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Induction of cytochrome P450 1A by cow milk-based formula: a comparative study between human milk and formula

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1 During the treatment of neonatal apnea, formula-fed infants, compared to breastfed infants, show nearly three-fold increase in clearance of caffeine, a substrate of cytochrome P450 1A (CYP1A) and in part CYP3A4. However, human milk is known to contain higher concentrations of environmental pollutants than infant formula, which are potent CYP1A inducers. To gain insight into the mechanism underlying this apparent contradiction, we characterized CYP1A and CYP3A4 induction by human milk and cow milk-based infant formula.

2 The mRNA and protein expression of CYP1A1/1A2 were significantly induced by cow milk-based formula, but not by human milk, in HepG2 cells.

3 Luciferase reporter assay demonstrated that cow milk-based formula but not human milk activated aryl hydrocarbon receptor (AhR) significantly. The cotreatment of 3,4-dimethoxyflavone, an AhR antagonist, abolished the formula-induced CYP1A expression. In addition, AhR activation by dibenzo[*a*,*h*]anthracene, a potent AhR agonist, was significantly suppressed by infant formula and even more by human milk.

4 In contrast, CYP3A4 mRNA expression was only mildly induced by formula and human milk. Consistently, neither formula nor human milk substantially activated pregnane X receptor (PXR).

5 Effects of whey and soy protein-based formulas on the AhR–CYP1A and the PXR–CYP3A4 pathways were similar to those of cow milk-based formula.

6 In conclusion, infant formula, but not human milk, enhances *in vitro* CYP1A expression *via* an AhR-mediated pathway, providing a potential mechanistic basis for the increased caffeine elimination in formula-fed infants.
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Abbreviations: AhR, aryl hydrocarbon receptor; CYP1A, cytochrome P450 1A; DBA, dibenzo[*a*,*h*]anthracene; 3,4-DMF, 3,4-dimethoxyflavone; DRE, dioxin response element; F1, formula 1; F2, formula 2; F3, formula 3; F4, formula 4; F5, formula 5; FP1, powdered formula 1; FP2, powdered formula 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAH, polycyclic aromatic hydrocarbons; PBS, phosphate-buffered saline; PCB, polychlorinated biphenyls; PEC, potency equivalency concentration; PXR, pregnane X receptor; TCDD, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin; TEQ, toxic equivalent

Introduction

Exclusive breastfeeding is strongly recommended for the first 6 months of life because of its tangible beneficial effects (American Academy of Pediatrics, 1997), such as containing essential nutrients, cells, enzymes, trace elements, vitamins, growth factors, hormones, and immune factors. Despite the benefits of human milk, infant formulas are often used for medical and socio-economic reasons. Most infant formulas are based on cow milk-derived casein, but whey- or soy-based formulas are also available as alternatives (American Academy of Pediatrics, 1998).

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Concerns have been expressed on the contamination of human milk with carcinogenic and mutagenic environmental pollutants, such as halogenated aromatic hydrocarbons (e.g., dioxin and polychlorinated biphenyls (PCB)) and polycyclic aromatic hydrocarbons (PAH) (Somogyi & Beck, 1993; Schecter *et al*., 1994; Hooper & McDonald 2000). Concentrations of these compounds are higher in human milk than in formula, which leads to higher levels of pollutant intake in the breastfed infants (Schecter *et al*., 1994; Abraham *et al*., 1996). As a consequence, the intake and body burden of these contaminants are higher in breastfed infants than in formula-fed infants (Abraham *et al*., 1996; Karmaus *et al*., 2001). Recently, the rapidly rising milk levels of polybrominated

diphenyl ethers, a family of brominated flame retardants classified also as persistent organic pollutants, are becoming challenging problems (Hooper & McDonald, 2000).

These environmental pollutants, as well as many other natural dietary aryl hydrocarbon receptor (AhR) ligands, selectively bind to AhR, a nuclear receptor (Machala *et al*., 2001; Jeuken *et al*., 2003). This intracellular protein is activated by ligand binding, and subsequently undergoes heterodimerization and translocation, triggering the downstream events that include transcriptional induction of the cytochrome P450 1A (CYP1A), one of the major drug-metabolizing enzymes (Merchant *et al*., 1992). The CYP1A is involved in metabolism of a wide range of drugs and chemicals, such as theophylline (Ha *et al*., 1995), caffeine (Ha *et al*., 1996), propranolol (Walle *et al*., 1987), verapamil (Nielsen-Kudsk *et al*., 1990), and polyaromatic hydrocarbons (Ioannides & Parke, 1990). Some of the well-recognized CYP1A inducers are in our regular diet and environment, such as char-grilled meat (Fontana *et al*., 1999), broccoli (Vang *et al*., 2001), cigarette smoke (Parsons & Neims, 1978), and persistent environmental pollutants (Nebert *et al*., 2004). Although the exact mechanism remains to be elucidated, AhR activation is undoubtedly the initial step of this cascade. At present, it is not known if levels of these environmental pollutants in human milk are high enough to activate AhR.

Caffeine, a drug for treating apnea of prematurity (Comer *et al*., 2001), is extensively metabolized by CYP1A and partly by CYP3A4 (Ha *et al*., 1996), a major hepatic CYP regulated mainly by pregnane X receptor (PXR). During the treatment of apnea of prematurity, the elimination of caffeine has been found nearly three-fold faster in the formula-fed than in the breast-fed infants (Le Guennec & Billon, 1987; Blake *et al*., 2004), the underlying mechanism(s) of which is unknown.

Given the reportedly higher concentration of environmental pollutants in human milk, the above clinical observation is intriguing. This may suggest that infant formula contains AhR agonists unaccounted for by specific chemical analyses. We, therefore, characterized effects of cow milk-based infant formula and human milk on CYP1A expressions in a biological system: HepG2 cells, a human hepatoma cell line, which is an established *in vitro* hepatic cell model. Our findings show that cow milk-based formula, but not human milk, strongly induced the expression of the two isoforms of CYP1A, CYP1A1 and CYP1A2, through AhR activation.

Methods

Human milk samples and infant formulas

We used milk samples from 29 healthy nursing mothers in a breastfeeding follow-up clinic. The minimally restrictive inclusion criteria were used, so that life style variations influencing the experimental end points can be captured, if any. The criteria were (1) between 2 weeks and 6 months postpartum and (2) no medications within last 2 weeks. The protocol was approved by the institutional review board. After written consents were obtained, 5–10 ml of the manually expressed milk samples was collected, stripped of identities, and stored at −80°C until use. We avoid pooling the milk samples to preserve individual variations in their experimental effects. Each milk sample was evaluated for its effect on CYP1A1, CYP1A2, and CYP3A4 expressions, and AhR and hPXR activations. Brands of cow milk-, whey-, and soy protein-based formula were purchased from retail stores and stored at −80°C until use (Table 1). Powdered formulas were diluted fresh at the time of experiments according to the manufacturer's direction.

Materials

Dibenzo[*a*,*h*]anthracene (DBA) and 3,4-dimethoxyflavone (3,4-DMF) were purchased (Sigma Chemical Co, St Louis, MO, U.S.A.). All DBA and 3,4-DMF used in experiments were 10 μM unless otherwise mentioned. pGUDluc4.1 plasmid and [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were generous gifts from Dr Michael S. Denison and Dr Allan B. Okey, respectively. Other chemicals were purchased from Sigma-Aldrich Ltd, St Louis, MO, U.S.A. Culture-related materials were obtained from Gibco-BRL, Grand Island, NY, U.S.A.

Cell culture and treatment

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The cells were maintained in α-minimal essential medium containing 10% fetal bovine serum and 1% antibiotics (penicillin–streptomycin) under an atmosphere of 95% air–5% CO2 at 37°C, and subcultured weekly using 0.05% trypsin. The HepG2 cells contain

Table 1 Information about the composition of studied infant formulas (per 100 g)

	F1[a]	*F2*[a]	*FP1*[a]	*FP2*[a]	*F3*[a]	*F4*[b]	*F5*[a]
Product form	[c]	[c]	[d]	[d]	[c]	[c]	[c]
Source of protein	[e]	[e]	[e]	[e]	[f]	[g]	[g]
Protein (g)	1.38	1.36	10.8	10.89	1.45	1.9	1.61
Total lipid (g)	3.5	3.55	27	28.87	3.37	3.4	3.59
Carbohydrates (g)	6.45	6.92	56.2	54.73	7.39	7.4	6.6
Cholesterol (mg)	1	2	17	14	4	[h]	0
Total sugar (g)	7.18	6.9	56	54.73	5	[h]	6.7

[a]Data referred from USDA National Nutrient Database for Standard Reference, Release 17 (2004).
[b]Data referred from the nutrition table of the product.
[c]Liquid.
[d]Powder
[e]Cow milk-based.
[f]Whey protein-based.
[g]Soy protein-based.
[h]No information.

inducible CYP1A (Roberts *et al*., 2000) and CYP3A subfamilies (Sumida *et al*., 1999), and resemble fetal and early neonatal liver with regard to their CYP1A and CYP3A4 profiles. The cells at 80–90% confluence were incubated with human milk, or formula at the final concentrations of up to 20% v v^{-1} for 24 h under the same culture conditions as above. To minimize the potential dilution effects of formula or milk treatment, the controls were also similarly diluted with phosphate-buffered saline (PBS). In a set of control experiments, the cells were grown on culture inserts with permeable membranes (Costar, Corning Inc., Corning, NY, U.S.A.) placed in six-well plates, and the experiments were conducted by adding milk or formula to the bottom compartment, so that the cells could face components of milk or formula from underneath without any potential sediments on the cell surface.

Cell viability assay

The HepG2 cells grown in 24-well plates were similarly treated for 72 h (see above). After replacing the media with that containing 10% AlamarBlue solution (TREK Diagnostic Systems, Cleveland, OH, U.S.A.), the cells were incubated in the dark for 3 h, and then rinsed thoroughly with PBS. Fluorescence was determined by excitation at 540 nm and emission at 590 nm using a SpectraMax Gemni microplate reader (GMI Inc., Albertville, MN, U.S.A.).

Reverse transcription, polymerase chain reaction and real-time PCR

The isolation of total mRNA and reverse transcription–polymerase chain reaction (RT–PCR) were performed as described previously, with slight modifications (Li *et al*., 1998). Briefly, cells were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Total mRNA was extracted and 10 μg of total mRNA was used for RT. Published primers were chosen for PCR amplifications of CYP1A1 (Jaiswal *et al*., 1985), CYP1A2 (Jaiswal *et al*., 1986), and β-actin (Giannone *et al*., 1998). Specific primers for AhR and CYP3A4 were designed as shown in Table 2. A 2 μl RT product was used for PCR amplification in a 20 μl reaction. The annealing temperatures were 49°C for human CYP1A1, 52°C for human CYP1A2, and 61°C for β-actin, AhR, and CYP3A4. The PCR reactions were optimized to ensure that PCR products for each gene were obtained in the linear range of the reaction. Results were quantified on a 1.5% agarose gel and digitalized by Fluorchem (Innotech, San Leandro, CA, U.S.A.).

Commercially available primers for real-time PCR of CYP1A1, CYP1A2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems (Foster City, CA, U.S.A.) as shown in Table 2. Real-time PCR was performed using a TaqMan 7700 sequence detector system in a 96-well optical plate with a final reaction volume of 50 μl. All reactions were prepared with master mix consisting of 10 μl of TaqMan Universal PCR Master Mix, No Amp Erase® UNG (Applied Biosystems, Foster City, CA, U.S.A.), 1 μl 20 × gene expression assay mix, and 100 ng of cDNA. The specific fluorescent probes were labeled at the 5′ end with FAM™. Thermal cycler conditions included an initial setup of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min according to the TaqMan Universal PCR Protocol (Applied Biosystems, Foster City, CA, U.S.A.). Results were analyzed as described previously (Livak & Schmittgen, 2001).

Immunoblotting

Microsome fraction of HepG2 cells was prepared in HEGD buffer (25 mM HEPES pH 7.5, 1 mM EDTA, 10% v v^{-1} glycerol, 1 mM dithiothreitol), as previously described with some modifications (Chen & Bunce, 2003). Briefly, trypsinized cells were rinsed with 1 × PBS and homogenized manually with a glass homogenizer. Lysed cells were centrifuged at 8944 × *g* at 4°C for 15 min. The supernatant was further centrifuged at 105,000 × *g* for 1 h at 4°C. Microsome pellet were stored at −80°C until experiments. Protein concentrations were measured with the Bio-Rad Protein Assay kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA, U.S.A.). A 50 μg portion of protein (5 μg protein for DBA-treated group for CYP1A1 to reduce overloading) and BenchMark™ protein ladder (Invitrogen, Carlsbad, CA, U.S.A.) were electrophoresed in a 10% SDS–polyacrylamide gel, electrotransferred and blotted with CYP1A1 and CYP1A2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The membrane was subsequently visualized by exposing to Kodak X-OMAT film and digitalized by Fluorchem.

AhR-responsive luciferase reporter assay

The pGUDluc4.1 plasmid contains the firefly luciferase reporter gene under control of the mouse Cyp1a1 promoter

Table 2 List of PCR primers

	Sense	*Antisense*
Primers for regular PCR		
CYP1A1	TCTTTCTCTTCCTGGCTATC	CTGTCTCTTCCCTTCACTCT
CYP1A2	CCAACGTCATTGGTGCCATG	GTGATGTCCCGGACACTGTTC
CYP3A4	GCAAAGAGCAACACAGAGCTG	GTGATAGCCAGCACAGGCTG
AhR	CTACAATGAGCTGCGTGTGG	TAGCTCTTCTCCAGGGAGGA
hPXR	GCCCAGTGTCAACGCAGATG	CCCTGATCATCATCCGCTGC
β-Actin	CTACAATGAGCTGCGTGTGG	TAGCTCTTCTCCAGGGAGGA
Catalogue numbers of commercial kits for real-time PCR (Applied Biosystems, Foster City, CA, U.S.A.)		
CYP1A1	Hs00153120_m1	
CYP1A2	Hs00167927_m1	
GAPDH	Hs99999905_m1	

and 1.2 kb of the 5′-flanking sequence (Walsh *et al*., 1996). To monitor the transactivation of endogenous AhR, pGUDluc4.1 and pRL-TK plasmids (Promega, Madison, WI, U.S.A.) containing a Renilla luciferase gene were cotransfected transiently into HepG2 cells as described previously (Roberts *et al*., 2000), with the following modifications. Briefly, 0.5 μg pGUDluc4.1 plasmid and 0.1 μg *Renilla* plasmid were co-transfected into 40% confluent HepG2 cells overnight using lipofectamine (Invitrogen, Carlsbad, CA, U.S.A.). Cells were further treated with various chemicals and milk or formula for 24 h. Whole-cell lysates were collected and luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, U.S.A.). The value of firefly luciferase activity was normalized by the corresponding *Renilla* luciferase value. Fold induction was obtained by dividing the normalized value from a treatment by that of the control group.

PXR-responsive luciferase reporter assay

The CYP3A4-XREM-Luc (XREM: Xenobiotic response element modulator) reporter plasmid (Zhang *et al*., 2001) containing the CYP3A4 distal enhancer region (XREM: −7836/−7208) and the promoter (−362/+53) was used with pRL-TK as a transfection control. To acquire accentuated PXR-mediated responses, we also used the pEF-hPXR expression plasmid (Tirona *et al*., 2003). The HepG2 cells were similarly transfected (see above) using a mixture containing the CYP3A4-XREM-Luc reporter plasmid (1 μg), pRL-TK (0.05 μg), and 0.05 μg of either pEF-hPXR or pEF as a negative PXR control. Cells were further treated with various chemicals and milk or formula for 24 h. Luciferase activity was measured as described above.

Electrophoretic mobility shift assay

The double-stranded oligonucleotides were designed as described previously (Harper *et al*., 1992). The digoxigenin (DIG)-labeled forward strand 5′-GAT CTG GCT CTT CTC ACG CAA CTC CG-3′ was synthesized by BioSource International (Camarillo, CA, U.S.A.) and was annealed with the reverse strand 5′-GAT CCG GAG TTG CGT GAG AAG AGC CA-3′. Cytosol from Hepa1c1c7 cells, a frequently used mouse hepatoma cell line for EMSA (Cuthill *et al*., 1987), was prepared in HEGD buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 10% v v^{-1} glycerol, 1 mM dithiothreitol), as described previously (Harper *et al*., 1992). A 20 μl portion of the cytosol preparation (20 mg ml^{-1}) was incubated with 5 μl of substrates at 4°C overnight in the presence or absence of the specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) against AhR (NCBI accession number: 3041653), which recognizes the dioxin response element (DRE) binding domain of AhR. The samples were further incubated at 24°C for 2 h. An aliquot of the cytosol containing 150 μg of protein was incubated with 150 ng of poly(dI-dC) (Roche Applied Science, Montreal, Quebec, Canada) in HEGD buffer containing 40 mM NaCl for 15 min and then with DIG-labeled probe for additional 15 min. Samples were then electrophoresed on a 6% DNA retardation gel (Invitrogen, Carlsbad, CA, U.S.A.) and transferred on to a nylon membrane (Roche Applied Science, Montreal, Quebec, Canada). The membrane was further blotted with anti-DIG antibody (Roche Applied Science, Montreal, Quebec, Canada) at 24°C for 30 min. The specific binding of AhR and DNA complexes was visualized by chemiluminescent detection using CSPD® solution (Roche Applied Science, Montreal, Quebec, Canada).

As most known AhR substrates are hydrophobic (Denison & Nagy, 2003), the lipid fraction was extracted from the formulas using the modified Mojonnier's method (Barbano *et al*., 1988). Briefly, 10 ml of the milk samples was alkalized with NH_4OH, mixed with 95% ethanol, ethylether, and petroleum ether, and vigorously shaken for 2 min. After centrifugation at 600 r.p.m. for 1 min, the upper organic layer was transferred to a tube, and reduced to 500 μl with nitrogen gas stream at room temperature. An aliquot (1% v v^{-1}) of the final lipid fraction was reconstituted in the same volume of DMSO for the assay.

Calculation of TCDD toxic equivalents

The relative potency of the AhR agonist activity of the cow milk-based formula was estimated, and expressed as the TCDD toxic equivalents (TEQs) as described previously with some modifications (Bovee *et al*., 1998; Koppen *et al*., 2001). The HepG2 cells transfected with the AhR-responsive luciferase reporter were exposed to human milk, formula, or TCDD at different concentrations for 24 h. The standard curve of TCDD dose–response for the AhR-reactive luciferase expression was established for the range between 1 pM and 10 nM by fitting the data to a sigmoid curve of the Hill equation using the least squares method. The derived equation was used to convert the luciferase activity elicited by formula or milk to the TCDD equivalent values (i.e., TCDD toxic equivalents: TEQs).

Statistical analysis

Comparative results of multiple groups were tested with analysis of variance (ANOVA), followed by Duncan multiple-range test for *post hoc* multiple comparisons among means. Data were expressed as mean ± standard deviation. *P*-value of less than 0.05 was considered significant.

Results

Effects of human milk and cow milk-based infant formula on CYP1A1 and CYP1A2 mRNA and protein expression

The AlamarBlue™ assay showed that the viability of HepG2 cells was not affected by milk or formula treatment at 20% concentration (v v^{-1}) up to 72 h (data not shown), validating the 24-h incubation protocol. Protein sediments of milk or formula precipitate on the cell surface in our experimental condition. Although unlikely, this may cause mechanical stress. Shearing force imposed on cells may cause transient AhR activation that subsides by 15 h (Mufti *et al*., 1995). Therefore, we conducted the same experiments with the HepG2 cells grown on permeable membranes and exposed them to milk or formula treatment from beneath. Results were similar to those with cells on plates, validating the experimental protocol (data not shown). Based on these findings, all remaining experiments were performed with cells grown on

culture plates at 5–20% $v\,v^{-1}$ concentrations of milk or formula for 24 h.

Regular RT–PCR demonstrated that 24 h treatments of infant formula at 20% concentration significantly increased the expressions of both CYP1A1 and CYP1A2 mRNA (Figure 1a), which were further quantified using real-time PCR (Figure 1b). The level of CYP1A mRNA induction by formula is approximately 1% of that by 10 μM DBA. The protein levels of CYP1A1 and CYP1A2 were then measured by Western blotting. As shown in Figure 1c, CYP1A1 and CYP1A2 protein expressions were increased significantly after 24 h incubation with formula. In contrast, human milk did not alter CYP1A1 or CYP1A2 expression at mRNA or protein levels.

Effects of human milk and cow milk-based formula on AhR activation

Involvement of the AhR pathway in the observed CYP1A induction was verified using the luciferase-based reporter responsive to activated AhR. Expression levels of AhR itself were not altered by the treatments of formula or human milk (data not shown). HepG2 cells were transiently transfected with pGUDluc4.1 plasmid and pRL-TK plasmid overnight. The cells were then treated with milk treatments for 24 h. As shown in Figure 2a, the firefly luciferase activity was significantly increased by two- to six-fold following the formula treatments (formula 1 (F1): $P<0.05$; formula 2 (F2): $P<0.05$). To examine if the findings are due to manufacturing processes specific to liquid formulas, we tested powder forms of these cow milk-based formulas. The powder forms of F1 (FP1) and F2 (FP2) also induced the luciferase activity significantly (FP1: $P<0.05$; FP2: $P<0.05$), indicating that both liquid and powder formulations have similar effects on AhR activation. In contrast, human milk did not enhance AhR activity (Figure 2a). Formula-induced AhR activation was further confirmed in dose-dependency experiments

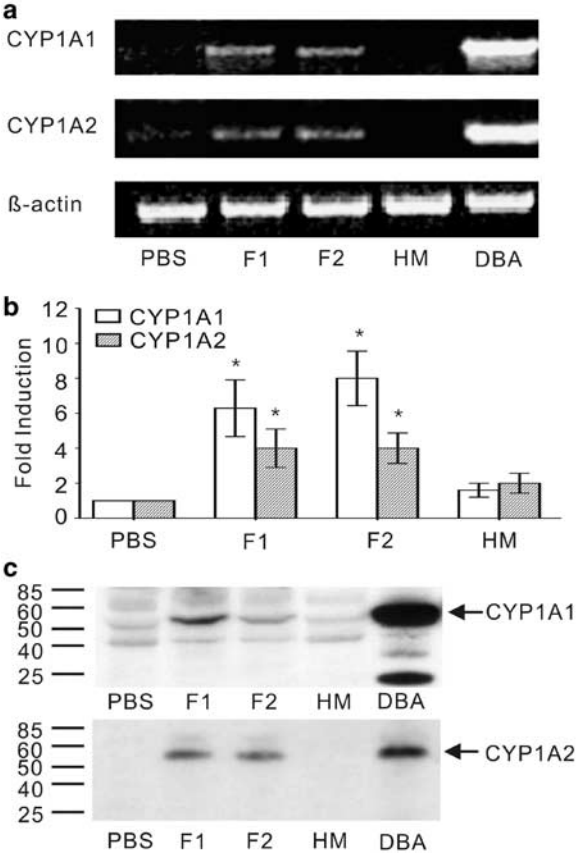

Figure 1 Effects of cow milk-based formula and human milk on the expression of CYP1A. (a) RT–PCR quantification of CYP1A1 and CYP1A2 mRNA expression following 24 h treatment of 20% human milk, 20% cow milk-based F1 and F2, and controls. (b) The real-time PCR quantification of CYP1A1 and CYP1A2 mRNA expression following the same treatment as described in (a). All values are expressed as mean $\pm$ standard deviation of at least six independent experiments. Significant differences among the groups were determined as described in Methods. $^*P<0.05$: significantly higher than the PBS-treated group. (c) Immunoblot of CYP1A1 and CYP1A2 protein after 24 h treatment with formula or human milk. HepG2 cells were treated as described in (a). A representative of at least three independent experiments is shown.

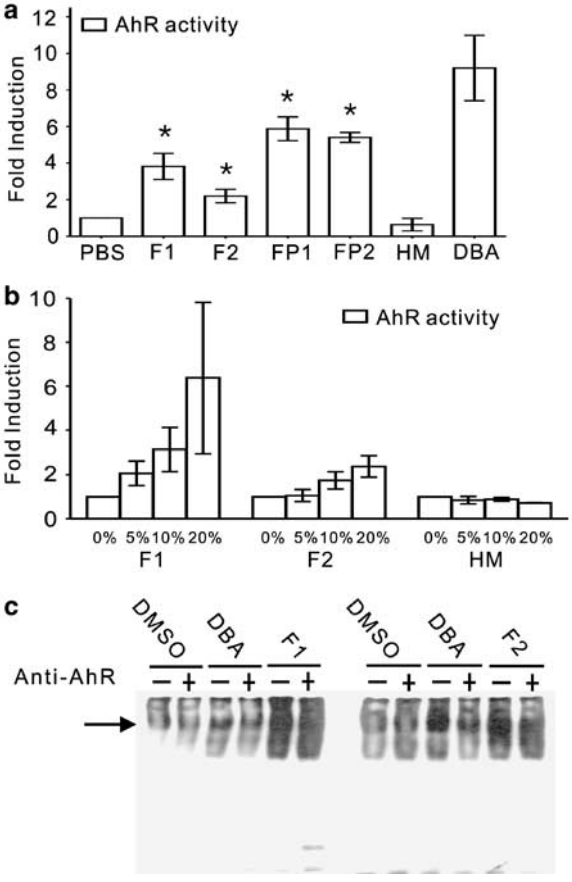

Figure 2 Effects of human milk and formula on the AhR activity in HepG2 cells. (a) AhR activation by milk treatments. All values are expressed as mean $\pm$ standard deviation of at least three separate determinations. Significant differences among the groups were determined as described in Methods. $^*P<0.05$: significantly higher than the PBS-treated group. (b) Dose response of AhR activity to milk treatment. HepG2 cells were transfected and exposed to different concentrations of formulas and human milk for 24 h. The data are expressed as mean $\pm$ standard deviation of at least three separate determinations. (c) EMSA. Cytosolic fraction of Hepa1c1c7 cells ($\sim 20\,\mu g\,\mu l^{-1}$) was treated with the lipid fraction of formula, DMSO, or DBA as described in Methods. An arrow indicates the substrate-inducible AhR–DRE complex. A representative of three separate determinations is shown.

(Figure 2b; F1: $r^2=0.64$, $P<0.05$; F2: $r^2=0.77$, $P<0.05$). Electrophoretic mobility shift assay (EMSA) indicated that the incubation of the cytosol of Hepa1c1c7 cells with the lipid extract of the formulas resulted in formation of AhR–DRE complexes (Figure 2c). This finding confirms the presence of the AhR ligand(s) in the infant formulas that does not require activation by intact cells.

We next examined if antagonizing AhR activity could abolish the CYP1A induction by cow milk-based formula. 3,4-DMF is a potent AhR antagonist (Lee & Safe, 2000). HepG2 cells were pre-exposed to 10 μM 3,4-DMF for 3 h to avoid potential interference by simultaneous formula treatments. The cells were then treated with formula or with controls for another 24 h. As shown in Figure 3a, the inductions of AhR activity by formula were completely blocked in the 3,4-DMF pretreated groups (F1: $P<0.05$; F2: $P<0.05$). RT–PCR further demonstrated that 3,4-DMF significantly inhibited the induction of CYP1A1 mRNA (F1: $P<0.05$; F2: $P<0.05$) and CYP1A2 mRNA (F1: $P<0.05$; F2: $P<0.05$) by formula (Figure 3b). These data demonstrate that activation of the AhR-mediated pathway is the primary mechanism for the induction of CYP1A by formula.

TCDD toxic equivalents of formula and human milk

The lower quantitation limit for a response of the HepG2 AhR activity to TCDD was 10 pM TCDD. The potency equivalency concentrations (PECs) to TCDD of the cow milk-based formulas (F1: PEC = 19.7 ± 3.3 pM; F2: PEC = 18.7 ± 3.7 pM) were significantly higher than the quantitation limit ($n=4$; $P<0.05$). The TCDD TEQs were further calculated from the PECs (F1: TEQ = 6.4 ± 1.1 pg Teq g^{-1} wet weight; F2: 6.3 ± 2.0 pg Teq g^{-1} wet weight). Assuming a 4 kg infant consuming 800 ml day^{-1} of formula, we estimated the infant's AhR agonist intake through the cow milk-based formulas to be greater than 1000 pg TEQ kg^{-1} day^{-1} (F1: 1270 ± 210 pg Teq kg^{-1} day^{-1}; F2: 1260 ± 235 pg Teq kg^{-1} day^{-1}). In contrast, the PEC of human milk was less than the quantitation limit (10 pM TCDD equivalent).

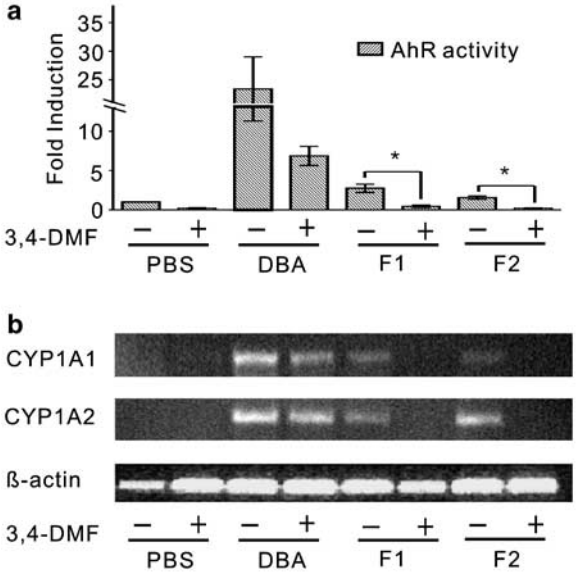

Figure 3 Inhibitory effects of DMF on AhR activity and CYP1A expression in HepG2 cells. (a) Inhibition of formula-induced AhR activation by 3,4-DMF. The transfected HepG2 cells were treated with or without 10 μM 3,4-DMF for 3 h, followed by further incubation with F1, F2, PBS, or 50 nM DBA for 24 h. All values are expressed as mean ± standard deviation of at least three separate determinations. Significant differences were determined as described in Methods. *$P<0.05$: significantly higher than the PBS-treated group. (b) 3,4-DMF inhibition of the formula-induced CYP1A mRNA expressions. The HepG2 cells were treated as described in (a) without transfection and mRNA expressions were quantified by RT–PCR.

Induction of whey- and soy protein-based formula on CYP1A expression via *AhR pathway*

To evaluate the effects of other types of infant formulas on CYP1A1/2 expression, we similarly treated HepG2 cells with 20% whey-based (formula 3 (F3)) or soy protein-based formulas (formula 4 (F4) and formula 5 (F5)) for 24 h. Both CYP1A1 and CYP1A2 mRNA were induced significantly (Figure 4a). Consistently, CYP1A1 and CYP1A2 protein levels were increased after the same treatments (Figure 4b), and the AhR was activated significantly (Figure 4c).

Inhibition of DBA-induced AhR activation by formula and human milk

The data so far indicate that formula, but no human milk, has AhR agonistic activity. We then examined if they possess any antagonistic activity against AhR. Representative brands of formulas were chosen from cow milk-based (F1) and soy-based formula (F4) for the experiments. The HepG2 cells transfected with the luciferase reporter were cultured in the medium containing 0.5 μM DBA for 6 h, and then the media were removed, the cells were rinsed, and the media were changed to those containing F1 (cow milk-based), F4 (soy-based), human milk, or PBS at 10–20% concentrations. The cells were cultured for another 18 h, and then the luciferase activity was measured. As shown in Figure 4d, infant formula significantly inhibited the DBA-induced AhR activation (10% F1: $P<0.05$; 20% F1: $P<0.05$; 10% F2: $P<0.05$; 20% F2: $P<0.05$). Interestingly, despite the lack of overall intrinsic agonistic function, human milk clearly inhibited the DBA-induced AhR activation (10% human milk: $P<0.05$; 20% human milk: $P<0.05$).

Effects of infant formulas and human milk on CYP3A expression

The HepG2 cells were treated with formulas or human milk (20% v v^{-1}), or 10 μM dexamethasone for 24 h. CYP3A4 mRNA expression measured by the semiquantitative RT–PCR was significantly, but only mildly, increased by human milk and most infant formula treatments over PBS control (Figure 5). This finding prompted us to characterize the effects of milk and formula treatments on CYP3A4 using the luciferase-based reporter construct (CYP3A4-XREM-Luc reporter) under control of the human CYP3A4 promoter and an upstream XREM, which was reported previously elsewhere (Zhang *et al*., 2001).

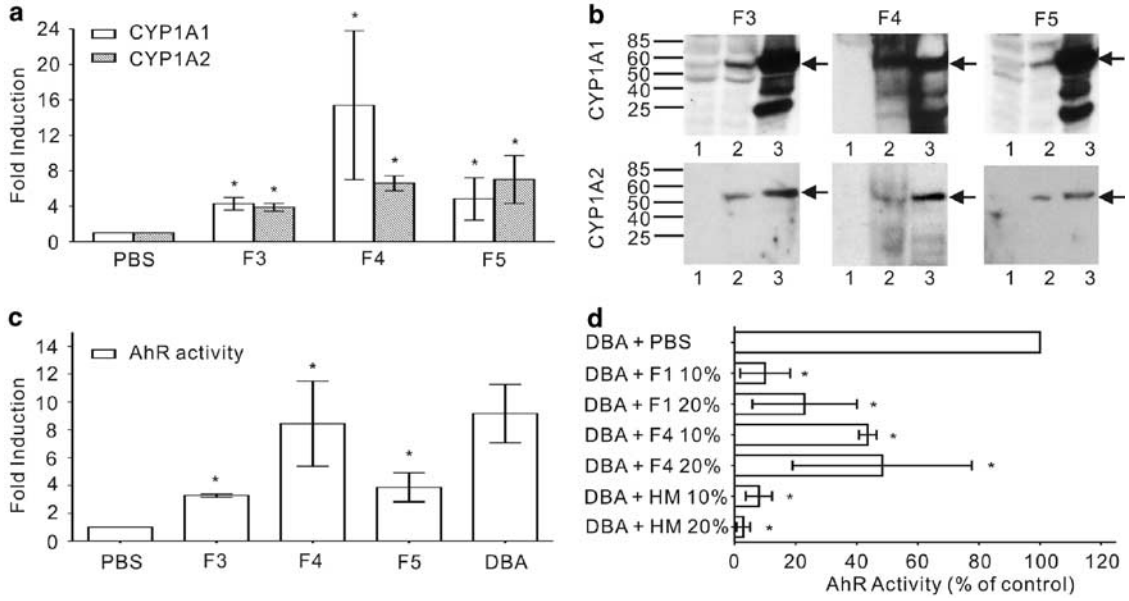

Figure 4 Induction of HepG2 CYP1A1 and CYP1A2 by whey- and soy protein-based formula *via* an AhR-mediated pathway. (a) Real-time PCR quantification of CYP1A1 and CYP1A2 mRNA expression in HepG2 cells following 24 h treatment with whey-based formula (F3) or soy-based formulas (F4 and F5). (b) Western blot of CYP1A1 and CYP1A2 protein following the treatments described in (a). Lane 1: PBS; lane 2: formula treatment; lane 3: DBA. (c) Activation of AhR following the treatments described in (a). (d) Inhibitory effects of formulas and human milk on DBA-induced AhR activation. All values are expressed as mean ± standard deviation. Significant differences among the groups were determined as described in Methods. $*P<0.05$: significant difference from the PBS-treated control group. Data shown were analyzed from at least three independent experiments.

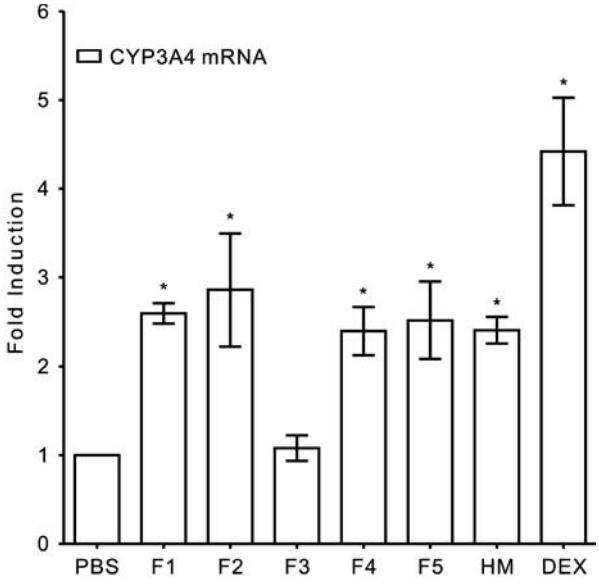

Figure 5 Effects of infant formula and human milk on the mRNA expression of CYP3A4 in HepG2 cells. HepG2 cells were treated with 20% human milk, cow milk-based formulas (F1 and F2), whey-based formula (F3), soy-based formulas (F4 and F5), or controls for 24 h. Total mRNA was isolated and analyzed by RT–PCR for the expression of CYP3A4 and β-actin. All values are expressed as mean ± standard deviation of at least three separate determinations. Significant differences among the groups were determined as described in Methods. $*P<0.05$: significantly higher than the PBS-treated group.

Effects of human milk and formula treatment on CYP3A4-XREM-Luc reporter activity

The HepG2 cells were cotransfected with a CYP3A4-XREM-Luc reporter construct and either an hPXR expression plasmid (pEF-hPXR) or an empty plasmid (pEF). In the pEF group, no significant activation of endogenous PXR was found in either human milk or formula treatment groups with the exception of F5. With pEF-hPXR cotransfection, 10 μM rifampicin resulted in a 50-fold increase in the reporter activity over PBS control, which was about 10-fold higher than in the cells transfected with pEF empty vector alone ($P<0.05$; Figure 6), indicating its potent induction of the PXR–CYP3A4 pathway. Similarly, 100 μM dexamethasone showed approximately three-fold increase in the luciferase activity, from 1.8-fold induction with pEF to 5.1-fold induction with pEF-PXR ($P<0.05$). However, most formula treatments other than F5 showed no significant increase in the luciferase activity in the presence of pEF-hPXR, compared to the ones transfected only with pEF. Powder forms of the cow milk-based formulas showed no increase either (data not shown). Human milk also showed no significant PXR activation effect. These data suggest that the mild induction of CYP3A4 mRNA by infant formula and human milk is not mainly mediated *via* the PXR pathway.

Discussion

Our results demonstrate for the first time that cow milk-based infant formula but not human milk increases CYP1A expression *in vitro*, and that the effects are mediated mainly by AhR. Moreover, the effects are also shared with whey- and soy-based infant formulas. It provides a clue to a clinical observation by Le Guebbec & Billon (1987), who discovered that caffeine half-life was significantly shorter in formula-fed infants than that in breastfed infants, which was confirmed recently (Blake *et al*., 2004). In the study by Le Guebbec & Billon (1987), it was hypothesized that some components in

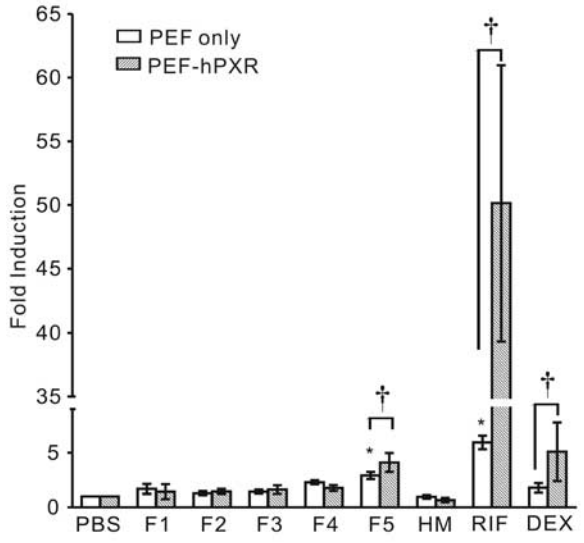

Figure 6 Effects of human milk and formula treatments on CYP3A4-XREM-Luc reporter activity with or without cotransfection of hPXR expression plasmid. HepG2 cells transfected with the CYP3A4 reporter construct with cotransfection of hPXR expression plasmid or with an empty plasmid were treated with 20% human milk or formulas for 24 h. Data are expressed as mean ± standard deviation of at least three independent measurements. Significant differences among the groups were determined as described in Methods. $^{*}P<0.05$: significantly different from the control group; †significantly different between hPXR and the empty vector cotransfected groups.

human milk inhibit the postnatal maturation of caffeine disposition by hepatic CYP enzymes. Although this notion remains to be tested, our results provide an alternative explanation that infant formula may increase the metabolism of caffeine by inducing CYP1A expression. Because of the extremely low constitutive expression levels of CYP1A in neonates (Hokkola *et al*., 1994; Sonnier & Cresteil, 1998), even a mild induction may bear significant pharmacokinetic consequences.

Interestingly, our results showed that none of the human milk samples caused induction of CYP1A1/2 expression or the activation of AhR, which indicates that the levels of environmental pollutants, even if higher than those in formula as reported, may not be sufficient to cause a significant activation of AhR *in vitro*. This is consistent with the TEQ value in Canadian human milk collected in 2002, which is 9.2 pg TEQ g^{-1} lipid (Chen & Bunce, 2003). Namely, assuming that milk contains about 3.5 g lipid 100 g^{-1} wet weight, 10 pg TEQ g^{-1} lipid may be translated to approximately 2.9 pg TEQ g^{-1} milk (about 10 pM). This is in the vicinity of the lower quantitation limit of our TEQ assay based on the AhR-responsive luciferase reporter. Although human milk did not induce or significantly inhibit the baseline activity of AhR (Figure 2a and b), the inhibition of the DBA-induced AhR activation by human milk (Figure 4d) suggests the possibility that human milk contains AhR antagonist(s). Women's diet may be the likely source, but it necessitates further studies.

In this study, we used the minimally restrictive inclusion criteria to collect human milk samples, which would allow us to capture interindividual variations stemming from life style differences, if any. Because about one in 10 pregnant women may be smokers (Mathews, 2001), we speculate in our study that the milk samples were donated mostly by nonsmoking women. Even if some of our human milk samples were from smokers, none showed AhR activation in our *in vitro* cell model. Whether human milk from smokers activates AhR awaits further study.

Our data show that the formula overall functions as an AhR partial agonist and human milk as an AhR antagonist. It is most likely that they contain mixtures of individual antagonists and agonists. Identity of these AhR ligands is unknown at present. Most of the studies that compared the levels of the environmental pollutants between human milk and formula have so far measured individual compounds by using highly specific analytical methods (Schecter *et al*., 1994; Abraham *et al*., 1996). This approach is specific and accurate, but does not address other AhR agonists or antagonists that were not the measurement targets. For example, even if the level of dioxin-like compounds is low in infant formulas, AhR-activating plant flavonoids that cows consume may be present in cow milk-based formula. Many plant extracts have been shown to activate AhR (Jeuken *et al*., 2003). Heating processes of organic materials may produce CYP1A inducers, such as PAH and aromatic amines (Sinha *et al*., 1994; Halperin *et al*., 1995). Similarly, heating procedures during manufacturing processes for infant formula may create an AhR ligand(s).

The exposure of infants to formula with partial agonistic effect on AhR is of clinical interest, as it may increase infantile hepatic CYP1A expression and accelerate the biotransformation of medications that are CYP1A substrates. The exact role of CYP1A in mutagenesis is currently under debate. By converting certain procarcinogens into their ultimate carcinogenic metabolites, CYP1A may promote mutagenesis (Mimura & Fujii-Kuriyama, 2003). On the other hand, concomitant induction of different CYP1A subfamilies and conjugating enzymes may have a chemopreventive effect by eliminating the toxic chemicals and their reactive metabolites (Vang *et al*., 2001).

In contrast to CYP1A, formula induction of CYP3A4 mRNA was only two- to three-fold. Increase in CYP3A4 protein expression could not be confirmed in our study because of the predominant CYP3A7 expression in HepG2 cells and crossreactivity of antibodies (data not shown). Further experiments suggested that PXR pathway appears to be dispensable in this phenomenon, because the presence or absence of the PXR expression plasmid made little difference in their responses, except for a soy-based formula. As CYP3A4 serves only as a minor catalyst of caffeine biotransformation, acting mainly at relatively high substrate concentrations (Ha *et al*., 1996), it would require a dramatic induction to be responsible for the three-fold higher elimination of caffeine observed in infants fed with formula. Although the effects of formula on CYP3A4 do not explain the differential influences of cow milk-based formula and human milk demonstrated *in vivo*, evaluating the effect of milk on its expression in the future may be of clinical interest, as CYP3A4 makes a pivotal contribution to metabolism of numerous medications (Wrighton *et al*., 2000).

We conclude that formula, but not human milk, induces CYP1As *in vitro via* the AhR-mediated pathway, suggesting the presence of an AhR agonist(s) in infant formula. On the other hand, overall human milk lacks AhR agonistic activity, and rather exerts antagonistic effects on the AhR pathway. Our findings provide insight into the mechanism behind the clinical phenomenon of accelerated caffeine elimination in

formula-fed infants. Future study on the identification of the active constituents in the infant formula and human milk will be essential for the elucidation of the mechanism behind this clinical phenomenon, which may require a combination of biochemical and clinical approaches.

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References

ABRAHAM, K., KNOLL, A., ENDE, M., PAPKE, O. & HELGE, H. (1996). Intake, fecal excretion, and body burden of polychlorinated dibenzo-*p*-dioxins and dibenzofurans in breast-fed and formula-fed infants. *Pediatr. Res.,* **40,** 671–679.

AMERICAN ACADEMY OF PEDIATRICS WORK GROUP ON BREASTFEEDING (1997). Breastfeeding and the use of human milk. *Pediatrics,* **100,** 1035–1039.

AMERICAN ACADEMY OF PEDIATRICS. COMMITTEE ON NUTRITION (1998). Soy protein-based formulas: recommendations for use in infant feeding. *Pediatrics,* **101,** 148–153.

BARBANO, D.M., CLARK, J.L. & DUNHAM, C.E. (1988). Comparison of Babcock and ether extraction methods for determination of fat content of milk: collaborative study. *J. Assoc. Off. Anal. Chem.,* **71,** 898–914.

BLAKE, M.J., ABDEL-RAHMAN, S.M. & KEARNS, G.L. (2004). Effect of diet on caffeine elimination in healthy infants. *Clin. Pharmacol. Ther.,* **75,** 50 [abstract].

BOVEE, T.F., HOOGENBOOM, L.A., HAMERS, A.R., TRAAG, W.A., ZUIDEMA, T., AARTS, J.M., BROUWER, A. & KUIPER, H.A. (1998). Validation and use of the CALUX-bioassay for the determination of dioxins and PCBs in bovine milk. *Food Addit. Contam.,* **15,** 863–875.

CHEN, G. & BUNCE, N.J. (2003). Polybrominated diphenyl ethers as Ah receptor agonists and antagonists. *Toxicol. Sci.,* **76,** 310–320.

COMER, A.M., PERRY, C.M. & FIGGITT, D.P. (2001). Caffeine citrate: a review of its use in apnoea of prematurity. *Paediatr. Drugs,* **3,** 61–79.

CUTHILL, S., POELLINGER, L. & GUSTAFSSON, J.A. (1987). The receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the mouse hepatoma cell line Hepa 1c1c7. A comparison with the glucocorticoid receptor and the mouse and rat hepatic dioxin receptors. *J. Biol. Chem.,* **262,** 3477–3481.

DENISON, M.S. & NAGY, S.R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol.,* **43,** 309–334.

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. (1999). Effects of a chargrilled meat diet on expression of CYP3A, CYP1A, and P-glycoprotein levels in healthy volunteers. *Gastroenterology,* **117,** 89–98.

GIANNONE, J.V., LI, W., PROBST, M. & OKEY, A.B. (1998). Prolonged depletion of AH receptor without alteration of receptor mRNA levels after treatment of cells in culture with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biochem. Pharmacol.,* **55,** 489–497.

HA, H.R., CHEN, J., FREIBURGHAUS, A.U. & FOLLATH, F. (1995). Metabolism of theophylline by cDNA-expressed human cytochromes P50. *Br. J. Clin. Pharmacol.,* **39,** 321–326.

HA, H.R., CHEN, J., KRAHENBUHL, S. & FOLLATH, F. (1996). Biotransformation of caffeine by cDNA-expressed human cytochromes P-450. *Eur. J. Clin. Pharmacol.,* **49,** 309–315.

HALPERIN, W., KALOW, W., SWEENEY, M.H., TANG, B.K., FINGERHUT, M., TIMPKINS, B. & WILLE, K. (1995). Induction of P-450 in workers exposed to dioxin. *Occup. Environ. Med.,* **52,** 86–91.

HARPER, P., GIANNONE, J., OKEY, A.B. & DENISON, M.S. (1992). *In vitro* transformation of the human Ah Receptor and its binding to a dioxin response element. *Mol. Pharmacol.,* **42,** 603–612.

HOKKOLA, J., PASANEN, M., PURKUNEN, R., SAARIKOSKI, S., PELKONEN, O., MAENPAA, J., RANE, A. & RAUNIO, H. (1994). Expression of xenobiotic-metabolizing cytochrome P450 forms in human adult and fetal liver. *Biochem. Pharmacol.,* **48,** 59–64.

HOOPER, K. & MCDONALD, T.A. (2000). The PBDEs: an emerging environmental challenge and another reason for breast-milk monitoring programs. *Environ. Health Perspect.,* **108,** 387–392.

IOANNIDES, C. & PARKE, D.V. (1990). The cytochrome P450 I gene family of microsomal hemoproteins and their role in the metabolic activation of chemicals. *Drug Metab. Rev.,* **22,** 1–85.

JAISWAL, A.K., GONZALEZ, F.J. & NEBERT, D.W. (1985). Human dioxin-inducible cytochrome P1-450: complementary DNA and amino acid sequence. *Science,* **228,** 80–83.

JAISWAL, A.K., NEBERT, D.W. & GONZALEZ, F.J. (1986). Human P3(450): cDNA and complete amino acid sequence. *Nucleic Acids Res.,* **14,** 6773–6774.

JEUKEN, A., KESER, B.J., KHAN, E., BROUWER, A., KOEMAN, J. & DENISON, M.S. (2003). Activation of the Ah receptor by extracts of dietary herbal supplements, vegetables, and fruits. *J. Agric. Food Chem.,* **51,** 5478–5487.

KARMAUS, W., DEKONING, E.P., KRUSE, H., WITTEN, J. & OSIUS, N. (2001). Early childhood determinants of organochlorine concentrations in school-aged children. *Pediatr. Res.,* **50,** 331–336.

KOPPEN, G., COVACI, A., VAN CLEUVENBERGEN, R., SCHEPENS, P., WINNEKE, G., NELEN, V. & SCHOETERS, G. (2001). Comparison of CALUX-TEQ values with PCB and PCDD/F measurements in human serum of the Flanders Environmental and Health Study (FLEHS). *Toxicol. Lett.,* **123,** 59–67.

LE, GUENNEC J.C. & BILLON, B. (1987). Delay in caffeine elimination in breast-fed infants. *Pediatrics,* **79,** 264–268.

LEE, J.E. & SAFE, S. (2000). 3′,4′-Dimethoxyflavone as an aryl hydrocarbon receptor antagonist in human breast cancer cells. *Toxicol. Sci.,* **58,** 235–242.

LI, W., HARPER, P.A., TANG, B.K. & OKEY, A.B. (1998). Regulation of cytochrome P450 enzymes by aryl hydrocarbon receptor in human cells: CYP1A2 expression in the LS180 colon carcinoma cell line after treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or 3-methylcholanthrene. *Biochem. Pharmacol.,* **56,** 599–612.

LIVAK, K. & SCHMITTGEN, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta *C*(*T*)) method. *Methods,* **25,** 402–408.

MACHALA, M., VONDRACEK, J., BLAHA, L., CIGANEK, M. & NECA, J.V. (2001). Aryl hydrocarbon receptor-mediated activity of mutagenic polycyclic aromatic hydrocarbons determined using *in vitro* reporter gene assay. *Mutat. Res.,* **497,** 49–62.

MATHEWS, T. (2001). Smoking during pregnancy in 1990s. *Natl. Vital Stat. Rep.,* **49,** 1–14.

MERCHANT, M., WANG, X., KAMPS, C., ROSENGREN, R., MORRISON, V. & SAFE, S. (1992). Mechanism of benzo[a]pyrene-induced Cyp1a-1 gene expression in mouse Hepa 1c1c7 cells: role of the nuclear 6 s and 4 s proteins. *Arch. Biochem. Biophys.,* **292,** 250–257.

MIMURA, J. & FUJII-KURIYAMA, Y. (2003). Functional role of AhR in the expression of toxic effects by TCDD. *Biochim. Biophys. Acta,* **1619,** 263–268.

MUFTI, N.A., BLECKWENN, N.A., BABISH, J.G. & SHULER, M.L. (1995). Possible involvement of the Ah receptor in the induction of cytochrome P-450IA1 under conditions of hydrodynamic shear in microcarrier-attached hepatoma cell lines. *Biochem. Biophys. Res. Commun.,* **208,** 144–152.

NEBERT, D.W., DALTON, T.P., OKEY, A.B. & GONZALEZ, F.J. (2004). Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J. Biol. Chem.,* **279,** 23847–23850.

NIELSEN-KUDSK, J.E., BUHL, J.S. & JOHANNESSEN, A.C. (1990). Verapamil-induced inhibition of theophylline elimination in healthy humans. *Pharmacol. Toxicol.,* **66,** 101–103.

PARSONS, W.D. & NEIMS, A.H. (1978). Effect of smoking on caffeine clearance. *Clin. Pharmacol. Ther.,* **24,** 40–45.

ROBERTS, E.A., HARPER, P.A., WONG, J.M., WANG, Y. & YANG, S. (2000). Failure of Ah receptor to mediate induction of cytochromes P450 in the CYP1 family in the human hepatoma line SK-Hep-1. *Arch. Biochem. Biophys.,* **384,** 190–198.

SCHECTER, A., STARTIN, J., WRIGHT, C., KELLY, M., PAPKE, O., LIS, A., BALL, M. & OLSON, J.R. (1994). Congener-specific levels of dioxins and dibenzofurans in US food and estimated daily dioxin toxic equivalent intake. *Environ. Health Perspect.,* **102,** 962–966.

SINHA, R., ROTHMAN, N., BROWN, E.D., MARK, S.D., HOOVER, R.N., CAPORASO, N.E., LEVANDER, O.A., KNIZE, M.G., LANG, N.P. & KADLUBAR, F.F. (1994). Pan-fried meat containing high levels of heterocyclic aromatic amines but low levels of polycyclic aromatic hydrocarbons induces cytochrome P4501A2 activity in humans. *Cancer Res.,* **54,** 6154–6159.

SOMOGYI, A. & BECK, H. (1993). Nurturing and breast-feeding: exposure to chemicals in breast milk. *Environ. Health Perspect.,* **101** (Suppl. 2), 45–52.

SONNIER, M. & CRESTEIL, T. (1998). Delayed ontogenesis of CYP1A2 in the human liver. *Eur. J. Biochem.,* **251,** 893–898.

SUMIDA, A., YAMAMOTO, I., ZHOU, Q., MORISAKI, T. & AZUMA, J. (1999). Evaluation of induction of CYP3A mRNA using the HepG2 cell line and reverse transcription–PCR. *Biol. Pharm. Bull.,* **22,** 61–65.

TIRONA, R.G., LEE, W., LEAKE, B.F., LAN, L., CLINE, C.B., LAMBA, V., PARVIZ, F., DUNCAN, S.A., INOUE, Y., GONZALEZ, F.J., SCHUETZ, E. & KIM, R.B. (2003). The orphan nuclear receptor HNF4 determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat. Med.,* **9,** 220–224.

U.S. DEPARTMENT OF AGRICULTURE AND AGRICULTURAL RESEARCH SERVICE (2004). USDA Nutrient Database for Standard Reference, Release 17. Nutrient Data Laboratory Home Page. http://www.nal.usda.gov/fnic/foodcomp.

VANG, O., FRANDSEN, H., HANSEN, K.T., SORENSEN, J.N., SORENSEN, H. & ERSEN, O. (2001). Biochemical effects of dietary intakes of different broccoli samples. I. Differential modulation of cytochrome P-450 activities in rat liver, kidney, and colon. *Metabolism,* **50,** 1123–1129.

WALLE, U.K., WALLE, T., COWART, T.D., CONRADI, E.C. & GAFFNEY, T.E. (1987). Selective induction of propranolol metabolism by smoking: additional effects on renal clearance of metabolites. *J. Pharmacol. Exp. Ther.,* **241,** 928–933.

WALSH, A.A., TULLIS, K., RICE, R.H. & DENISON, M.S. (1996). Identification of a novel *cis*-acting negative regulatory element affecting expression of the CYP1A1 gene in rat epidermal cells. *J. Biol. Chem.,* **271,** 22746–22753.

WRIGHTON, S.A., SCHUETZ, E.G., THUMMEL, K.E., SHEN, D.D., KORZEKWA, K.R. & WATKINS, P.B. (2000). The human CYP3A subfamily: practical considerations. *Drug Metab. Rev.,* **32,** 339–361.

ZHANG, J., KUEHL, P., GREEN, E., TOUCHMAN, J.W., WATKINS, P.B., DALY A HALL, S.D., MAUREL, P., RELLING, M., BRIMER, C., YASUDA, K., WRIGHTON, S.A., HANCOCK, M., KIM, R.B., STROM, S., THUMMEL, K., RUSSELL, C.G., HUDSON, J.R., SCHUETZ, E.G. & BOGUSKI, M.S. (2001). The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. *Pharmacogenetics,* **11,** 555–572.

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