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Quercetin, Resveratrol, and Curcumin Are Indirect Activators of the Aryl Hydrocarbon Receptor (AHR)

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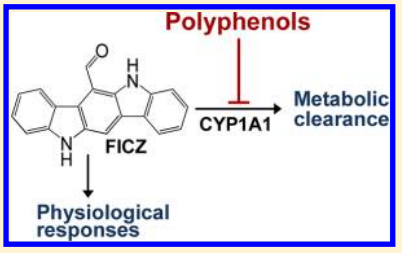
Supporting Information

ABSTRACT: Several polyphenols have been shown to activate the aryl hydrocarbon receptor (AHR) in spite of the fact that they bind to the receptor with low affinity. The aim of this study was to investigate whether quercetin (QUE), resveratrol (RES), and curcumin (CUR) interfere with the metabolic degradation of the suggested endogenous AHR ligand 6-formylindolo[3,2-*b*]carbazole (FICZ) and thereby indirectly activate the AHR. Using recombinant human enzyme, we confirmed earlier reported inhibitory effects of the polyphenols on cytochrome P4501A1 (CYP1A1) activity, and inhibition of metabolic clearance of FICZ was documented in FICZ-treated immortalized human keratinocytes (HaCaT). CYP1A1 activity was induced in HaCaT cells by all three compounds, and when they were added together with FICZ, a prolonged activation was observed after a dose-dependent inhibition period. The same pattern of responses was seen at the transcriptional level as determined with a CYP1A1 reporter assay in human liver hepatoma (HepG2) cells. To test the ability of the polyphenols to activate the AHR in the absence of FICZ, the cells were treated in medium, which in contrast to commercial batches of medium did not contain background levels of FICZ. Importantly, AHR activation was only observed in the commercial medium. Taken together, these findings suggest that QUE, RES, and CUR induce CYP1A1 in an indirect manner by inhibiting the metabolic turnover of FICZ. Humans are exposed to these compounds through the diet and nutritional supplements, and we propose that altered systemic levels of FICZ caused by such compounds may have physiological consequences.



INTRODUCTION

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor belonging to the basic helix−loop−helix/Per-Arnt-Sim (bHLH/PAS) protein family.[1] In recent years, the focus of AHR research has shifted from studies of its roles in mediating toxicity of xenobiotics to its roles in normal homeostasis, immune functions, and the development that occurs in utero.[2−4] AHR activation by endogenous ligands seems to be essential for the proper development and function of mammals since its absence in AHR-null mice leads to severe phenotypic abnormalities.[5] The highly toxic compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other xenobiotic ligands with high affinity for binding to the AHR have been used to assess the biological functions of the AHR to understand intrinsic AHR physiology. In this way, more than 100 AHR downstream genes with various functions as well as a multitude of AHR-mediated cross-talks between the AHR and other signaling pathways have been described.[3,6] The most highly up-regulated genes are those that encode xenobiotic metabolizing enzymes belonging to the first family of cytochrome P450 (CYP) enzymes including CYP1A1, CYP1A2, and CYP1B1. The CYP1 enzymes have been examined extensively for their capacities to activate compounds with carcinogenic properties, but their endogenous homeostatic functions are far from fully understood. The existence of a possible endogenous CYP1A1 substrate with roles in normal cellular processes was suggested early,[7] but its identity was never established.

In studies to find and characterize endogenous AHR ligands, several candidates have been suggested,[8] but most of them exhibit low affinity for binding to the receptor. However, one endogenous substance, 6-formylindolo[3,2-*b*]carbazole (FICZ), a photoproduct that is formed from the amino acid tryptophan (Trp), is receiving increasing attention. FICZ has been found to distribute systemically in mice exposed topically and to cause pronounced gene expression in tissues distant from the site of application.[9] It influences the circadian timing system[10] and the organization of the genome.[11] In recent in vivo studies, FICZ has also been shown to activate T-cells and influence responses to myelin peptides, dextran sodium sulfate, or ovalbumin in experimental mouse models of multiple sclerosis, inflammatory bowel disease, and asthma, respectively, when 30−50 μg per kilo body weight were given orally or intraperitoneally.[12−15]

FICZ has been detected in cell culture media and aqueous solutions of Trp exposed to visible and UV light,[16−18] and

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FICZ-derived sulfate conjugates have been confirmed in human urine.[18] It exhibits the highest affinity of all compounds tested for AHR binding, including the model anthropogenic AHR ligand TCDD,[8,18,19] and it is efficiently metabolized by CYP1 enzymes and conjugating enzymes into inactive forms that no longer bind to the AHR.[18,20] The high substrate specificity and catalytic efficiency of CYP1A1 toward FICZ make this substance a likely endogenous CYP1A1 substrate as well as an endogenous AHR activator.

Several classes of natural small molecular weight compounds have been shown to both antagonize TCDD-activated AHR signaling and to activate AHR signaling by themselves in spite of the fact that they bind to the receptor with low affinity.[21] Many polyphenolic compounds demonstrate such properties.[22,23] Quercetin (QUE), resveratrol (RES), and curcumin (CUR) have been described to function as AHR antagonists by inhibiting TCDD-dependent activation of the receptor[24−26] and to induce CYP1A1 transcription when added to cells in vitro.[25,27,28] In vivo, these compounds are rapidly metabolized, mainly into conjugates that are eliminated in urine, but when added as glucosides or as polymeric implants, higher plasma levels are obtained, and such forms of QUE and CUR stimulate CYP1A1 expression and activity.[29,30]

The polyphenols QUE, RES, and CUR have also been shown to inhibit CYP1A1 enzyme activity.[25,31−34] Therefore, our hypothesis is that they may obstruct the tightly regulated turnover of FICZ. In this study, we attempted to determine if these compounds can inhibit the metabolic clearance of FICZ and if this can explain how QUE, RES, and CUR, having no or low affinity for binding to the AHR, can activate AHR-mediated responses.

MATERIALS AND METHODS

Materials. Compounds were obtained from the following suppliers: FICZ from Syntastic AB (Sweden); β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), QUE, RES, CUR, and 7-ethoxyresorufin (EOR) from Sigma-Aldrich (Germany); lysis buffer from Promega (Madison, WI); DC protein assay kit from Bio Rad; and D-luciferin from BioTherma (Sweden). All cell culture reagents and the media RPMI 1640 (#21875), DMEM (#21068), and DMEM without Trp (custom order based on #21068) and Almar Blue assay kit were purchased from Invitrogen (Carlsbad, CA).

Inhibition of Recombinant Human CYP1A1. The bicistronic plasmid encoding human CYP1A1 and human NADPH-450 reductase (NOR) was kindly provided by F. P. Guengerich (Vanderbilt University School of Medicine, Nashville). The CYP1A1 and NOR vector were recombinantly expressed in *Escherichia coli*, and membrane fractions containing the enzymes were prepared according to a procedure established by Guengerich and colleagues.[35] The CYP1A1 content was determined by reduced CO-difference spectroscopy.[36] The inhibition of EROD activity was measured as follows: A mixture of 0.5 μM EOR, 40 nM CYP1A1, and 0−60 μM inhibitor in TE-enzymatic buffer (0.1 M Tris-HCl, pH 7.4, with 1 mM EDTA) was incubated at 37 °C for 10 min. The reaction was initiated by the addition of 0.5 mM NADPH, and the increase in fluorescence was recorded at excitation/emission wavelengths 535/590 nm over time. The activity was calculated as a percent of an uninhibited control.

Cell Culture Conditions and Treatments. The immortalized human keratinocyte cell line HaCaT was kindly provided by N. E. Fusenig (DKFZ, Heidelberg, Germany). HaCaT cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 IU/mL penicillin under an atmosphere containing 5% CO_2 at 37 °C.

The human hepatoma HepG2-derived cell line HepG2-XRE-Luc,[37] containing a pTX.DIR-luciferase reporter under the control of two XRE sequences of the rat CYP1A1 gene, was kindly provided by K. Gradin, Karolinska Institutet. The cells were cultured in RPMI 1640 or DMEM medium supplemented with 10% FBS, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 800 μg/mL geneticin under an atmosphere containing 5% CO_2 at 37 °C.

To investigate the influence of trace levels of FICZ in cell culture media, the cells were treated in either commercial DMEM 21068 or DMEM 21068-like medium free from Trp, which had been supplemented with purified Trp prior to each use. Trp was purified and recrystallized with absolute ethanol as described previously.[9] In short, the crystals were collected and washed in absolute ethanol and dried under vacuum, and the quality of the recrystallized Trp was confirmed by HPLC analysis showing that FICZ in the recrystallized Trp was below the detection level (<5 fmol, corresponding to <3 fmol/16 mg Trp). To avoid light-dependent formation of FICZ during experiments, the cell culture media were handled at all times in the dark and covered with aluminum foil, and all procedures were carried out protected from light.

All compounds were dissolved in DMSO and used at concentrations that did not cause cell toxicity (Figure S1 in the Supporting Information). Treatments were started by replacing the growth medium with fresh medium containing different concentrations of QUE, RES, or CUR alone or in combination with FICZ. The final concentration of DMSO was 0.1% (v/v).

HPLC Analyses of Cellular FICZ Content. HaCaT cells were seeded onto 60 mm dishes and cultured and treated as described above. At the time points indicated (0, 0.5, 1.5, 3, and 6 h), samples were taken by removing the medium, rapidly washing with ice-cold PBS, and then harvesting the cells in distilled water. The cells were sonicated on ice and stored at −20 °C until further analysis by means of HPLC. The levels of FICZ in whole cell lysates were analyzed using an in-line solid-phase extraction column coupled to a reverse-phase C18 column, as previously described.[18] In brief, the cell lysates were injected onto the extraction column, washed with water, and thereafter separated on the analytical C18 column (Alltech Alltima; 250 mm × 4.6 mm). The separation was performed using a mobile phase consisting of acetonitrile and water, both supplemented with 1.5 mM formic acid. FICZ was detected using excitation and emission wavelengths of 390 and 525 nm, respectively. Finally, the amount of FICZ was normalized to cellular protein content determined by the DC protein assay kit.

CYP1A1 Enzyme Activity. CYP1A1-dependent ethoxyresorufin-*O*-deethylase (EROD) activity was measured in HaCaT cells essentially as described by Hestermann et al.[38] Briefly, cells were seeded at high density in 96-well plates and grown to confluence. At the indicated time points (1.5, 3, 6, 24, and 48 h) after the start of treatment, the treatments were terminated by removing the medium and rinsing the cells with PBS. The EROD reaction was initiated by addition of 2 μM EOR in sodium phosphate buffer (50 mM pH 8.0). After 20 min of incubation at 37 °C, the formation of resorufin was measured using a Genios Pro plate reader (Tecan) with the excitation/emission wavelengths 535/590 nm, and data were normalized to cellular protein content determined as above.

CYP1A1 Reporter Gene Activation. HepG2-XRE-Luc cells were grown in 24-well plates to 80% confluence. At the indicated time points (0.5, 5, 7.5, 24, and 48 h) after the start of treatment, the medium was removed, and the cells were briefly washed with ice-cold PBS and harvested in 50 μL of cell culture lysis buffer. The luciferase activity was measured in 30 μL cell extracts after the addition of 40 μL of ATP and 40 μL of D-luciferin using a Genios Pro plate reader (Tecan). The luciferase activity was normalized to the cellular protein content determined as above.

Statistical Analysis. All measurements were performed with replicates (N = 3−4, unless otherwise indicated). Dose− and time−course studies of EROD activity, luciferase reporter activity, and clearance of FICZ were performed at least in two independent experiments. All results are expressed as means ± SDs and show results from one representative experiment. For comparison between the experimental groups, either two-way analysis of variance or two-

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tailed t tests with Bonferroni correction were used. Probability p values <0.05 were considered statistically significant.

RESULTS

Polyphenols Inhibit Human Recombinant CYP1A1. The effects of polyphenols on CYP1A1 activity were determined with recombinantly expressed human CYP1A1 using the standard substrate EOR (Figure 1). QUE was found to be a strong inhibitor with an IC_{50} value of 1.2 μM, while RES and CUR were slightly less efficient with IC_{50} values of 11.8 and 7.3 μM, respectively.

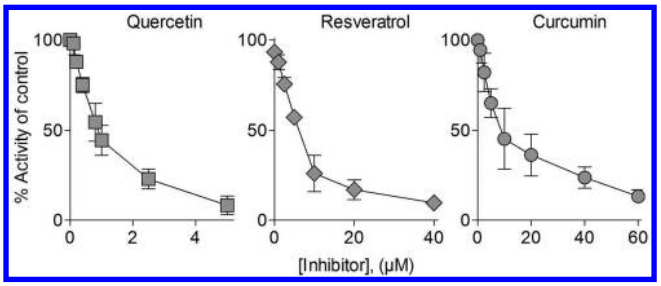

Figure 1. Inhibition of recombinantly expressed human CYP1A1 activity by QUE, RES, or CUR. The reaction was initiated by the addition of 0.5 mM NADPH, and EROD activity (pmol resorufin/mg protein/min) was measured. The activity is presented as percent of uninhibited control.

Effects of QUE, RES, and CUR on Metabolic Clearance of FICZ. Intracellular levels of FICZ in HaCaT cells treated with FICZ alone or in combination with different concentrations of QUE, RES, and CUR were determined by HPLC. All three cotreatments resulted in a reduced rate of clearance of FICZ in a dose-dependent manner (Figure 2).

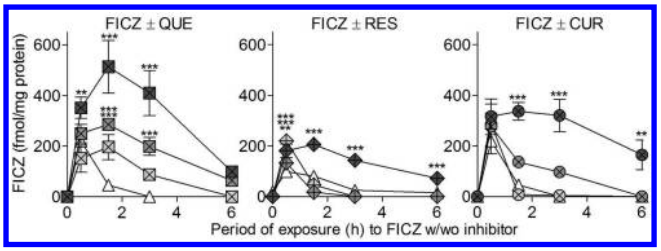

Figure 2. Metabolic clearance of FICZ in HaCaT cells treated with QUE, RES, and CUR. Cells were treated with 150 pM FICZ (unfilled triangle) alone or in combination with 1, 10, or 50 μM QUE (crossed light, medium, and dark gray squares, respectively); 1, 10, or 20 μM RES (crossed light, medium, and dark gray diamonds, respectively); or 1, 10, or 20 μM CUR (crossed light, medium, and dark gray circles, respectively). At the indicated time points, cell lysates were collected, and intracellular FICZ levels were analyzed by HPLC. Data are expressed as means ± SDs; $n \geq 2$. Asterisks indicate significant differences (**P < 0.01 and ***P < 0.001) between coexposed cells vs cells exposed to FICZ alone.

Impact of QUE, RES, and CUR on CYP1A1 Enzyme Activity and AHR Activation in Cultured Cells. Treatment of HaCaT cells with the polyphenols led to a significant dose-dependent induction of CYP1A1 enzyme activity at 24−48 h (Figure 3 A), whereas cells treated with 150 pM FICZ showed an early and transient induction of enzyme activity with maximal induction at 3−5 h (Figure 3B). The combined treatment with QUE, RES, or CUR and FICZ led to a dose-dependent transient attenuation of FICZ-dependent induction of CYP1A1 enzyme activity at early time points (Figure S2 in the Supporting Information) followed by a prolonged induction at later time points (Figure 3B).

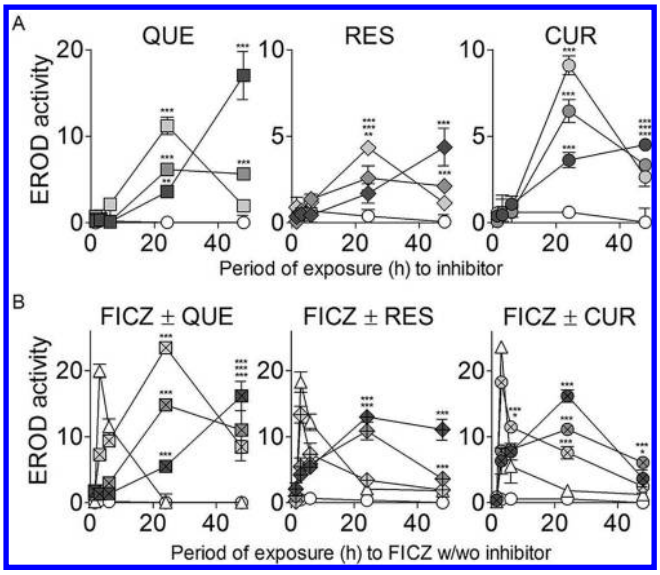

Figure 3. CYP1A1 enzyme activity in HaCaT cells treated with QUE, RES, or CUR alone (A) or in combination with FICZ (B). Symbols: vehicle (DMSO, unfilled circles); 150 pM FICZ (unfilled triangles); 1, 10, or 50 μM QUE (light, medium, and dark gray squares, respectively); 1, 10, or 20 μM RES (light, medium, and dark gray diamonds, respectively); 1, 10, or 20 μM CUR (light, medium, and dark gray circles, respectively). Cotreatments with FICZ and QUE, RES, or CUR are indicated by crossed squares, diamonds, or circles, respectively. Treatments were terminated at the indicated time points, and the EROD activity (pmol resorufin/mg protein) was measured. Data are expressed as means ± SDs. Asterisks denote significantly higher induction (*P < 0.05, **P < 0.01, and ***P < 0.001) as compared to DMSO- or FICZ-treated cells. Inhibitions at early time points are shown in greater resolution in Figure S2 in the Supporting Information.

To investigate whether the observed increased CYP1A1 enzyme activity was caused by increased transcriptional regulation, a reporter assay with HepG2-XRE-Luc cells was employed. All three polyphenols activated transcription of the reporter gene by themselves (Figure 4A) and prolonged the FICZ-dependent induction (Figure 4 B). Similar to the results with the HaCaT cells, the pattern of responses was highly influenced by the strength and duration of the inhibition caused by the polyphenols (Figure S3 in the Supporting Information). At the lowest concentrations applied, QUE, RES, and CUR increased the transcriptional activity when added together with FICZ (Figure 4B).

As compared to the strong exogenous agonist TCDD, the concentrations needed for comparable activation of the AHR by QUE, RES, or CUR was at least 3 orders of magnitude higher (Figure S4D in the Supporting Information).

Medium-Dependent Activation of AHR by QUE, RES, and CUR. Our previous studies have shown that low levels of Trp-derived FICZ are present in commercial DMEM medium and that this background level of FICZ is sufficient to activate an AHR response if the metabolic clearance of FICZ is compromised.[9] We now set out to determine whether QUE, RES, and CUR were able to activate the AHR in such an indirect manner. Cells were treated with the respective

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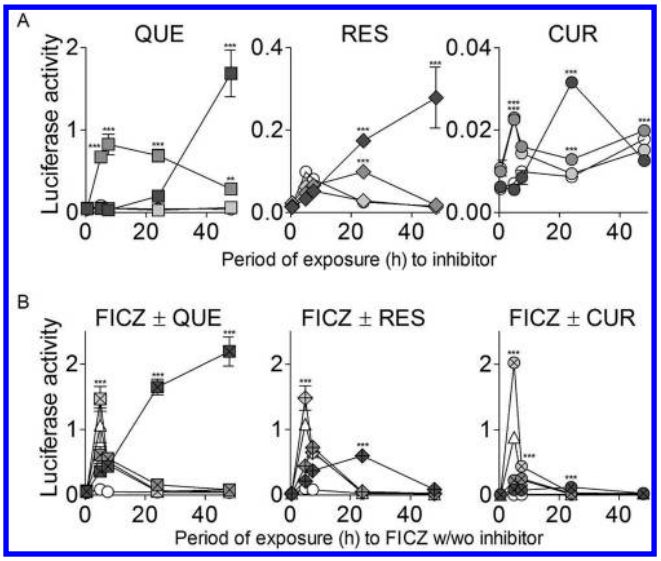

Figure 4. AHR-driven XRE-luciferase reporter activity measured in HepG2-XRE-Luc cells. Cells were treated with QUE, RES, or CUR in RPMI medium containing 10% FBS, alone (A) or in combination with FICZ (B). Treatments: vehicle (DMSO, unfilled circles); 150 pM FICZ (unfilled triangles); 1, 10, or 50 μM QUE (light, medium, and dark gray squares, respectively); 1, 10, or 20 μM RES (light, medium, and dark gray diamonds, respectively); 1, 10, or 20 μM CUR (light, medium, and dark gray circles, respectively). Cotreatments with FICZ and QUE, RES, or CUR are indicated by crossed squares, diamonds, or circles, respectively. Luciferase activities (unit luciferin/mg protein $\times 10^{-3}$) are expressed as means ± SDs. Asterisks denote significantly higher induction (*P < 0.05, and ***P < 0.001) as compared to DMSO- or FICZ-treated cells. Inhibition at early time points is shown in greater resolution in Figure S3 in the Supporting Information.

compounds in a commercial DMEM medium and a DMEM prepared with Trp that had been recrystallized and thereby uncontaminated with FICZ. The EROD assay data, derived using HaCaT cells, clearly show that QUE, RES, and CUR were able to induce CYP1A1 enzyme activity but only in the commercial medium (Figure 5). To confirm the EROD data, HepG2-XRE-Luc cells were treated with different concentrations of polyphenols in both media for 24 h, and the

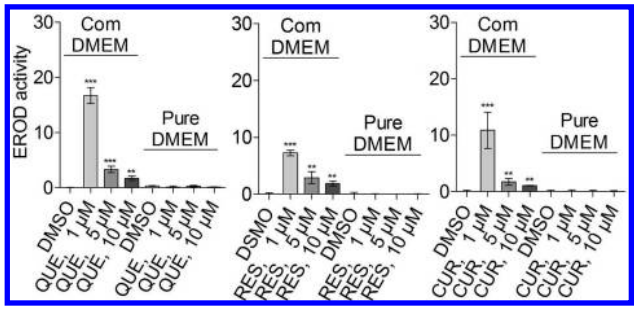

Figure 5. CYP1A1 enzyme activity in HaCaT cells treated with QUE, RES, or CUR in commercial (Com) DMEM medium or FICZ-free (Pure) DMEM medium. Cells were treated for 24 h with vehicle (DMSO) or 1, 5, or 10 μM QUE; 1, 5, or 10 μM RES; or 1, 5, or 10 μM CUR in commercial medium or Trp-free medium supplemented with purified Trp. Treatments were terminated at the indicated time points, and the EROD activity (pmol resorufin/mg protein) was measured. Data are expressed as means ± SDs. Asterisks denote significant differences (** P < 0.01, and *** P < 0.001) between the same treatments in commercial and pure medium.

luciferase activity was determined. In agreement with the data obtained with the EROD assay, all three polyphenols were inactive in the FICZ-free medium (Figure 6).

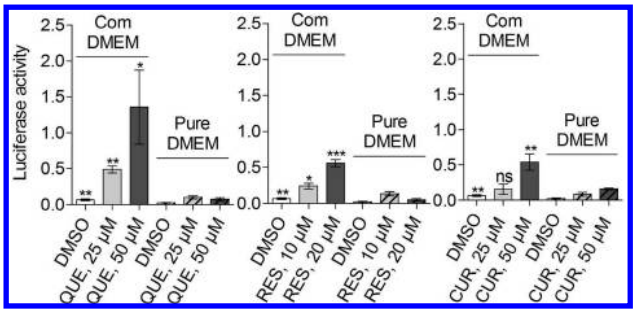

Figure 6. AHR-driven XRE-luciferase reporter activity in HepG2-XRE-Luc cells treated with QUE, RES, or CUR in commercial (Com) DMEM medium and FICZ-free (Pure) DMEM medium. Cells were treated for 24 h with vehicle (DMSO) or 1, 10, or 50 μM QUE; 1, 10, or 20 μM RES; or 1, 10, or 20 μM CUR in commercial medium and free Trp medium supplemented with purified Trp. Luciferase activities (unit luciferin/mg protein $\times 10^{-3}$) are expressed as means ± SDs. Asterisks denote significant differences (*P < 0.05, **P < 0.01, and ***P < 0.001) between the same treatments in commercial and pure medium.

Effects of FBS on CYP1A1 Enzyme Activity and AHR Activation. The presence of serum was found to lower the responses in the EROD assay in preliminary experiments. We therefore compared the responses to FICZ, QUE, RES, or CUR in incubations with or without 10% FBS. Induction of EROD activity by FICZ or QUE was reduced by 50% in HaCaT cells treated in DMEM supplemented with 10% FBS as compared to DMEM without serum (Figure S3A,B in the Supporting Information). In the same manner, the luciferase activity induced by FICZ, QUE, RES, or CUR was significantly attenuated when the treatments were performed in medium that contained serum (Figure S4C,D in the Supporting Information). All experiments, except the experiments presented in Figures 4 and S4 in the Supporting Information, were conducted in growth medium that lacked serum.

DISCUSSION

A large number of structurally divergent chemical substances can activate the AHR.[21] One interesting group of AHR activators, the polyphenolic compounds, has gained a lot of attention. Most people are exposed to them through dietary intake of fruits and vegetables, and they are also common in herbal additives because of their wide range of proposed pharmacological properties. These include anticarcinogenic, cardioprotective, and antioxidant properties,[39] although antioxidant effects of polyphenols in vivo have not been well documented.[40] Polyphenols have been suggested as both agonists and antagonists of the AHR.[23] In studies of competitive binding to AHR, QUE at 50 μM was reported to compete with [^{3}H]TCDD for binding to cytosolic proteins from human MCF7 cells;[28] RES competed with 2-azido-3-[^{125}I]iodo-7,8-dibromodibenzo-*p*-dioxin for binding to cytosolic lysates prepared from MCF7 cells[41] or with [^{3}H]TCDD for binding to rabbit liver cytosols,[42] giving EC_{50} values of 5 and 6 μM, respectively, but was inactive at 50 μM in displacing [^{3}H]TCDD from cytosolic lysates derived from HepG2 cells;[43] CUR at 10 μM partly displaced [^{3}H]TCDD from cytosolic proteins prepared from MCF7 cells[25] and [^{3}H]3-methylcho-

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lanthrene from rat liver cytosolic fractions.[44] Our results confirm earlier reports of AHR activating capacity of these polyphenols.[25,27,28] All three activated the AHR already at the lowest concentration tested, which was 1 μM. However, when the experiments were performed in a medium that lacks background levels of the endogenous AHR activator FICZ, all three compounds were inactive. To clarify how the AHR is activated by the polyphenols, some pieces of information should be taken into account that relate to what has sometimes been described as nonligand activation of the AHR. First of all, induction of CYP1A1 without addition of AHR ligands has been reported in several studies following the first report in 1985 by Nebert and colleagues.[45] Second, the cell culture medium itself has been found to induce CYP1A1,[46−48] and third, if the cell culture medium is exposed to normal laboratory light or to sunlight, FICZ is formed, which can contribute to background AHR activation and significantly induce CYP1A1 activity.[16,17] Thus, it is plausible that the reported AHR activation by polyphenols is explained by the presence of the natural agonist FICZ in the medium, since even in batches of medium that have been protected from light, low levels of FICZ (0.1 pM) were detected.[9]

In 2009, we suggested that the capacity of the bioactive polyphenol α-naphthoflavone to inhibit CYP1A1 could explain its observed AHR activation,[18] and in a recent publication, we show that UVB, H_2O_2, and 3′-methoxy-4′-nitroflavone (MNF) also activate the AHR through an indirect mechanism.[9] In accordance with these earlier results, we now show that inhibition of CYP1A1 by QUE, RES, and CUR results in prolonged presence of FICZ in the cells. QUE was the most efficient inhibitor of the expressed human CYP1A1 enzyme with an IC_{50} value of 1.2 μM, and this compound was also the most efficient activator of AHR responses. It has been demonstrated that polyphenols via interaction with transition metal ions have pro-oxidant properties,[40,49] and we propose that they can inhibit CYP1A1 gene expression and enzyme activity via generation of reactive oxygen species and that the mechanism of inhibition is similar to the inhibition shown for other oxidants.[50,51] In addition, we have earlier observed that oxidants strongly sensitize the AHR to ligand-dependent activation,[52] and we suggest that such pro-oxidant effects of the polyphenols contribute to the late responses observed at high concentrations of QUE, RES, and CUR.

The AHR plays an important role in normal development and in many physiological processes including cellular homeostasis, reproduction, immunity, circadian rhythms, skin physiology, and endocrine functions.[4] Because the suggested endogenous AHR ligand FICZ is a very bioactive compound that may influence both AHR-dependent as well as AHR-independent pathways, the physiological concentrations of FICZ need to be tightly regulated. In this study, we have shown that the autoregulatory feedback control of FICZ by the CYP1A1 enzyme can be targeted by some biologically active polyphenols. Humans are exposed to these compounds through the diet and nutritional supplements, and we propose that altered systemic levels of FICZ caused by such compounds may have physiological consequences such as impaired immune regulation.

■ ASSOCIATED CONTENT

Supporting Information

Figures of viability of HaCaT cells exposed to QUE, RES, or CUR alone or in combination with FICZ; CYP1A1 enzyme activity at early time points in HaCaT cells; AHR-driven XRE-luciferase reporter activity at early time points in HepG2-XRE-Luc cells; and CYP1A1 enzyme activity in HaCaT cells and AHR-driven XRE-luciferase reporter activity in HepG2-XRE-Luc cells treated in medium with or without FBS. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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