbon receptor; B[*a*]P, benzo[*a*]pyrene; CAT, chloramphenicol acetyltransferase; CYP1A1, cytochrome P450 1A; DMBA, dimethylbenz[*a*]anthracene; EMSA, electrophoretic mobility-shift assay; EROD, ethoxyresorufin-*O*-de-ethylase; GPDH, glyceraldehyde-3-phosphate dehydrogenase; PAH, polycyclic aromatic hydrocarbon; RT–PCR, reverse-transcriptase-mediated PCR; TBE, Tris/borate/EDTA; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic-responsive element.

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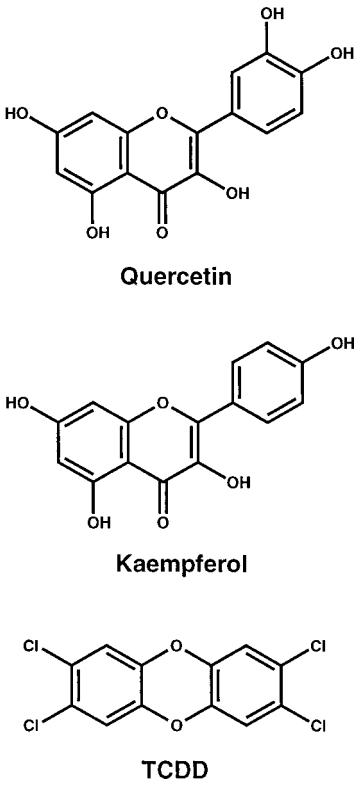

Figure 1 Structures of quercetin, kaempferol and TCDD

such as the flavonoids might be natural ligands of the AhR. This is based on two sets of data: natural ligands of the AhR, indolo[3,2-*b*]carbazole and curcumin, are dietary polyphenolic compounds; and several synthetic derivatives of flavone, the parent structure of flavonoids, are known to interact with the AhR, either as antagonists or as agonists [9,27,28]. To test this hypothesis we examined the effect of the most common and widely distributed flavonoids, quercetin and kaempferol, on *CYP1A1* transcription mediated by the AhR in MCF-7 human breast cancer cells. These cells were chosen as a model system because the function of AhR in these cells has been well characterized [29–31]. We demonstrate that quercetin induces *CYP1A1* transcription by activating the AhR. Although kaempferol does not induce *CYP1A1* transcription, it too interacts with the AhR, and can act as an antagonist of *CYP1A1* transcription induced by TCDD.

MATERIALS AND METHODS

Materials

MCF-7 cells were from the American Type Culture Collection (Rockville, MD, U.S.A.). RPMI 1640, glutamine, fetal bovine serum, trypsin/EDTA, PBS and Tris/borate/EDTA (TBE) buffer were from BioFluids (Rockville, MD, U.S.A.). Quercetin and kaempferol were from Indofine (Somerville, NJ, U.S.A.). Actinomycin D, benzo[*a*]pyrene (B[*a*]P), dimethylbenz[*a*]-anthracene (DMBA), EDTA, dithiothreitol, glycerol, Hepes, polydeoxyinosinic-deoxycytidylic acid, sodium molybdate, ethoxyresorufin, resorufin, Tris/HCl, salmon sperm DNA, DMSO and protease inhibitors were from Sigma (St. Louis, MO, U.S.A.). [^{32}P]dCTP and [^{32}P]dATP were from DuPont NEN (Boston, MA, U.S.A.). [^{3}H]TCDD (specific radioactivity 28.4 Ci/mmol) was from ChemSyn (Lenexa, KS, U.S.A.). Reverse-transcriptase-mediated PCR (RT–PCR) was performed with a kit from Stratagene (La Jolla, CA, U.S.A.). TBE gels, TBE running buffer and high-density sample buffer were from Novex (San Diego, CA, U.S.A.). Primers for glyceraldehyde-3-phosphate dehydrogenase (GPDH) PCR and β-galactosidase-containing reporter vector were from Clontech (Palo Alto, CA, U.S.A.). Bradford protein assay kit was from Bio-Rad (Hercules, CA, U.S.A.). Trizol reagent and LipofectAmine were from Gibco BRL (Gaithersburg, MD, U.S.A.). Chloramphenicol acetyltransferase (CAT) ELISA assay kit was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Polyclonal antibody against AhR was a gift from Dr. Alan Poland (University of Wisconsin, Madison, WI, U.S.A.).

Cell culture

MCF-7 cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine and 10 % (v/v) fetal bovine serum. Cell were subcultured weekly with 0.25 % trypsin/0.05 % EDTA. All experiments were performed on confluent cultures in growth medium, unless otherwise noted.

RT–PCR

Stock solutions of all chemicals (except where indicated) were made up in DMSO and stored at −20 °C. Control cultures received an amount of DMSO equal to the treated cultures (the final concentration of DMSO was 0.1 %). After incubation the cells were washed twice with PBS and total RNA was isolated with Trizol reagent as directed. Semi-quantitative RT–PCR for CYP1A1 mRNA was performed in the presence of 1.5 μCi of [^{32}P]dATP with the primer sequences and conditions of Dohr et al. [29]. cDNA was synthesized from 10 μg of total RNA with the use of a RT–PCR kit as instructed. The optimum cycle number that fell within the exponential range of response for both CYP1A1 (23 cycles) and GPDH (19 cycles) was used. After PCR, 5 μl of high-density sample buffer was added to the samples and they were subjected to electrophoresis on a 10 % (w/v) gel in 1 × TBE running buffer. The gel was dried and the results were detected and quantified on a Bio-Rad GS-363 Molecular Imaging System (Hercules, CA, U.S.A.). Graphs of the resulting data were generated by normalizing CYP1A1 to GPDH.

Transient transfections

MCF-7 cells were plated at 60000 cells per well in 24-well plates. After 24 h the cells were transiently co-transfected with 12.0 μg of a CAT reporter vector containing the full-length rat *CYP1A1* promoter [32] and 1.0 μg of a vector containing β-galactosidase with the use of LipofectAmine as directed. The amount of CAT transcription was determined with an ELISA assay as directed. β-Galactosidase activity was determined by the method of Rosenthal [33].

CYP1A1 activity in intact MCF-7 cells

Ethoxyresorufin-*O*-de-ethylase (EROD) activity, which is a specific assay of the bioactivation capacity of CYP1A1, was determined in intact MCF-7 cells grown in 24-well plates as described by Kennedy and Jones [34], with 5 μM ethoxyresorufin in growth medium as a substrate in the presence of 1.5 mM salicylamide to inhibit conjugating enzymes. The assay was performed at 37 °C. The fluorescence of resorufin generated by the conversion of ethoxyresorufin by CYP1A1 was measured every 10 min for 60 min in a CytoFlor II multi-well fluorescence plate reader (PerSeptive Biosystems, Framingham, MA, U.S.A.),

with excitation at 530 nm and emission at 590 nm. A standard curve was generated with resorufin.

The AhR-deficient MCF-7 cell line used to determine EROD activity in Figure 5(C) was derived from the parent MCF-7 cells by long-term culture (more than 6 months) in increasing concentrations of the aryl hydrocarbon B[*a*]P. This resulted in the generation of a B[*a*]P-resistant MCF-7 cell line that expresses only approx. 20 % of the AhR of the wild-type cells, as measured at the protein (Western blotting) and mRNA (RT–PCR) levels. EROD activity is not up-regulated in these cells in response to most AhR ligands except high concentrations (10 nM) of the most potent ligand, TCDD. A paper describing these cells is currently in preparation (H. P. Ciolino and G. C. Yeh, unpublished work).

Electrophoretic mobility-shift assay (EMSA)

Confluent cultures of MCF-7 cells were treated as described in the figure legends in growth medium for 3 h. Nuclear protein was isolated and EMSA was performed by the method of Denison et al. [35]. Synthetic oligonucleotides containing the AhR-binding site of the XRE [36] were labelled with [^{32}P]dCTP. The binding reactions were performed for 30 min at room temperature and contained 5 μg of nuclear protein, 1 μg of polydeoxyinosinic-deoxycytidylic acid, 500 ng of salmon sperm DNA and approx. 50000 c.p.m. of labelled probe in a final volume of 20 μl of binding buffer [25 mM Tris/HCl (pH 7.9)/50 mM KCl/1 mM $MgCl_2$/1.5 mM EDTA/0.5 mM dithiothreitol/5 % (v/v) glycerol]. To determine the specificity of binding to the oligonucleotide, a 50-fold excess of unlabelled specific probe, a 50-fold excess of unlabelled non-specific probe of the transcription factor AP-2 or 0.864 μg of anti-AhR polyclonal antibody were incubated with the nuclear extract of quercetin (10 μM)-treated cells on ice for 15 min. DNA–protein complexes were separated under non-denaturing conditions on a 6 % (w/v) polyacrylamide gel with 0.5 × TBE (45 mM Tris borate/45 mM boric acid/2 mM EDTA) as a running buffer. The gels were dried and the DNA–protein complexes were detected and quantified with a Bio-Rad GS-363 Molecular Imaging System.

AhR ligand binding assay

MCF-7 cells were grown to confluence in 175 cm^2 flasks. The cells were washed once in PBS, harvested by treatment with trypsin, and pelleted by centrifugation at 800 ***g*** for 10 min at 4 °C. The pellet was washed once in cold PBS, repelleted as above and resuspended in cold buffer [25 mM Hepes/1 mM EDTA/1 mM dithiothreitol/20 mM sodium molybdate/10 % (v/v) glycerol (pH 7.4)] containing protease inhibitors (100 μg/ml PMSF, 300 μg/ml EDTA, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin and 0.7 μg/ml Pepstatin A). The cells were homogenized by 30 strokes with a Dounce glass homogenizer on ice and the homogenate was centrifuged at 100000 ***g*** for 60 min at 4 °C. The supernatant (cytosol) was removed and protein content was determined by the Bradford method [37]. The cytosol was used immediately or divided into aliquots, stored at −70 °C and used within 24 h. Specific binding to the AHR was measured by sucrose density-gradient centrifugation as described by Raha et al. [38]. Cytosolic protein (1.0 mg) was incubated with 10 nM [^{3}H]TCDD in the presence of DMSO (control), 10 μM unlabelled TCDD (positive control) or 50 μM quercetin or kaempferol in a total volume of 500 μl of the above buffer for 2 h at 4 °C. Samples were applied to 5–30 % (w/v) linear sucrose density gradients in 12 ml Beckman Quick-Seal rotor tubes. The gradients were centrifuged for 2 h at 63000 rev./min (372000 ***g***) in a Beckman VTI-65-1 rotor; 25 fractions of seven drops each (approx. 500 μl) were collected from the bottom of the tubes and assayed for radioactivity with Aquasure scintillation fluid. Specific binding to the AhR was also measured by hydroxyapatite absorption chromatography by a modification of the method of Poellinger et al. [39] as described [13].

Statistical analysis

Statistical analyses were performed with STATVIEW Statistical Analysis software (SAS Institute, San Francisco, CA, U.S.A.). Differences between group mean values were determined by a one-factor analysis of variance (ANOVA), followed by Fisher PSLD post-hoc analysis for pairwise comparison of means.

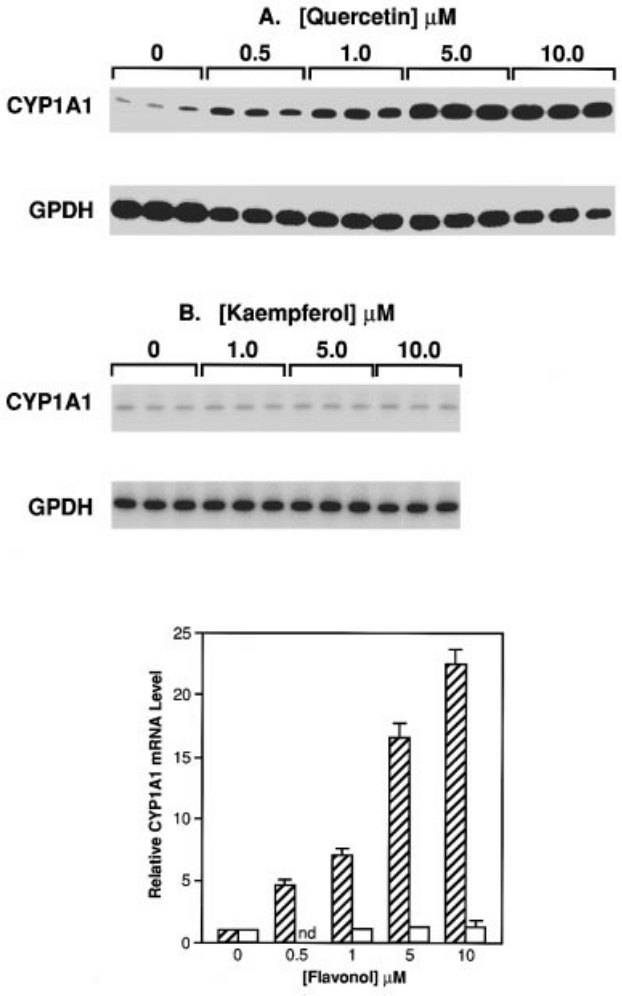

Figure 2 Concentration response of CYP1A1 mRNA to quercetin (A) and kaempferol (B)

MCF-7 cells were treated with the indicated concentration of quercetin (**A**) or kaempferol (**B**) for 24 h RT–PCR for CYP1A1 and GPDH mRNA was performed as described in the Materials and methods section and the results were detected and quantified by phosphorimaging. For the bar chart, the amount of CYP1A1 was normalized to the GPDH level. Hatched bars, quercetin; open bars, kaempferol. Abbreviation: nd, not determined. The level of CYP1A1 mRNA in all quercetin-treated cells was significantly different from control cells ($P < 0.05$).

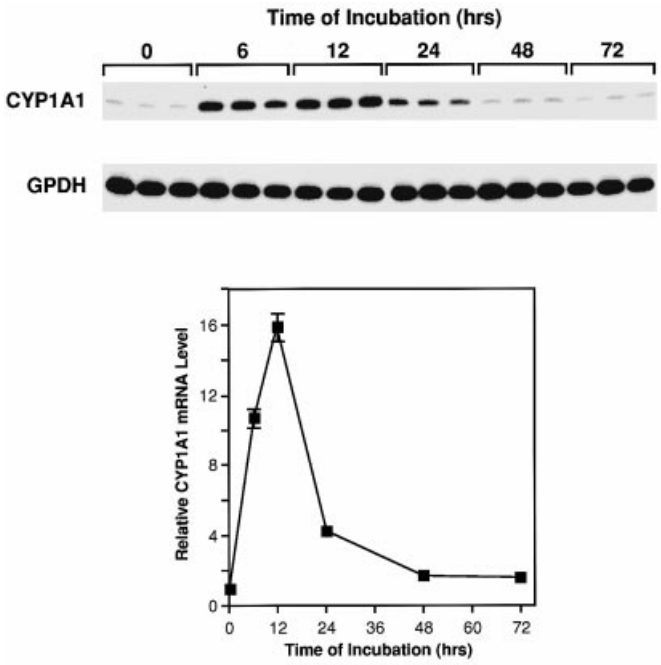

Figure 3 Time course of CYP1A1 mRNA increase caused by quercetin

MCF-7 cells were treated with 0.5 μM quercetin for the durations indicated. CYP1A1 and GPDH mRNA were determined by RT–PCR. For the graph, the amount of CYP1A1 mRNA was normalized to GPDH levels. The level of CYP1A1 mRNA was significantly increased compared with controls after 6, 12 and 24 h of incubation with quercetin ($P < 0.05$).

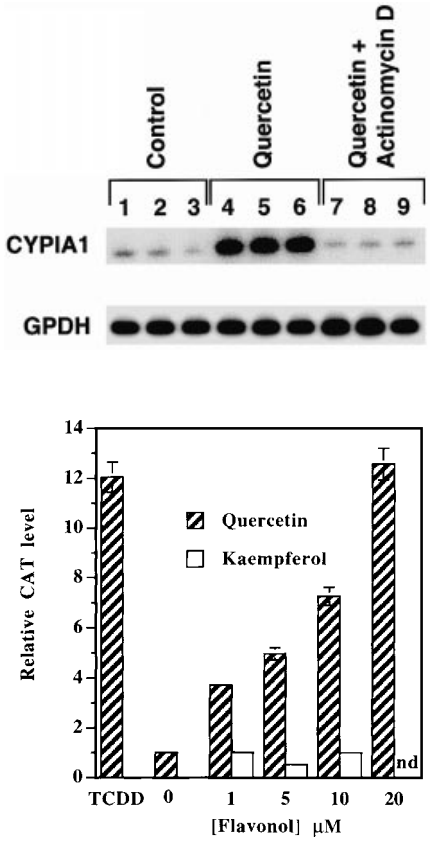

Figure 4 Effect of quercetin or kaempferol on *CYP1A1* transcription

Upper panel: MCF-7 cells were treated for 1 h with ethanol (control) or actinomycin D (5 μg/ml) followed by DMSO (control) or 5 μM quercetin for 6 h; the amount of CYP1A1 and GPDH mRNA was measured by RT–PCR as described. The level of CYP1A1 mRNA in cells treated with quercetin in the presence of actinomycin D was not significantly different from that in control cells. Lower panel: MCF-7 cells were transfected with the aryl hydrocarbon-responsive vector pMC6.3, which contains the *CYP1A1* promoter, and a vector containing β-Gal. Transfected cells were treated with the indicated concentrations of quercetin or kaempferol for 24 h The amount of CAT transcription was normalized to the amount of β-Gal transcribed. Abbreviation: nd, not determined. CAT transcription in all quercetin-treated samples was significantly increased over that in controls ($P < 0.05$).

RESULTS

Effect of quercetin and kaempferol on the expression of *CYP1A1*

MCF-7 cells were treated with quercetin or kaempferol for 24 h and the amount of CYP1A1 mRNA was measured by semi-quantitative RT–PCR. Quercetin caused a concentration-dependent increase in the amount of CYP1A1 mRNA (Figure 2A), whereas kaempferol had no effect on CYP1A1 mRNA (Figure 2B). Quercetin caused a rapid increase in CYP1A1 transcript that reached a maximum after 12 h of treatment but was still significantly increased after 24 h (Figure 3).

Pretreatment of the cells with the transcription inhibitor actinomycin D abolished the induction of CYP1A1 mRNA caused by quercetin (Figure 4, upper panel).

MCF-7 cells were transfected with a CAT reporter vector containing the full-length *CYP1A1* promoter. Treatment of transfected cells with 1 nM TCDD for 6 h resulted in an increase in CAT transcription of approx. 12-fold over the DMSO control (Figure 4, lower panel). CAT transcription was also increased by treatment with the AhR ligands B[*a*]P, DMBA and 3-methylcholanthrene (results not shown). Quercetin, but not kaempferol, caused a concentration-dependent increase in CAT transcription. This increase reached the approximate level of induction seen in cells treated with 1 nM TCDD (approx. 12-fold over control levels) in cells treated with 20 μM quercetin.

The enzymic activity of CYP1A1 in intact MCF-7 cells treated with quercetin or kaempferol was assayed by measuring EROD activity. Incubation of the cells with quercetin for 48 h caused a concentration-dependent increase in EROD activity over the range of concentrations tested, whereas kaempferol had no effect on EROD activity in the cells (Figure 5A). The quercetin-induced increase in EROD activity was maximal at 48 h but still significantly increased compared with controls after 72 h of incubation (Figure 5B). Wild-type and AhR-deficient MCF-7 cells were incubated with TCDD, B[*a*]P or quercetin for 24 h and the EROD activity was measured after 24 h. Although all three compounds induced EROD activity in varying amounts in wild-type cells, B[*a*]P and quercetin failed to induce EROD activity in AhR-deficient cells, and a high concentration (10 nM) of TCDD induced only approx. 25% of the activity in deficient cells compared with wild-type cells (Figure 5C).

Effect of quercetin on AhR activation

The effect of quercetin on the translocation of the AhR to the nucleus and binding to the XRE of *CYP1A1* was measured by EMSA. Cells were treated with the indicated concentrations of quercetin for 3 h and their nuclear extracts were subjected to EMSA. Extracts from TCDD-treated cells were run as a positive control. Quercetin caused a concentration-dependent increase in the DNA-binding capacity of nuclear AhR, as shown by the

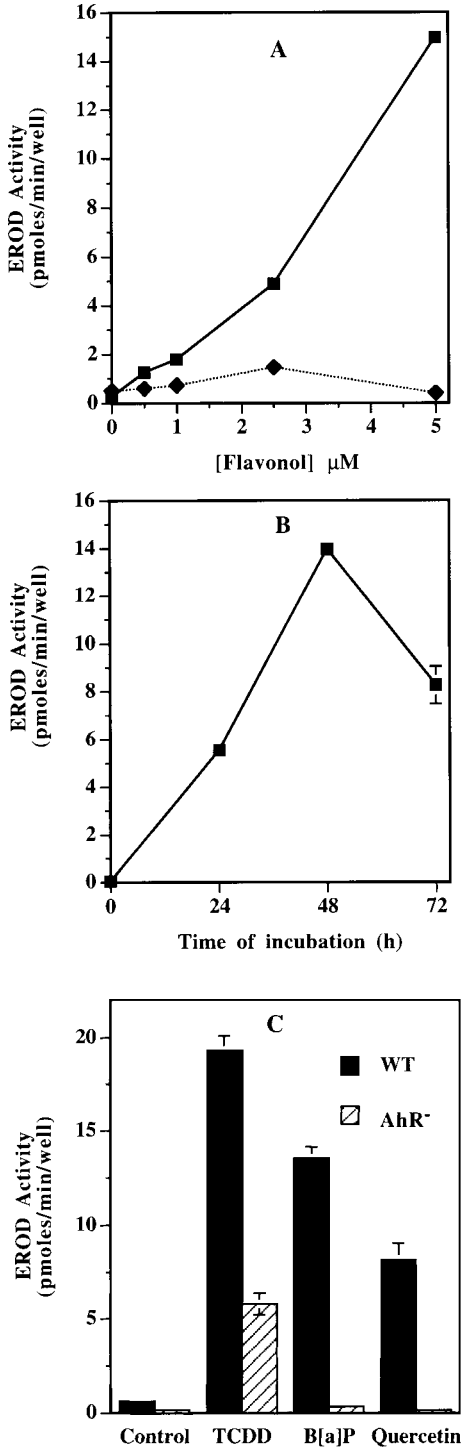

Figure 5 Effect of quercetin or kaempferol on CYP1A1 activity

The activity of CYP1A1 in intact MCF-7 cells was determined by EROD assay. (**A**) Cells were treated with the indicated concentrations of quercetin (■) or kaempferol (◆) for 48 h. (**B**) Cells were treated with 5 μM quercetin for the times indicated. (**C**) Wild-type (WT) and AhR-deficient (AhR$^-$) MCF-7 cells were incubated with DMSO (control), 10 nM TCDD, 1 μM B[*a*]P or 5 μM quercetin for 24 h. Each point or bar is the mean $\pm$ S.E.M. for four determinations. EROD activity in wild-type cells (**A**, **B**) treated with quercetin was significantly different from that in controls at all concentrations and time points tested ($P < 0.05$). There was no significant difference in EROD activity in AhR$^-$ cells treated with B[*a*]P or quercetin compared with that in controls.

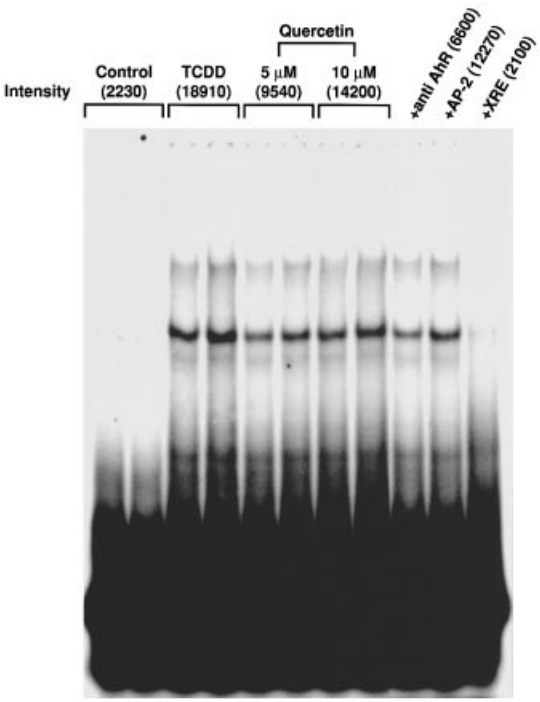

Figure 6 Effect of quercetin on DNA-binding activity of nuclear AhR

Cells were treated with DMSO (control), 10 nM TCDD or the indicated concentrations of quercetin for 3 h. Nuclear extracts were isolated, incubated with labelled XRE sequence and subjected to EMSA. Competition was performed with nuclear extract treated with 10 μM quercetin pretreated with an excess of unlabelled XRE, an oligonucleotide containing the AP-2 sequence, or a polyclonal anti-AhR antibody. The bands were detected and the band intensities quantified by phosphorimaging. The average intensity of each band signal is shown at the top in arbitrary units.

band intensity (arbitrary units) shown at the top of the gel (Figure 6). The specificity of this band shift was examined by pretreating nuclear extract from cells treated with 10 μM quercetin with unlabelled XRE probe, or with a non-specific probe containing the binding site of the transcription factor AP-2. The band shift was abolished in the presence of excess unlabelled XRE but was diminished only slightly in the presence of AP-2 probe. Nuclear extract from quercetin-treated cells was also incubated with a polyclonal antibody against the AhR, which decreased the band intensity by more than 50%. Attempts to super-shift the band with this antibody were unsuccessful.

Effect of quercetin and kaempferol on the binding of ligand to the AhR

The ability of quercetin and kaempferol to compete with the prototypical AhR ligand TCDD for binding to the AhR was measured. Cytosol isolated from MCF-7 cells was incubated with [^{3}H]TCDD in the presence of a 1000-fold excess of unlabelled TCDD (positive control) or a 5000-fold excess of quercetin or kaempferol for 3 h. As shown in Figure 7, unlabelled TCDD inhibited [^{3}H]TCDD binding. Quercetin, and to a smaller extent kaempferol, also inhibited [^{3}H]TCDD binding (see Figure 9). These results were confirmed by using hydroxyapatite chromatography to separate specific from non-specific [^{3}H]TCDD binding (results not shown).

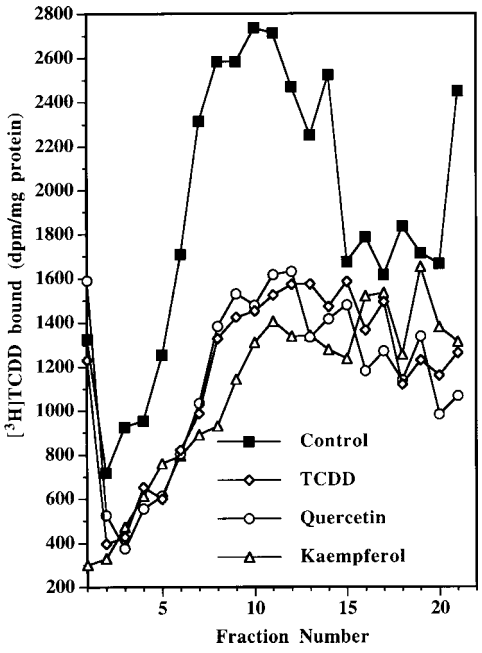

Figure 7 Effect of quercetin or kaempferol on the binding of [^{3}H]TCDD to the AhR

Cytosol isolated from MCF-7 cells was incubated with 10 nM [^{3}H]TCDD in the presence of DMSO (control), a 1000-fold excess of unlabelled TCDD or a 5000-fold excess of quercetin or kaempferol. Ligand-binding activity by the receptor was analysed by sedimentation through 5–30% (w/v) sucrose density gradients; bound [^{3}H]TCDD was measured by liquid-scintillation counting. The figure shows a representative experiment of three.

Effect of kaempferol on the TCDD-induced expression of *CYP1A1*

Although kaempferol did not induce the expression of *CYP1A1*, the results of the ligand binding assay (Figure 7) indicate that it might inhibit the binding of TCDD to the AhR. We therefore tested whether kaempferol could affect the expression of *CYP1A1* induced by TCDD. Treatment of cells with 1 nM TCDD for 6 h caused a 24-fold increase in CYP1A1 transcript compared with that in DMSO-treated cells (Figure 8, top and middle panels). Treatment with TCDD and kaempferol together resulted in an inhibition of TCDD-induced CYP1A1 mRNA in a concentration-dependent manner (Figure 8, top and middle panels). We also examined the effect of kaempferol on *CYP1A1*-promoter-driven CAT transcription. Cells were transfected with the PAH-responsive CAT vector and treated for 6 h with TCDD and kaempferol together. Kaempferol inhibited the TCDD-induced increase in CAT transcription in a concentration-dependent manner (Figure 8, lower panel). The increase in band shift of the XRE caused by TCDD was completely abolished in the presence of kaempferol (Figure 9).

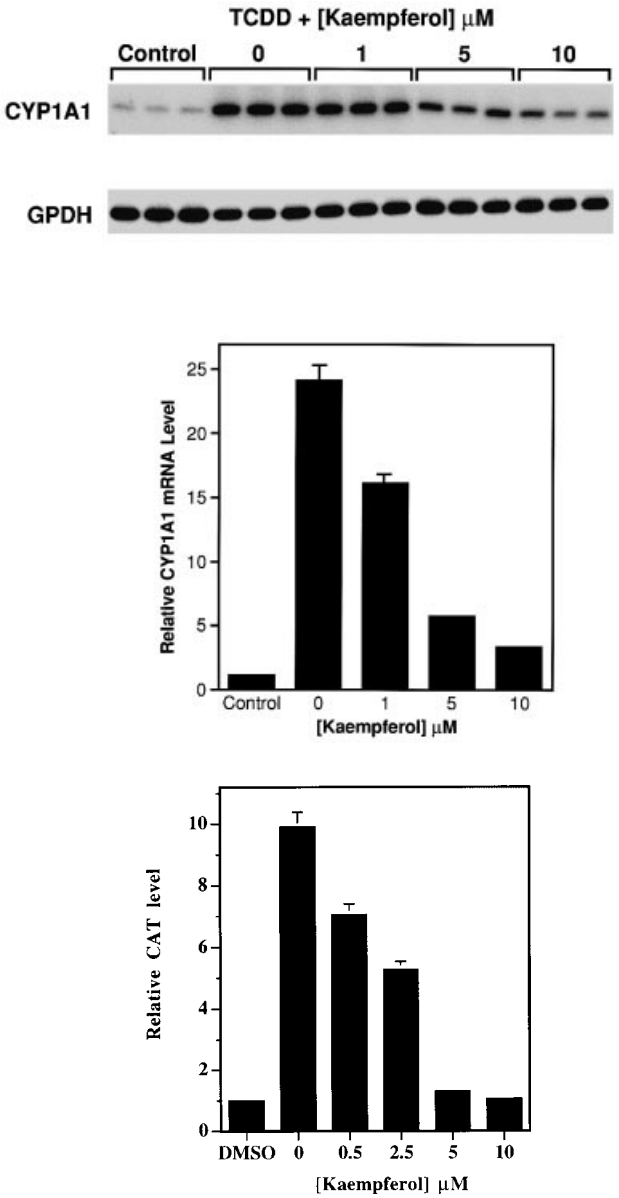

Figure 8 Effect of kaempferol on TCDD-induced CYP1A1 mRNA and transcription

MCF-7 cells were treated with DMSO (control) or 1 nM TCDD in the presence of the indicated concentrations of kaempferol for 6 h. Top and middle panels: CYP1A1 and GPDH mRNA were measured by RT–PCR. Middle panel: the amount of CYP1A1 mRNA was normalized to GPDH mRNA levels. The level of CYP1A1 mRNA was significantly decreased in all samples treated with kaempferol compared to that in cells treated with TCDD alone ($P < 0.05$). Bottom panel: MCF-7 cells were transiently transfected and treated as described above. CAT transcription was normalized to β-Gal transcription. CAT transcription was significantly decreased in all samples treated with kaempferol compared with that in samples treated with TCDD alone ($P < 0.05$).

DISCUSSION

Known ligands of the AhR are mainly man-made; natural ligands of the AhR have remained elusive. Two plant-derived dietary compounds, indolo[3,2]carbazole and curcumin, have been shown to be AhR ligands [10–13] and it is therefore likely that the AhR and the pathway that it mediates evolved in response to dietary xenobiotics. If this is so, one would expect at least some of the thousands of chemicals naturally present in the diet to be AhR ligands too. In the present study we have examined the effects of the dietary compounds quercetin and kaempferol on AhR function. These members of the flavonol class of flavonoids are far more widely distributed in the plant kingdom than the compounds mentioned above and are therefore among the most abundant phytochemicals in human diets. Although it has been established that synthetic derivatives of flavone, the parent structure of all flavonoids, might interact with the AhR [9,27,28], the effect of naturally occurring flavonoids

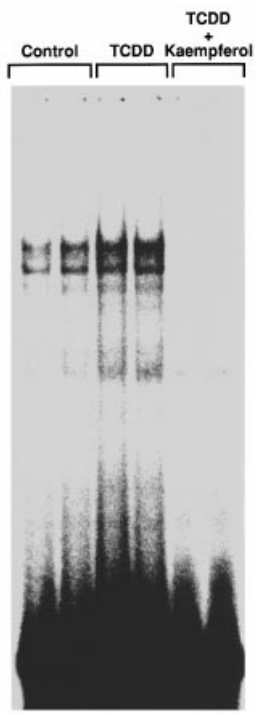

Figure 9 Effect of kaempferol on TCDD-induced DNA-binding activity of nuclear AhR

Cells were treated with DMSO (control), 10 nM TCDD or TCDD and 10 μM kaempferol for 3 h Nuclear extracts were isolated, incubated with labelled XRE sequence and subjected to EMSA. Bands were detected by phosphorimaging.

on the AhR is largely unexplored. Unfortunately, despite extensive interest in the effects of flavonoids on human health, little is known about the physiologically relevant concentrations of individual flavonoids attainable in human plasma and tissue, but recent experiments have confirmed the absorption of quercetin and kaempferol in humans [19]. Moreover, the concentrations used in this study correspond to plasma levels found in rats fed with a flavonoid-enriched diet [40].

We began by examining the effect of quercetin and kaempferol on the expression of *CYP1A1*. Quercetin induced a concentration-dependent increase in the amount of CYP1A1 mRNA present in MCF-7 cells (Figure 2A). The increase in CYP1A1 mRNA caused by quercetin was rapid but transient, reaching a maximum after 12 h and declining by 24 h (Figure 3). Pretreatment of the cells with the RNA polymerase inhibitor actinomycin D completely blocked the increase in mRNA, indicating that RNA synthesis *de novo* resulting from the transcriptional activation of *CYP1A1* is required for quercetin to exert its effect (Figure 4, upper panel). We examined the effect of quercetin or kaempferol on the transcriptional activation of a CAT reporter vector controlled by the full-length *CYP1A1* promoter. In transient transfection experiments, this vector responded to the prototypical AhR ligand TCDD as well as to other ligands (B[*a*]P, DMBA and 3-methylcholanthrene; results not shown) with an increase in CAT transcription. Quercetin caused a concentration-dependent increase in CAT transcription (Figure 4, lower panel), although it was much less potent an inducer than TCDD.

CYP1A1 encodes the enzyme CYP1A1, the primary carcinogen-activating enzyme in MCF-7 cells under conditions of AhR activation [41]. The enzymic activity of CYP1A1 was measured by EROD assay, the best measurement of its bioactivation capacity. MCF-7 cells also express *CYP1B1* in response to TCDD, but it has been reported that the CYP1B1 enzyme possesses little [42] or no EROD activity [29]. Treatment of MCF-7 cells with quercetin resulted in a concentration- and time-dependent increase in EROD activity in the intact cells (Figures 5A and 5B respectively). EROD activity reached a maximum 48 h after the addition of quercetin; it began to decline after 72 h. As one would expect, the increase in EROD activity follows the increase in CYP1A1 mRNA. Enzyme activity persists much longer than the increase in mRNA, probably reflecting the stability of the enzyme compared with the mRNA. The increases in CYP1A1 mRNA, CYP1A1 enzyme activity and *CYP1A1* promoter-driven transcription indicate that quercetin induces the expression of *CYP1A1*. As shown in Figures 2(B), 4 (lower panel) and 5(A), kaempferol, despite its structural similarity to quercetin, did not affect *CYP1A1* expression.

Because *CYP1A1* transcription is regulated by the AhR, we investigated whether quercetin is a ligand of the receptor. We performed three types of experiment to determine whether quercetin is an AhR ligand. First, we examined the induction of EROD activity in AhR-deficient MCF-7 cells that we have developed and characterized (H. P. Ciolino and G. C. Yeh, unpublished work). These cells express only approx. 20% of the AhR compared with wild-type cells (results not shown). EROD activity in these cells increases only slightly in response to TCDD, the most potent ligand of the AhR, and not at all to other ligands such as B[*a*]P. As shown in Figure 5(C), quercetin failed to induce EROD activity in the AhR-deficient cells, indicating that the AhR is required for quercetin to exert its effect on *CYP1A1* expression. Secondly, we examined the ability of quercetin to transform the cytosolic receptor to its nuclear, DNA-binding, form. As shown in the EMSA in Figure 6, treatment of cells with quercetin resulted in a concentration-dependent increase in the amount of nuclear AhR DNA-binding capacity for an oligonucleotide containing the XRE of the *CYP1A1* promoter. That this band shift was specific for activated AhR is demonstrated by the specific competition of XRE binding of nuclear extracts of quercetin-treated cells with unlabelled XRE probe or anti-AhR antibody. The band also shifted to the same position as that caused by TCDD. Thirdly, we tested the ability of quercetin to compete with TCDD for AhR binding. At a 5000-fold excess, quercetin partly inhibited the binding of [^{3}H]TCDD to the cytosolic AhR (Figure 7). Although the affinity of quercetin for the receptor is therefore low compared with that of TCDD, this result indicates that quercetin interacts directly with the AhR. Taken together, these results demonstrate that quercetin is a ligand of the AhR.

Interestingly, kaempferol also inhibited the binding of TCDD (Figure 7), indicating that it does interact with the AhR. We therefore hypothesized that because kaempferol interacts with the ligand-binding site of the AhR without itself up-regulating transcription, it would antagonize *CYP1A1* transcription induced by TCDD. Treatment of cells with kaempferol and TCDD together resulted in a concentration-dependent decrease in the TCDD-induced increase in both CYP1A1 mRNA (Figure 8, top and middle panels) and CAT transcription (Figure 8, bottom panel). Furthermore, kaempferol completely abolishes the activation of the XRE-binding capacity of the AhR induced by TCDD, as shown in Figure 9. This indicates that kaempferol does in fact interact with the receptor, and therefore is a ligand of the receptor because it functions as an AhR antagonist. It has been shown previously that compounds with weak to moderate binding affinity for the AhR might exhibit partial antagonistic

activity. For example, α-naphthoflavone, a synthetic flavone, inhibits TCDD-induced *CYP1A1* transcription at less than 10 μM, but acts as an agonist at higher concentrations [43]. Similar results were recently obtained with another synthetic flavone, PD98050 [27]. We detected no agonist activity of kaempferol, although concentrations greater than 10 μM were not tested. The mechanism by which kaempferol antagonizes the AhR without any agonistic activity awaits further experimentation.

It is interesting that two compounds so similar in structure as quercetin and kaempferol have such different effects on AhR function. Both compounds fit the profile of AhR ligands: they are polycyclic, planar and hydrophobic. On the basis of computer modelling of known AhR agonists such as TCDD, Kleman et al. [44] determined the molecular structure that allows these compounds to interact tightly with the AhR. AhR ligands were determined to fit a hypothetical rectangle of 6.8 Å × 13.7 Å. This result was confirmed by Lee et al. [45]. Despite the structural similarity of quercetin and kaempferol, it might be that the absence of the extra hydroxy group on the B-ring (Figure 1) prevents kaempferol from achieving an optimal fit into this site, preventing transcriptional activation, while blocking other ligands such as TCDD from binding. Because the induction of *CYP1A1* via the AhR is associated with mutagenic activity of many carcinogens, kaempferol might therefore prove to be an effective chemopreventive agent. In contrast, whether *CYP1A1* induction is harmful or helpful to the organism is a complex question that has not been resolved. One could argue that the induction of *CYP1A1* by quercetin might increase the rate of detoxification of PAHs, because PAH metabolites are better substrates for Phase II enzymes. Therefore quercetin might be chemopreventive, especially if it causes a co-ordinate induction of both *CYP1A1* and the Phase II enzymes, several of which are known to be regulated by the AhR [46].

In this study we have demonstrated that quercetin and kaempferol are natural, dietary ligands of the AhR. In general, most inducers of *CYP1A1* are metabolized by the enzyme that it encodes, TCDD being one prominent exception. If this pathway has evolved in response to such phytochemicals, one could hypothesize that they would be catabolized by CYP1A1. Because quercetin, like B[*a*]P or DMBA (results not shown), induces a transient increase in CYP1A1 mRNA and EROD activity in MCF-7 cells, it might be undergoing catabolic breakdown. Whether this activity is due directly to the activity of CYP1A1 is currently under investigation.

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