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Cytochrome P450 1A1 Expression and Activity in Caco-2 Cells: Modulation by Apple Juice Extract and Certain Apple Polyphenols

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Cytochrome P450 (CYP) 1A1 plays a role in drug metabolism of intestinal cells (e.g., by activating certain chemical carcinogens such as polycyclic aromatic hydrocarbons into carcinogenic metabolites). In the human colon carcinoma cell line Caco-2, we investigated the effects of a defined polyphenolic apple juice extract (AJE), the major principle flavonoid/dihydrochalkone constituents quercetin and phloretin, and the corresponding prototype glycosides rutin and phlorizin on CYP1A1 expression and activity. Incubations were carried out with or without the potent aryl hydrocarbon receptor agonist/CYP1A1 inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). AJE and quercetin acted as weak inducers of CYP1A1 mRNA and protein, and AJE, quercetin, and phlorizin led to a slight induction of CYP1A1-catalyzed 7-ethoxyresorufin *O*-deethylase (EROD) activity. However, AJE, quercetin, and phloretin were highly effective in suppressing CYP1A1 induction in co-incubations of the cells with 1 nM TCDD. The antagonistic effects were seen on the levels of mRNA, enzyme protein, and catalytic activity. In contrast, the related glycosides rutin and phlorizin were inactive as inducers or inhibitors. Inhibition of CYP1A1 induction was not related to general cytotoxicity, which could be completely abolished by the addition of ascorbic acid/α-tocopherol. AJE, quercetin, and phloretin also antagonized the TCDD-mediated induction of a reporter gene driven by a regulatory sequence of the human CYP1A1 gene promoter. Our findings suggest that apple juice extract can antagonize TCDD-mediated CYP1A1 induction by interfering with AhR-dependent gene transcription and by inhibiting the catalytic activity of CYP1A1. These effects may result in reduced metabolic activation of certain chemical carcinogens, in particular, under conditions of sustained AhR activation.

KEYWORDS: Apple juice; arylhydrocarbon receptor; cytochrome P450; flavonoids; phloretin; phlorizin; polyphenols; quercetin; rutin

INTRODUCTION

Colorectal cancer is the most important contributor to cancer mortality in many industrialized countries (*1*). Among various causative factors, the role of food contaminants and/or tobacco smoke constituents such as polycyclic aromatic hydrocarbons and heterocyclic aromatic amines is widely discussed (*2*). The mutagenic and carcinogenic members of these classes of chemicals require metabolic activation into electrophilic intermediates that can modify DNA. The members of subfamily 1 of the cytochrome P450 (CYP) gene family play an outstanding role in the catalysis of such metabolic activation (*3*). Therefore, the intestinal levels and activities of these enzymes, most notably of CYP 1A1, have been investigated and are widely considered as a parameter for the capacity of intestinal tissues and/or cells to activate putative carcinogenic food contaminants and/or tobacco smoke constituents (*4*, *5*). The expression of CYP1A1, related CYP enzymes, and a variety of other genes is under the control of the aryl hydrocarbon receptor (AhR) (*6*) present in many cell types. In the human colon, constitutive CYP1A1 expression in general is low but shows considerable interindividual variation (*7*). Smoking, certain drugs (*8*), or consumption of charcoal-grilled meat (*9*) can lead to enhanced levels of CYP1A1 in the duodenum, while no comparable data are available for the large intestine. In addition, epidemiological evidence suggests that certain genetic polymorphisms of CYP1A1 influence the risk of colorectal cancer (*4*).

Flavonoids and dihydrochalcones are secondary plant constituents belonging to the large group of plant polyphenols. While most flavonoids and dihydrochalcones are present in the intact plant as glycosides bearing saccharide residues, hydrolysis leading to aglycones can occur during food processing and storage. The highest polyphenol levels are found in fruits and

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vegetables and related food products that contain various amounts of both glycosides and aglycones. Apples are a major nutritional source of flavonoids and dihydrochalcones, the average daily intake from all sources being estimated as 10−100 mg (*10*, *11*). Apple juice extract was shown previously to interact with the epidermal growth factor receptor (*12*) and to modulate the expression of genes involved in the biotransformation of xenobiotics (*13*) in HT29 cells. Cloudy apple juice decreased DNA damage, hyperproliferation, and aberrant crypt foci development in the colon of dimethylhydrazine-treated rats (*14*).

The information available on the interaction of major apple flavonoids with CYP1 enzymes provides a somewhat complex picture. For the flavonol quercetin induction of CYP1A1, gene expression mediated by the aryl hydrocarbon receptor (AhR) was reported (*15*, *16*). This effect of quercetin, however, appears to depend on the cell line investigated (*17*). On the other hand, the addition of quercetin to rat liver microsomes was reported to inhibit the catalytic activity of the enzyme (*18*−*20*). Our study in the human colonic cell line Caco-2, used as a surrogate for intestinal epithelial cells, was aimed at the investigation of the possible interaction of a defined apple juice extract (AJE) and major flavonoids thereof with both AhR-dependent expression of CYP1A1 and its catalytic activity. We found that unknown AJE constituent(s) and the aglycone quercetin act as weak AhR agonists. AJE, quercetin, and phloretin effectively inhibited CYP1 catalytic activity. Furthermore, both compounds as well as AJE suppressed the induction of CYP1A1 by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) the most toxic AhR agonist.

MATERIALS AND METHODS

Compounds. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, nonessential amino acid mixture, penicillin/streptomycin, trypsin, and EDTA were from PAA Laboratories (Cölbe, Germany). 7-Ethoxyresorufin, phloretin, phlorizin, and rutin were purchased from Sigma (Steinheim, Germany), and quercetin was from ICN Biochemicals (Aurora, OH). Sodium ascorbate and α-tocopherol were from Roth (Karslruhe, Germany). All other chemicals were of the highest purity commercially available.

Apple juice extract was prepared from native apple juice as described elsewhere (*12*). The extract was devoid of major fruit acids and sugars. The dry powder was easily soluble in ethanol. Major constituents were analyzed by HPLC as described previously (*12*). Data on major constituents of the extract are given in **Table 1**.

Rabbit polyclonal anti-CYP1A1 antibodies (H-70) were from Santa Cruz (Heidelberg, Germany), and secondary goat anti-rabbit IgG horseradish peroxidase conjugates were from Sigma (Steinheim, Germany).

Cell Culture. Caco-2 cells (HTB 37) were obtained from the American Type Culture Collection (Rockville, MD). Cells were seeded at a density of 1.5×10^6 cells/plate on 60 mm Petri dishes (60 000/cm^2) (Greiner bio-one, Frickenhausen, Germany) in DMEM with 10% FBS, 1% penicillin/streptomycin, and 1% nonessential amino acid mixture (100×). Cells were cultured under standard conditions at 37 °C under air (8% CO_2). After 3 h, the medium was replaced by fresh medium. Then, 24 h after seeding, cultures were semiconfluent and were treated with vehicle only (ethanol or DMSO) or with the test compounds dissolved in ethanol (AJE, phloretin, and phlorizin) or DMSO (quercetin and rutin). The DMSO concentration did not exceed 0.1% of the incubation volume. Cells were harvested after 48 h treatment.

Cytotoxicity Assays. Cytotoxicity was either tested by measuring the reductive cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) according to Mosmann (*21*) or by the Alamar Blue assay according to the manufacturer's (BioSource International, Camarillo, CA) instructions. The latter is based on intracellular reduction of resazurin to the fluorescent product resorufin. Fluorescence was measured in a fluroescence spectrophotometer (Labsystems, Dreieich, Germany) using excitation at 544 nm and detection of emission at 590 nm. For both assays, the reducing capacity was expressed as percent of vehicle (ethanol or DMSO)-treated controls.

Table 1. Major Constituents of Apple Juice Extract (AJE) According to the Sequence of their Elution from the HPLC Column

constituent	concentration (mg/g)
procyanidine B1	2.9
chlorogenic acid	171.8
3-coumaroyl quinic acid	16.0
5-coumaroyl quinic acid	7.0
procyanidine B2	16.0
caffeic acid	102.7
epicatechin	11.8
4-coumaroyl quinic acid	72.4
phloretin glycoside 1[a]	16.6[b]
phloretin glycoside 2[a]	9.4[b]
phloretin-2-xyloglucoside	42.7
phlorizin	34.7
quercetin-3-rutinoside	1.8
quercetin-3-galactoside	0.9
quercetin-3-glucoside	1.4
quercetin-3-rhamnoside	3.2

[a] Characterized based on UV spectra only. [b] Estimates based on the absorption coefficient for phlorizin.

RNA Isolation and RT−-PCR Analysis. After removal of medium, cells were washed twice with ice-cold saline and treated with 500 μL of guanidinium thiocyanate solution, and total RNA was prepared by chloroform−phenol extraction according to the method of Chomczynski and Sacchi (*22*). Total RNA was dissolved in pure water (LiChrosolv, Merck, Germany), quantified spectrophotometrically using a Nanodrop analyzer (Nanodrop, Wilmington, DE), and diluted to 100 ng/μL. Aliquots of RNA were analyzed by agarose/formaldehyde gel electrophoresis to control RNA integrity.

Real-time quantitative RT−-PCR was performed using an iCycler iQ Real-Time PCR Detection System and iCycler Software version 2.2 (Bio-Rad, Munich, Germany). The RT reaction iScript-Kit (Bio-Rad) was used according to the manufacturer's protocol with 100 ng of RNA in a final volume of 20 μL. Samples were then assayed in 25 μL of reaction mixture using iQ SYBR Green Supermix (Bio-Rad), 6.25 ng of cDNA per reaction, and primers (MWG Biotech, Gelsenkirchen, Germany). The primers used were 5′-CCA TCC CCC ACA GCA CAA CAA GA-3′ (hCYP1A1, forward); 5′-CAGATG GGT TGA CCC ATA GCT TCT-3′ (hCYP1A1, reverse); 5′-TGC ACC ACC AAC TGC TTA GC-3′ (hGAPDH, forward); and 5′-GGC ATG GAC TGT GGT CAT GAG-3′ (hGAPDH, reverse). The iCycler was programmed as follows: 95 °C 3 min; 40 × (95 °C 1 min, 59 °C 1 min, 72 °C 1 min); 95 °C 1 min; and 55 °C 1 min. A melting curve emerging in a gradient from 55 to 95 °C in increasing steps of 0.5 °C verified the single PCR product. C_t values of the target gene were normalized to the housekeeping gene hGAPDH, and treated groups were related to the untreated control according to the equation of Pfaffl (*23*).

Microsome Preparation, EROD Assays, and Western Blotting. A total of 48 h after treatment, the medium was removed, and the cells were washed twice with ice-cold saline. After the addition of 1 mL of ice-cold Tris/sucrose, cells were scraped off and centrifuged at 4000*g* at 4 °C for 5 min. The pellet was resuspended in 800 μL of Tris/sucrose and ultrasound-treated for 10 s. After centrifugation at 5000*g* at 4 °C for 10 min, the supernatants were transferred to Eppendorf vials and centrifuged at 17 500*g* at 4 °C for 30 min. The pellet was resuspended in 100 μL of Tris/sucrose and stored at −80 °C.

After removal of the medium, cells were washed twice with saline and treated with 600 μL of swelling buffer (aqueous solution of 10 mM Tris/HCl, 10 mM NaCl, 1.5 mM $MgCl_2 \times 6H_2O$, 0.05% NaN_3, pH 7.4, supplemented with 1% of each DTT, PMSF, aprotinin, pepstatin, and leupeptin) for 1 min. Then, cells were scraped off, transferred to pre-cooled Eppendorf vials, and centrifuged at 4000*g* at 4 °C for 5 min. The pellet was resuspended in 800 μL of swelling

buffer and ultrasound-treated (50 W) for 10 s. After centrifugation at 5000*g* and 4 °C for 10 min, the supernatants were transferred to ultracentrifugation vials and centrifuged at 100 000*g* at 4 °C for 30 min. The pellet was resuspended in 25 μL of solubilizing buffer (a preparation of an aqueous solution of Tris/HCl 20 mM, NaCl 150 mM, EDTA 1 mM, Triton X-100 1%, NaN_3, pH 7.4, supplemented with 1% of each DTT, PMSF, aprotinin, pepstatin, and leupeptin) and stored at −80 °C. 7-Ethoxyresorufin dealkylase (EROD) activity was determined in microsomes as described earlier (*24*).

EROD activity and protein levels were also determined directly in the cell cultures according to Kennedy et al. (*25*) using a Fluorescent Ascent FL plate reader (Labsystems, Dreieich, Germany).

For Western blotting, 25 μg of microsomal protein was used. After separation by SDS polyacrylamide electrophoresis, proteins were blotted on polyvinylidene fluoride membranes and visualized by immunodetection as described (*24*).

Transfection and Reporter Gene Analysis. For the preparation of competent *Escherichia coli*, XL-10 Gold cells of the QuikChange XL Site-Directed Mutagensis Kit (Stratagene, Heidelberg, Germany) were used and were made $CaCl_2$ competent according to the manufacturer's protocol.

For transformation, 10 ng of plasmid and 10 μL of ligation mix (Stratagene, Heidelberg, Germany) were added to 100 μL of a suspension of the $CaCl_2$ competent bacteria. After the addition of 900 μL of 0.1 M $CaCl_2$ solution, the cells were kept on ice for 30 min. After keeping the cells for 1 min at 42 °C, and subsequent cooling on ice for 2−3 min, the mixture was centrifuged at 6000 rpm for 1 min. The pellet was resuspended in 300 μL of 2YT medium (10 g of Baco-Tryptone, 10 g of NaCl, 5 g of yeast extract at 1 L with bi-destillated water, and 100 μg/mL ampicillin after autoclaving) and incubated at 37 °C under agitation for 10 min. Then, the bacteria were plated on ampicillin-supplemented LB agar and incubated overnight at 37 °C. The ampicillin-resistent colonies were picked and transferred into 5 mL ampicillin-supplemented LB medium and incubated overnight under agitation (200 rpm) at 37 °C. The plasmid was isolated using the Gen Elute Endotoxin-free Plasmid Maxiprep Kit (Sigma, Deisenhofen, Germany) according to the manufacturer's protocol. The 1 mL of plasmid-containing bacteria was mixed with 225 μL of glycerol and stored at −80 °C.

The pGL3 enhancer vector (Promega, Mannheim, Germany) was used for transfection. The reporter gene plasmid contained a 485 bp fragment of the rat CYP1A1 gene including two xenobiotic-responsive elements as previously described (*24*), a modified firefly luciferase gene (luc^+), an SV40 enhancer, and a sequence conferring ampicillin resistance. For standardization, the pRL-SV40 plasmid (Promega, Mannheim, Germany) expressing Renilla luciferase under the control of a SV40 promoter was cotransfected.

Caco-2 cells were seeded on six-well plates at a density of 100 000 cells/well. For transfection, 250 μL of plasmid solution (37 μL of 2 M $CaCl_2$ solution, 5 μg of reporter gene construct, and 1 μg of control plasmid filled up with bidestillated water to 250 μL) was mixed slowly with 250 μL of 2× HBS buffer (50 mM HEPES, 280 mM NaCl, 1.5 mM $Na_2HPO_4 \times 2\ H_20$, pH 7.4) under agitation. After 30 min at room temperature, the mixture was added slowly to one six-well plate. Thereafter, the plates were incubated for 7 h at 37 °C. After replacement of the medium by fresh medium, the test compounds were added, and the cells were washed twice with cold PBS and harvested after another 48 h. Then cells were lysed with 300 μL of 1× Passive Lysis Buffer (Promega, Mannheim, Germany) per plate. The cells were scraped off and centrifuged for 1 min at 10 000*g*. A total of 20 μL of each supernatant was transferred into a luminometer tube (Sarstedt, Nümbrecht, Germany) and mixed with 95 μL of Luciferase Assay Reagent II (Promega, Mannheim, Germany), and the light intensity was measured immediately in a Lumat LB 9507 luminometer (Berthold, Bad Wildbad, Germany) over 10 s. Then, 95 μL of Stop&GloTM reagent was added, and the light intensity of the control gene expression was measured. The ratio between both intensities was used as a parameter for relative reporter gene expression.

Statistical Analysis. From several independent measurements, means and standard deviations were calculated. Testing for significant differences between means was carried out using One-way ANOVA and Dunnett's post-test at a probability of error of 5 and 1%.

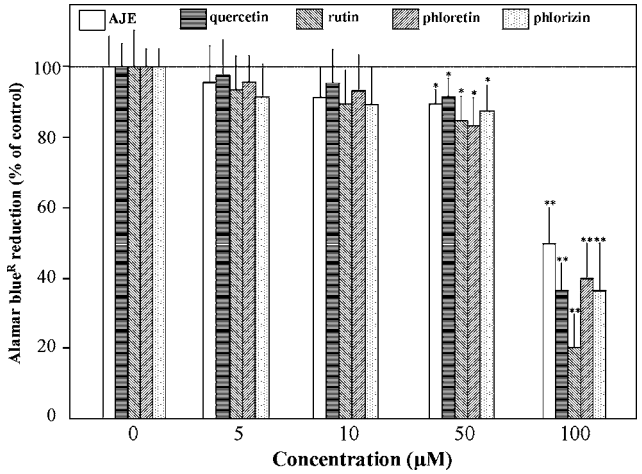

Figure 1. Cytotoxicity (reduction of Alamar blue^R) of apple juice extract (AJE) and the apple flavonoids quercetin, rutin, phloretin, and phlorizin in Caco-2 cells treated over 48 h. The concentration of AJE is expressed as phlorizin. *Significantly different ($p < 0.05$) or **very significantly ($p < 0.01$) different from the vehicle-treated control. Data are mean ± SD from $n = 3$ independent experiments.

RESULTS

Apple juice extract (AJE), quercetin, phloretin, and the glycosides rutin and phlorizin were added to semiconfluent cultures of Caco-2 cells, a common in vitro model for human intestinal mucosa cells of the colon. The AJE was devoid of major sugars found in apple juice such as fructose and glucose and of major fruit acids such as malic acid. Its major constituents were analyzed as described elsewhere (*12*) and are listed in **Table 1**. The major flavonoids/dihydrochalkones found were the dihydrochalcone phloretin, and the flavonol quercetin, which were mainly present as glycosides. Since polyphenol glycosides are subject to partial enzymatic hydrolysis of the glycosidic bond by gylcosidases of the intestinal mucosa, intestinal secretory glands, and the gut microflora (*26*), we investigated both the two prototype glycosides rutin and phlorizin and the corresponding aglycones quercetin and phloretin.

Surprisingly, AJE and the polyphenols including the glycosides showed a marked cytotoxicity, in particular, after an incubation time of 48 h (**Figure 1**). This effect was seen at a flavonoid concentration of 50 μM and was very pronounced at 100 μM. For AJE, the concentration was expressed as phlorizin, the major AJE flavonoid glycoside. Since the color of both the culture medium and the cells turned slowly into brown when AJE or individual flavonoids were added, we speculated that the formation of quinones in the presence of oxygen might be responsible for these cytotoxic effects. In fact, the addition of 0.25 mM sodium L-ascorbate together with 0.05 mM D,L-α-tocopherol completely prevented the formation of any brownish products as well as any cytotoxicity at all concentrations tested up to 100 μM (data not shown). Therefore, all further experiments were carried out with medium supplemented with L-ascorbate/D,L-α-tocopherol. Supplementation had no effect on the basic CYP1A1 levels.

AJE and quercetin led to an induction of CYP1A1 mRNA, the efficacy being about half of that of 1 nM TCDD (**Figure 2**). This effect was significant at 100 μM AJE (expressed as phlorizin) and at 50 or 100 μM quercetin; phloretin and the two glycosides rutin and phlorizin were inactive. A very similar

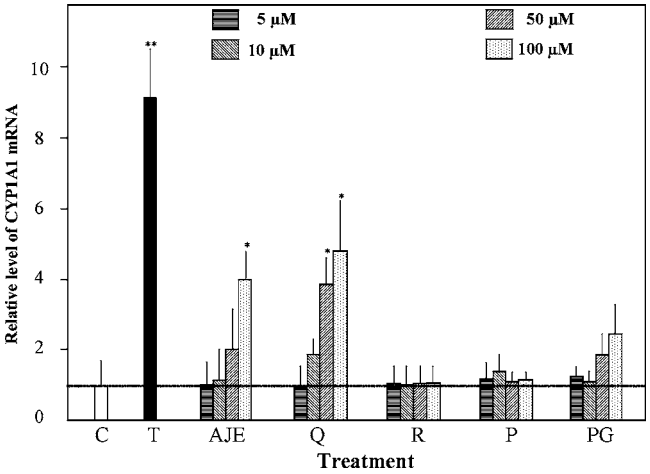

Figure 2. Effects of apple juice extract (AJE), quercetin (Q), rutin (R), phloretin (P), and phlorizin (PG) on CYP1A1 mRNA levels in Caco-2 cells treated over 48 h. Comparison with vehicle-treated control (C) and 1 nM TCDD (T). The concentration of AJE is expressed as phlorizin. *Significantly different ($p < 0.05$) or **very significantly ($p < 0.01$) different from the vehicle-treated control. Data are means ± SD from $n = 3$ independent experiments.

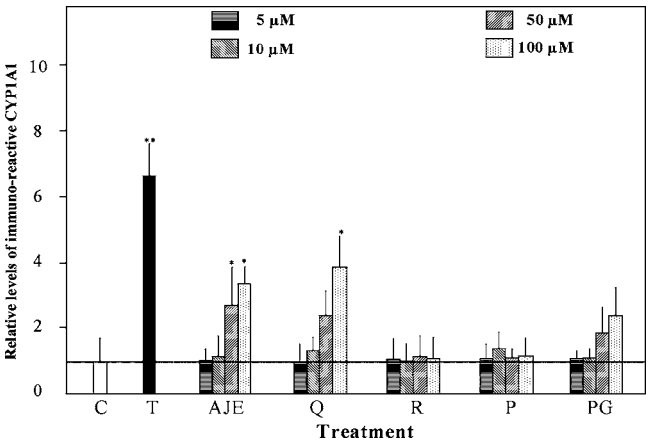

Figure 3. Effects of apple juice extract (AJE), quercetin (Q), rutin (R), phloretin (P), and phlorizin (PG) on immunoreactive CYP1A1 protein in Caco-2 cells treated over 48 h. Comparison with vehicle-treated control (C) and 1 nM TCDD (T). The concentration of AJE is expressed as phlorizin. *Significantly different ($p < 0.05$) or **very significantly ($p < 0.01$) different from the vehicle-treated control. Data are mean ± SD from $n = 3$ independent experiments.

result was obtained for the induction of immunoreactive CYP1A1 protein (**Figure 3**). When the catalytic activity of CYP1A1, 7-ethoxyresorufin *O*-deethylase (EROD), was measured in cells, a slight induction was found with AJE, quercetin, and phlorizin at the highest concentration but not with rutin or phloretin (**Table 2**). However, the inducing effect achieved not more than 7.3% (AJE), 4.5% (quercetin), and 4.1% (phlorizin) of the maximum response obtained with 1 nM TCDD.

These findings led us to the hypothesis that some of the substances tested may counteract induction at the level of catalytic activity of CYP1A1. In fact, AJE, quercetin, and phloretin efficiently inhibited EROD activity when added to microsomes isolated from TCDD-treated Caco-2 cells (**Table 3**). When co-incubated with 1 nM TCDD for 48 h, a marked suppression of EROD induction was also observed. These data allow the calculation of concentrations leading to a half-maximal inhibition of EROD activity in microsomes or of concentrations leading to half-maximal suppression of EROD induction in intact

Table 2. EROD Activity (pmol/min × mg of Protein) in Caco-2 Cells Treated over 48 h with AJE or Apple Polyphenols[a]

compound	−TCDD	+TCDD
control	4.0 ± 0.8	223 ± 35
	AJE[b]	
5 μM	4.5 ± 2.8	75 ± 22[c]
10 μM	4.9 ± 2.2	24 ± 13[c]
50 μM	6.1 ± 2.0	28 ± 16[c]
100 μM	16.2 ± 6.1[c]	21 ± 15[c]
	quercetin	
5 μM	3.3 ± 2.0	124 ± 44
10 μM	2.1 ± 1.9	75 ± 26[d]
50 μM	7.6 ± 3.2	38 ± 16[c]
100 μM	10.2 ± 2.8[d]	35 ± 19[c]
	rutin	
5 μM	3.6 ± 1.8	185 ± 31
10 μM	4.1 ± 1.3	150 ± 36
50 μM	7.0 ± 2.8	169 ± 40
100 μM	6.7 ± 3.1	153 ± 38
	phloretin	
5 μM	4.0 ± 2.8	72 ± 26[c]
10 μM	4.2 ± 2.7	42 ± 21[c]
50 μM	4.4 ± 2.5	39 ± 18[c]
100 μM	7.1 ± 2.8	21 ± 14[c]
	phlorizin	
5 μM	3.8 ± 2.3	142 ± 44
10 μM	5.3 ± 2.0	132 ± 56
50 μM	6.9 ± 3.1	152 ± 40
100 μM	9.1 ± 3.1[d]	158 ± 42

[a] Controls were treated with vehicle (DMSO or ethanol) only or with DMSO or ethanol and TCDD (1 nM). Data are mean ± SD from $n = 3$ independent experiments. [b] Expressed as phlorizin. [c] Very significantly different ($p < 0.01$) from the respective control (vehicle-treated or DMSO/TCDD-treated only). [d] Significantly different ($p < 0.05$) from the respective control (vehicle-treated or DMSO/TCDD-treated only).

Table 3. EC_{50} Values for the Suppression of EROD Induction in Caco-2 Cells Co-incubated with TCDD (1 nM) for 48 h versus the Inhibition of EROD Activity in Microsomes Prepared from Cells Pretreated with TCDD (1 nM) for 48 h[a]

compound	EC_{50} (μM) of suppression of induction	EC50 (μM) of inhibition in microsomes
AJE[b]	4.0 ± 0.8	3.5 ± 0.5
Quercetin	9.2 ± 1.2	0.3 ± 0.1
Rutin	n.d.[c]	n.d.
Phloretin	4.2 ± 0.9	0.5 ± 0.1
Phlorizin	n.d.	n.d.

[a] Data are mean ± SD from $n = 3$ independent experiments. [b] Expressed as phlorizin. [c] n.d. = not detectable.

cells. The EC_{50} values for suppression of EROD induction after addition to intact cells were higher by a factor of 30 (quercetin) or 8 (phloretin) than those for the inhibition of EROD activity after addition to microsomes. In contrast, AJE exhibited very similar EC_{50} values for the suppression of induction and inhibition of catalytic activity.

To test the possibility that the polyphenols and AJE may also affect induction of CYP1A1 gene expression, CYP1A1 mRNA and immunoreactive proteins were also analyzed after co-incubations with TCDD. The increases in mRNA (**Figure 4**) and CYP1A1 protein (**Figure 5**) were markedly suppressed by AJE, quercetin, and phloretin at concentrations of 50 and 100 μM, the intact glycosides being inactive. Finally, an XRE-driven reporter gene comprising core sequences of the human CYP1A1 promoter was transfected and used as a tool to study effects on

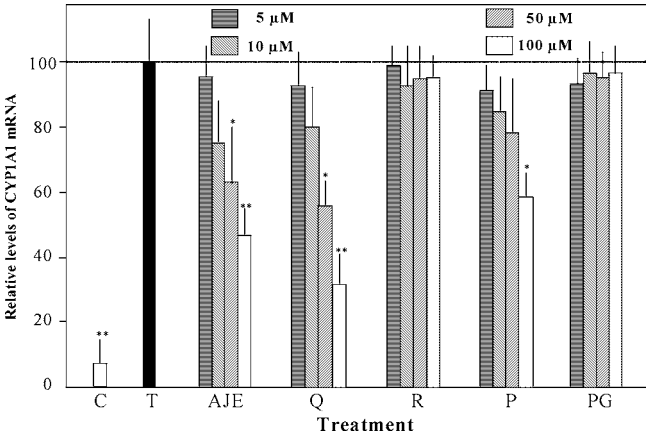

Figure 4. Effects of apple juice extract (AJE), quercetin (Q), rutin (R), phloretin (P), and phlorizin (PG) on CYP1A1 mRNA induction in Caco-2 cells co-incubated with 1 nM TCDD or treated with 1 nM TCDD only (T) over 48 h. The level in vehicle-treated controls (C) is shown for comparison. The concentration of AJE is expressed as phlorizin. *Significantly different ($p < 0.05$) or **very significantly ($p < 0.01$) different from the TCDD-treated cultures. Data are mean ± SD from $n = 3$ independent experiments.

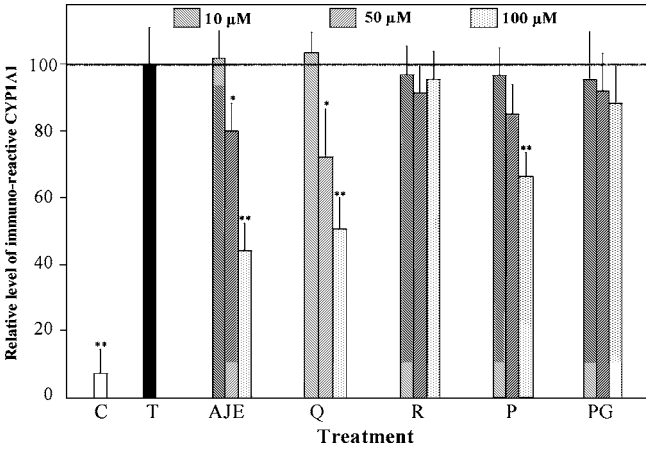

Figure 5. Effects of apple juice extract (AJE), quercetin (Q), rutin (R), phloretin (P), and phlorizin (PG) on immunoreactive CYP1A1 protein in Caco-2 cells co-incubated with 1 nM TCDD or treated with 1 nM TCDD only (T) over 48 h. The level in vehicle-treated controls (C) is shown for comparison. The concentration of AJE is expressed as phlorizin. The concentration of AJE is expressed as phlorizin. *Significantly different ($p < 0.05$) or **very significantly ($p < 0.01$) different from the TCDD-treated cultures. Data are mean ± SD from $n = 3$ independent experiments.

CYP1A1 gene transcription. AJE suppressed TCDD-induced reporter gene activity at 50 μM, quercetin and phloretin at 10 μM, and at higher concentrations (**Figure 6**). In particular with quercetin, the reporter gene activity could be reduced by about 90% of the corresponding level obtained with TCDD only.

DISCUSSION

In this study, we investigated the interaction of a well-defined polyphenol-rich apple juice extract (AJE) of the major principal apple flavonoid/dihydrochalkone quercetin and phloretin and of the corresponding glycosides rutin (a quercetin rutinoside) and phlorizin (a phloretin glucoside) on the aryl hydrocarbon receptor (AhR)-mediated induction of CYP1A1 in the human colon cell line Caco-2. Previous work with the same extract has shown modulation of expression of a variety of drug metabolism−-related genes in HT 29 human colon cancer cells

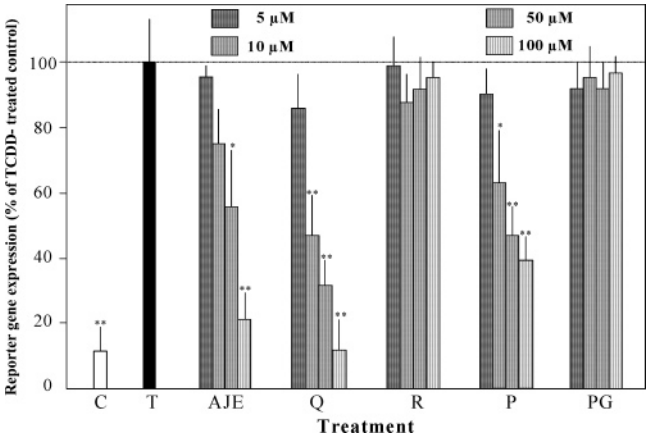

Figure 6. Effects of apple juice extract (AJE), quercetin (Q), rutin (R), phloretin (P), and phlorizin (PG) on XRE-driven reporter gene expression in Caco-2 cells co-incubated with 1 nM TCDD or treated with 1 nM TCDD only (T) over 48 h. The level in vehicle-treated controls (C) is shown for comparison. The concentration of AJE is expressed as phlorizin. *Significantly different ($p < 0.05$) or **very significantly ($p < 0.01$) different from the TCDD-treated cultures. Data are mean ± SD from $n = 3$ independent experiments.

(*13*). When added to the cell cultures at concentrations of 50 or 100 μM (the AJE level is expressed as phlorizin), all compounds tested exhibited a significant cytotoxicity. Since this effect was completely absent after the addition of the antioxidants L-ascorbate/α-tocopherol, we conclude that AJE constituents/flavonoids have been oxidized by oxygen to quinones that are able to bind to proteins and exert cytotoxic effects. A cytotoxic action may even occur after extracellular oxidation and subsequent binding of quinones to the cellular membrane. It appears unlikely that this mechanism is of relevance in vivo since micro-aerobic/anaerobic conditions most likely prevail in the intestinal lumen. Furthermore, intestinal mucus may probably bind eventually formed and/or ingested polyphenol-derived quinones since the latter may exhibit electrophilic properties and thus may bind covalently to proteins and other nucleophiles.

Our conclusion is confirmed by the observation that cultures without but not with L-ascorbate/α-tocopherol developed a brownish color after a few hours of incubation. Therefore, we carried out all subsequent experiments with anti-oxidative protection.

Our experiments can be categorized in three categories, namely, (i) induction of CYP1A1 gene expression, (ii) inhibition of CYP1A1 activity, and (iii) modulation of TCDD-mediated induction.

First, we found that AJE and quercetin were active as inducers of CYP1A1 gene expression at the level of mRNA, immunoreactive protein, and catalytic activity. The maximum response achieved with both compounds was in the range of 50% of the TCDD response, TCDD being one of the most potent and most effective AhR agonists. In contrast, both AJE and quercetin showed a very weak efficacy as inducers of EROD activity leading to a maximum induction of only 5−8% of that obtained with TCDD. These results are in agreement with previous studies, indicating that quercetin has weak AhR agonistic and/or CYP1A1 inducing activity in rat liver (*16*) and in the human cell line MCF-7 (*15*). In contrast, Zhang et al. (*17*) did not find CYP1A1 induction in quercetin-treated MCF-7 or HepG2 cells, and Veeriah et al. (*13*) did not see an effect of the same AJE on CYP1A1 according to cDNA array analysis.

In our study as in all other studies, the glycosides were inactive as inducers of CYP1A1 mRNA and protein, while

phlorizin led to a weak but significant increase in EROD activity. Since this effect was not seen with phloretin, cleavage of the glycosidic bond of phlorizin is unlikely to be a pre-condition for induction. In general, due to their high polarity and bulky structure, the intact glycosides are likely to be absorbed by the cells to a much lower extent than the aglycones. Furthermore, direct binding of glycosides to the AhR appears unlikely for similar reasons. The fact that we saw a slight induction of EROD activity but not of CYP1A1 mRNA or protein with phloretin may be due to the higher sensitivity of the EROD assay. Furthermore, AhR-independent induction of CYP1A1 was reported to occur (e.g., in Caco-2 cells (*27*)).

Second, the finding that AJE and quercetin showed a much stronger inducing effect (in the absence of TCDD) on mRNA and protein expression than on EROD activity led us to the hypothesis that these compounds may simultaneously induce CYP1A1 gene expression and inhibit CYP1A1 catalytic activity in Caco-2 cells. Previous reports have already shown that a number of flavonoids can inhibit CYP 1A1-mediated catalytic activities in other cell types (*15*−*18*). The hypothesis was confirmed in our study in a series of experiments with TCDD-treated Caco-2 cells. First, we addressed the question of enzyme inhibition in microsomes isolated from TCDD-treated cells and found that AJE, quercetin, and phloretin are potent inhibitors of EROD activity. In consequence, these compounds may suppress the metabolic activation of potent carcinogens such as benzo[*a*]pyrene or aflatoxin B_1 as was shown by others for certain flavonoids (*28*, *29*). Kang et al. (*29*) found a decrease in benzo[*a*]pyrene-related DNA adducts in HepG2 cells co-treated with quercetin. The finding of a 52% inhibition of EROD activity in cells treated with 10 μM quercetin is in excellent agreement with our finding of an EC_{50} of 9 μM quercetin in intact Caco-2 cells.

Third, we found that co-incubation of AJE, quercetin, or phloretin with TCDD was able to suppress EROD induction. In these experiments, the TCDD concentration of 1 nM leads to maximum induction of EROD activity. The EC_{50} value for the suppression of EROD induction in TCDD-treated cells was higher than the EC_{50} value for EROD inhibition in microsomes by a factor of 30 for quercetin and by a factor of 8 for phloretin, but both values were almost equal for AJE. These findings may be due to the fact that the intracellular availability of flavonoids is not complete (i.e., incubation at the same concentrations leads to lower effective concentrations at the site of action in intact cells as compared to microsomes). Furthermore, it can be speculated that this is not true for the (unknown) EROD inhibitor(s) active in AJE.

A more detailed picture was obtained when the effects on TCDD-induced CYP1A1 mRNA and protein expression were analyzed. There, it was found that AJE, quercetin, and phloretin also suppress CYP1A1 mRNA and protein induction. In a reporter gene assay, AJE, quercetin, and phloretin suppressed the trans-activating activity of TCDD measured as enhanced transcription of an XRE-driven reporter gene.

These findings demonstrate that AJE, quercetin, and phloretin not only inhibit CYP1A1 activity but also suppress its induction. Similar effects were reported for quercetin and kaempferol in MCF-7 cells (*17*). Ciolino et al. (*15*) could show that quercetin acts as a weak AhR agonist/antagonist and kaempferol as a pure antagonist by binding to the receptor and modulating its specific DNA binding elicited by TCDD treatment of MCF-7 cells.

For a number of polyphenols, a relatively low oral bioavailability was shown in humans (e.g., only a small portion of ingested polyphenols was recovered in plasma). Furthermore, extensive metabolism mainly via conjugation occurred, further reducing the levels of free polyphenols in blood. In contrast, the intestinal mucosa is exposed more directly to constituents of the diet. Recent work by Kahle et al. (*30*) has shown that certain polyphenols reach the human colon in considerable amounts when ingested via apple juice.

Taken together, our findings show that AJE and the flavonoids/dihydrochalkones quercetin and phloretin interfere with AhR-dependent regulation and catalytic activity of CYP1A1 in Caco-2 cells on several levels. First, quercetin and AJE act as partial AhR agonists/antagonists, while phloretin is a pure AhR antagonist. Our findings indicate that the CYP1A1 inducing effect of AJE is not due to quercetin (which is present in AJE in relatively low concentrations) or phloretin (which is inactive as an inducer). Therefore, unknown AJE constituents must be responsible for this effect. It is difficult to speculate on the identity of these constituents. Second, AJE, quercetin, and phloretin are potent inhibitors of CYP1A1 probably by direct interaction with the enzyme. This effect cannot be explained based on quercetin or phloretin contents only. Third, the three compounds effectively suppress TCDD-mediated CYP1A1 induction. This effect may be linked to AhR binding of quercetin, phloretin, and unknown AJE constituents that in the case of quercetin leads to partial antagonism, in the case of phloretin to full antagonism. It is feasible to assume that the unknown active constituent(s) may also belong to the class of polyphenols since phlorizin, which served as a reference compound in our experiments, accounted for less than 10% of the total polyphenols in the extract.

The most important of our findings is that unknown constituents of apple juice extract as well as quercetin and phloretin present in apple juice and many other types of fruits and vegetables lead to an overall antagonism of CYP1A1 induction elicited by strong agonists such as TCDD. These effects may be of relevance by suppressing the metabolic activation of mutagenic/carcinogenic substrates of CYP1A1 involved in colon carcinogenesis as well as by attenuating an over-production of reactive oxygen species as eventual byproducts of relevant CYP1A1 induction. In particular, phloretin, the major apple dihydrochalkone, may, after release from its glycosidic precursors, exert beneficial effects (e.g., under conditions of sustained CYP1A1 induction in the intestine resulting from chronic exposure to potent AhR agonists in tobacco smoke or in the diet).

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LITERATURE CITED

(1) EUCAN. *Cancer incidence, mortality and prevalence in the European Union*−*1998 estimates*; 2005; p 2.
(2) Cross, A. J; Sinha, R. Meat-related mutagens/carcinogens in the etiology of colorectal cancer. *Environ. Mol. Mutagen.* **2004**, *44*, 44−55.
(3) Nebert, D. W. Role of genetics and drug metabolism in human cancer risk. *Mutat. Res.* **1991**, *247*, 267−281.
(4) Murtaugh, M. A.; Sweeney, C.; Ma, K. N.; Caan, B. J.; Slattery, M. L. The CYP1A1 genotype may alter the association of meat consumption patterns and preparation with the risk of colorectal cancer in men and women. *J. Nutr.* **2005**, *135*, 179−186.
(5) Slattery, M. L.; Samowitz, W.; Ma, K.; Murtaugh, M.; Sweeney, C.; Levin, T. R.; Neuhausen, S. CYP1A1, cigarette smoking,

and colon and rectal cancer. *Am. J. Epidemiol.* **2004**, *160*, 842−852.
(6) Nebert, D. W.; Dalton, D. W.; Okey, A. B.; Gonzalez, F. J. Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J. Biol. Chem.* **2004**, *279*, 23847−23850.
(7) Mercurion, M. G.; Shiff, S. J.; Galbraith, R. A.; Sassa, S. Expression of cytochrome P450 mRNAs in the colon and the rectum in normal human subjects. *Biochem. Biophys. Res. Commun.* **1995**, *210*, 350−355.
(8) Buchthal, J.; Grund, K. E.; Buchmann, A.; Schrenk, D.; Beaune, P.; Bock, K. W. Induction of cytochrome P4501A by smoking or omeprazole in comparison with UDP-glucuronosyltransferase in biopsies of human duodenal mucosa. *Eur. J. Clin. Pharmacol.* **1995**, *47*, 431−435.
(9) Fontana, R. J.; Lown, K. S.; Paine, M. F.; Fortlage, L.; Santella, R. M.; Felton, J. S.; Knize, M. G.; Greenberg, A.; Watkins, P. B. Effects of a chargrilled meat diet on expression of CYP3A, CYP1A, and P-glycoprotein levels in healthy volunteers. *Gastroenterology* **1999**, *117*, 89−98.
(10) Aherne, S. A.; O'Brien, N. M. Dietary flavonols: chemistry, food content, and metabolism. *Nutrition* **2002**, *18*, 75−81.
(11) Price, K. R.; Prosser, T.; Richetin, A. M. F.; Rhodes, M. J. C. A comparison of the flavonol content and composition in dessert, cooking, and cider-making apples: distribution within the fruit and effect of juicing. *Food Chem.* **1999**, *66*, 489−494.
(12) Kern, M.; Tjaden, Z.; Ngiewih, Y.; Puppel, N.; Will, F.; Dietrich, H.; Pahlke, G.; Marko, D. Inhibitors of the epidermal growth factor receptor in apple juice extract. *Mol. Nutr. Food Res.* **2005**, *49*, 317−328.
(13) Veeriah, S.; Kautenburger, T.; Habermann, N.; Sauer, J.; Dietrich, H.; Will, F.; Pool-Zobel, B. L. Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics. *Mol. Carcinog.* **2006**, *45*, 164−174.
(14) Barth, S. W.; Fähndrich, C.; Bub, A.; Dietrich, H.; Watzl, B.; Will, F.; Briviba, K.; Rechkemmer, G. Cloudy apple juice decreases DNA damage, hyperprolifaration, and aberrant crypt foci development in the distal colon of DMH-initiated rats. *Carcinogenesis* **2005**, *26*, 1414−1421.
(15) Ciolino, H. P.; Daschner, P. J.; Yeh, G. C. Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. *Biochem. J.* **1999**, *340*, 715−722.
(16) Rahden-Staron, I.; Czeczot, H.; Szumilo, M. Induction of rat liver cytochrome P450 isoenzymes CYP 1A and CYP 2B by different fungicides, nitrofurans, and quercetin. *Mutat. Res.* **2001**, *498*, 57−66.
(17) Zhang, S.; Qin, C.; Safe, S. H. Flavonoids as aryl hydrocarbon receptor agonists/antagonists: effects of structure and cell context. *Environ. Health Perspect.* **2003**, *111*, 1877−1882.
(18) Zhai, S.; Dai, R.; Friedman, F. K.; Vestal, R. E. Comparative inhibition of human cytochromes P450 1A1 and 1A2 by flavonoids. *Drug Metab. Dispos.* **1998**, *26*, 989−992.
(19) Tsyrlov, I. B.; Mikhailenko, V. M.; Gelboin, H. V. Isozyme- and species-specific susceptibility of cDNA-expressed CYP1A P450s to different flavonoids. *Biochim. Biophys. Acta* **1994**, *5*, 325−335.
(20) Siess, M.-H.; Leclerc, J.; Canivenc-Lavier, M.-C.; Rat, P.; Suschetet, M. Heterogenous effects of natural flavonoids on monooxygenase activities in human and rat liver microsomes. *Toxicol. Appl. Pharmacol.* **1995**, *130*, 73−78.
(21) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.
(22) Chomczynski, P.; Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate−phenol−chloroform extraction. *Anal. Biochem.* **1987**, *162*, 156−159.
(23) Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acids Res.* **2001**, *29*, 2002−2007.
(24) Baumgart, A.; Schmidt, M.; Schmitz, H.-J.; Schrenk, D. Natural furocoumarins as inducers and inhibitors of cytochrome P450 1A1 in rat hepatocytes. *Biochem. Pharmacol.* **2005**, *69*, 657−667.
(25) Kennedy, S. W.; Jones, S. P.; Bastien, L. J. Efficient analysis of cytochrome P450 1A catalytic activity, porphyrins, and total proteins in chicken embryo hepatocyte cultures with a fluorescence plate reader. *Anal. Biochem.* **1995**, *226*, 362−370.
(26) Sesink, A. L. A.; Arts, I. C. W.; Faassen-Peters, M.; Hollman, P. C. H. Intestinal uptake of quercetin-3-glucoside in rats involves hydrolysis by lactase phlorizin hydrolase. *J. Nutr.* **2003**, *133*, 773−776.
(27) Guigal, N.; Seree, E.; Bourgarel-Rey, V.; Barra, Y. Induction of CYP1A1 by serum independent of AhR pathway. *Biochem. Biophys. Res. Commun.* **2000**, *267*, 572−576.
(28) Buening, M. K.; Chang, R. L.; Huang, M.-T.; Fortner, J. G.; Wood, A. W.; Conney, A. H. Activation and inhibition of benzo(a)pyrene and aflatoxin B1 metabolism in human liver microsomes by naturally occurring flavonoids. *Cancer Res.* **1981**, *41*, 67−72.
(29) Kang, Z. C.; Tsai, S.-J.; Lee, H. Quercetin inhibits benzo[*a*]pyrene-induced DNA adducts in human HepG2 cells by altering cytochrome P450 1A1 gene expression. *Nutr. Cancer* **1999**, *35*, 175−179.
(30) Kahle, K.; Kraus, M.; Scheppach, W.; Richling, E. Colonic availability of apple polyphenols—A study in ileostomy patients. *Mol. Nutr. Food Res.* **2005**, *49*, 1143−1150.

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