Diets Containing Soy Protein Isolate Increase Hepatic CYP3A Expression and Inducibility in Weanling Male Rats Exposed during Early Development[1,2]

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ABSTRACT Hepatic CYP3A enzymes were studied in weanling male Sprague-Dawley rats exposed to diets from gestational d 4 in which the sole protein source was either casein (CAS) or soy protein isolate (SPI). At age 25 d, rats were gavaged with corn oil or one of the CYP3A inducers, dexamethasone (DEX) and clotrimazole (CLT), at a dose of 50 mg/kg. Little CYP3A1 (CYP3A23), CYP3A2, or CYP3A9 mRNA was observed in CAS-fed weanling rats but CYP3A18 mRNA was readily detectable in Northern blots. In contrast, consumption of SPI without inducer treatment resulted in the expression of CYP3A1 (CYP3A23), and CYP3A2 mRNAs, expression of CYP3A apoprotein in hepatic microsomes, and a 2-fold greater turnover of the CYP3A substrate midazolam ($P < 0.05$). DEX induced CYP3A1, CYP3A2, and CYP3A9 ($P < 0.05$), but not CYP3A18 mRNA expression in rats fed both diets. Hepatic CYP3A apoprotein expression and midazolam 4-hydroxylation in SPI-fed rats was greater than that of CAS-fed rats after DEX treatment ($P < 0.05$). CLT also induced CYP3A2 mRNA 2-fold in rats fed both diets but CYP3A apoprotein expression in microsomes from SPI-fed CLT rats was double that of CLT-treated rats fed CAS ($P < 0.05$). The elevation of CYP3A apoprotein due to SPI and the CYP3A apoprotein induction by DEX and CLT treatment yielded no significant diet × inducer interaction. Analysis of heterologous nuclear RNA expression by RT-PCR using intron-specific primers for CYP3A1 revealed a 14-fold increase in RNA transcription in CAS-fed rats after treatment with DEX ($P < 0.05$) but no increase in rats fed SPI compared with rats fed CAS even though CYP3A1 mRNA and CYP3A apoprotein were significantly elevated. These data demonstrate that exposure to SPI during early development can increase CYP3A expression via post-transcriptional mechanisms and suggest that early soy consumption has potential effects on the metabolism of a wide variety of CYP3A substrates. J. Nutr. 134: 3270–3276, 2004.

KEY WORDS: • *soy protein isolate* • *CYP3A* • *induction* • *rats* • *development*

The CYP3A enzymes are the major phase I enzymes expressed in the adult human liver, accounting for between 30 and 60% of total spectrally determined cytochrome P_{450} (CYP)[4] content (1,2). They are also the major CYPs found in the livers of human fetuses and neonates (3). CYP3As are rate limiting in the oxidative metabolism of and may affect clearance of the majority of clinically utilized medications including many used in pediatric medicine (4–6). In addition, CYP3As play an important endogenous role in the degradation of bile acids and in the metabolism of estrogens (7–9). CYP3As have also been implicated in activation of procarcinogens such as aflatoxin B1 and food-derived heterocyclic amines, and it was suggested that high CYP3A4 activity may be a risk factor for breast cancer (10–12). There is wide interindividual variability in expression and activity of this enzyme system (13,14), and it is likely that a large proportion of this variability is related to differences in diet (15,16). We demonstrated previously that feeding semipurified diets based on the AIN-93G formulation incorporating soy protein isolate (SPI) as the sole protein source throughout development results in greater constitutive expression and glucocorticoid inducibility of CYP3A in the liver of adult male Sprague-Dawley rats relative to rats fed AIN-93G diets with casein (CAS) as the sole protein source (17). CYP3A is developmentally regulated in both rats and humans (3,6,18–21), and induction characteristics in young and old animals differ (22).

In rat liver, 4 major CYP3A enzymes are expressed: CYP3A1 and a closely related variant CYP3A23 [CYP3A1(CYP3A23)];

[1] Presented in part at Experimental Biology 02, April 2002, New Orleans, LA [Ronis, M.J.J., Hardy, H., Curtis, C., Badeaux, J., Hakkak, R. & Badger, T. M. (2002) Effects of dietary soy protein isolate on hepatic CYP3A activity, expression and inducibility in the 25 day old male Sprague-Dawley rat. FASEB J. 16: A643 (abs.)].

[2] Supported in part by the U.S. Department of Agriculture/Agricultural Research Service under Project #6251–51000–003.

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[4] Abbreviations used: CAS, casein; CLT, clotrimazole; CYP, cytochrome P_{450}; DEX, dexamethasone; Hn RNA, heterologous nuclear RNA; PXR, pregnane-X-receptor; SPI, soy protein isolate.

Manuscript received 14 July 2004. Initial review completed 27 August 2004. Revision accepted 20 September 2004.

CYP3A2; CYP3A18; and CYP3A9 (19). Although all 4 were shown to be inducible to some extent by glucocorticoids such as dexamethasone, their basal expression is under differential regulation during development and by sex hormones and growth hormone. For example, CYP3A2 is male specific in rat liver after puberty, whereas CYP3A9 is expressed at low levels predominantly in female liver (19). No data exist on the effects of feeding SPI on CYP3A enzyme expression and inducibility during early development. However, because SPI is the sole protein source used in soy infant formula, which is fed to 20–25% of U.S. formula-fed infants (>600,000) each year (18), our previous data showing enhanced CYP3A expression and activity in adult rats after soy feeding during development (17) raise important questions regarding the potential for increased clearance and reduced efficacy of pediatric medications, which are CYP3A substrates in soy-fed infants. In addition, these studies also suggested that soy feeding might affect bile acid and sex steroid metabolism and might in some circumstances increase cancer risk. In the current study, time-impregnated Sprague-Dawley rats were fed AIN-93G diets containing either CAS or SPI as the sole protein source beginning on gestational d 4. Dams and pups were fed the same diets until the offspring were killed at age 25 d. Constitutive expression of CYP3A enzymes and inducibility by the glucocorticoid dexamethasone (DEX) and the fungicide clotrimazole (CLT), two CYP3A inducers acting through different mechanisms, were examined in the male pups.

MATERIALS AND METHODS

Chemicals and reagents. Dexamethasone, clotrimazole, and testosterone were obtained from Sigma Chemical. 6β-Hydroxytestosterone standard was supplied by Steraloids. [^{14}C-testosterone] (2.1 GBq/mmol) was purchased from DuPont NEN. Mouse monoclonal antibodies against human CYP3A4/5 (Mab A254) and midazolam were obtained from Gestest, and rabbit polyclonal antibodies against rat CYP3A1 were the gift of Dr. Magnus Ingelman-Sundberg, Karolinska Institute, Stockholm, Sweden (23). Horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody was from Bio-Rad.

Oligonucleotides and cDNA probes. Oligonucleotide probes were synthesized by Bio-Synthesis based on the +68 to +80 bp sequences of rat CYP3A1 (CYP3A23), CYP3A2 and CYP3A18 published by Wright et al. (20). The cDNA against rat CYP3A9 was the gift of Dr. Henry Strobel (University of Texas, Houston, TX) (24). Oligonucleotide primers corresponding to bp 146–165 (15 bp of exon 1 + 5 bp of intron 1) and bp 334–354 (intron 1) of the rat CYP3A1 gene sequence (accession number #AB008377): forward: TGGTGCTCCTCTACGGGTGA and reverse: GAGTTGCATGTGGATGTTGG were used for RT-PCR analysis of CYP3A1 nuclear transcripts.

Animals and diets. The experiment received prior approval from the Institutional Animal Care and Use Committee at UAMS. All animals were housed in an AAALAC-approved animal facility at ACHRI, and all animal housing and husbandry conformed to USDA guidelines. Virus-free time-impregnated female Sprague-Dawley rats were purchased from Harlan Industries and entered the animal facility on gestational d 4. Rats were housed separately in plastic cages, kept at constant temperature (22°C) and humidity with lights on between 0600 and 1800 h, and given free access to water. Diets were formulated exactly as described by Reeves et al. (25) for AIN 93G except that soybean oil was replaced with corn oil, and soy protein isolate (SPI) replaced casein in 1 diet. Groups of 7–9 rats were fed a diet containing casein (CAS) as the protein source or one containing SPI as the sole protein source (DuPont Protein Technologies). The SPI diet had a total isoflavone concentration of 430 mg/kg diet, which consisted of 276 mg genistein and 132 mg daidzein/kg. At this level in the diet, 25-d-old male rats were calculated to consume a dose of 65 mg isoflavones/(kg · d). At birth, pups were culled to 5 males and 5 females per litter; lactating dams continued to consume their respective diets until the pups were weaned. At weaning (age 21 d) male pups had continued free access to their respective diets until the beginning of the induction experiment at age 25 d. Female pups also continued be fed their respective diets but were used for cancer studies reported elsewhere.

Induction experiment. At age 25 d, 2 male pups from each of the litters fed CAS or SPI diets were left untreated; 1 pup/litter was gavaged with 650 μL of corn oil vehicle; 1 pup/litter was gavaged with 50 mg dexamethasone (DEX)/kg in corn oil vehicle; and 1 pup/litter was gavaged with 50 mg clotrimazole/kg in corn oil vehicle at 1600 h, giving 7–9 rats per treatment group. At 0900 h the next morning, the rats were killed, livers removed and microsomes prepared by the differential ultracentrifugation method of Chipman et al. (26).

CYP3A-dependent monooxygenase activities. NADPH-dependent microsomal activity toward the 2 CYP3A substrates, testosterone and midazolam, was measured (27,28). In each case, monooxygenase assays had been standardized in the laboratory before the current study, and the incubation conditions described were within the linear range for incubation time and protein concentration. Testosterone 6β-hydroxylation was assayed as described previously (29). Midazolam metabolism was carried out using 0.055 mol midazolam/L in a total volume of 100 μL with 0.3 mg microsomal protein, 0.001 mol/L NADPH at 37°C for 40 min. The reaction was terminated with 200 μL of 0.1 mol/L sodium bicarbonate, pH 11.0. Substrate and products were extracted using 3 mL Supelclean LC-18 SPE-tubes (Supelco). Samples were eluted from the SPE tubes with methanol, evaporated to dryness using nitrogen and reconstituted in 250 μL of Buffer A (64% sodium acetate pH 4.7, 31% acetonitrile, 3% methanol, 2% tetrahydrofuran). For metabolite quantitation, 100 μL was injected onto a C18 reverse-phase HPLC column (15 cm $\times$ 4.6 mm, Supelco). The HPLC was run isocratically at 1 mL/min with diazepam as an internal standard. Midazolam had a retention time of 11.2 min, 1-OH midazolam of 8.5 min, 4-OH midazolam of 7.2 min and 1,4 dihydroxymidazolam of 5.3 min. Product identities were confirmed by LC-MS/MS (30).

Western immunoblot analysis. Western blotting of CYP3A apoprotein was conducted on microsomes using a mouse monoclonal antibody (Gentest) directed against human CYP3A4/5 (Mab A254) which cross-reacts with rat CYP3A1(CYP3A23) and CYP3A2 at a dilution of 1:500 and a microsomal concentration of 50 μg protein/well as described previously (17) except that the secondary antibody utilized was horseradish peroxidase-linked goat anti-mouse IgG at a concentration of 1:10,000 and immunoreactive apoprotein bands were visualized by enhanced chemiluminescence (Amersham). Because CYP3A enzymes are 80% homologous and cross-react to different degrees with different antibody preparations, the data were confirmed in separate blots using a rabbit anti-rat CYP3A1 polyclonal antibody that cross-reacts to some degree with both CYP3A2 and CYP3A9 (17,31). Protein concentrations were determined using bicinchoninic acid reagent (Pierce). Immunoquantitation was determined by densitometric scanning using a GS525 molecular imager (Bio-Rad). Densities of immunoreactive bands were normalized against a DEX-CAS sample, which was run on every gel and given an arbitrary value of 100.

Northern analysis. At present, 4 mRNA species belonging to the CYP3A subfamily have been isolated from rat liver: CYP3A1 (CYP3A23), CYP3A2, CYP3A9, and CYP3A18 (19). Steady-state mRNA levels were measured in Northern blots using specific antisense oligonucleotides against CYP3A1, CYP3A2, and CYP3A18 and using a cDNA probe against CYP3A9 as described previously (17). Bands were quantitated by densitometry of the autoradiographs and ethidium bromide–stained gel image (18S rRNA) using a GS525 molecular imager (Bio-Rad). The ratio of CYP3A mRNAs to 18S rRNA was determined and normalized to the mean value for CAS-fed rats, which was set at 100 except for CYP3A9, which was below detection limits in uninduced rats. For CYP3A9, mRNA:18S rRNA ratios were normalized to the mean value for DEX-induced CAS-fed rats.

RT-PCR analysis of CYP3A1 heterologous nuclear (Hn) RNA. The concentration of CYP3A1 Hn RNA was determined in isolated total RNA from CAS-fed, SPI-fed, and DEX-induced, CAS-fed rats prepared using TRI reagent as described above using RT-PCR. For

amplification of CYP3A1 nuclear transcripts, first-strand cDNA was synthesized from total RNA using the reverse transcription system with random primers according to the manufacturer's protocol (Applied Biosystems). PCR amplification was performed with PCR master mix (Promega) and 1 μL of RT product. The amplification cycle number was determined to keep amplification of the desired product in the linear range to avoid the "plateau effect" associated with increased numbers of PCR cycles. The PCR cycle was started at 95°C for 5 min followed by a 3-step cycling: 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s followed by 10 min of final extension at 72°C. Amplification cycle numbers were 30 for CYP3A1 Hn RNA and 26 for 18S rRNA primers (Ambion). The PCR products were run on a 2% agarose gel and visualized by staining with ethidium bromide (Sigma). The bands were analyzed by densitometry using a GS525 molecular imager (Bio-Rad). The relative values of CYP3A1 Hn RNA were calculated as the ratio of the CYP3A1 amplicon to that of 18S rRNA.

Statistical analysis. Data for Hn CYP3A1 mRNA and CYP3A apoprotein expression are presented as means ± SEM. Diet effects on the levels of CYP3A1 nuclear transcripts were determined using unpaired Student's *t* tests. For CYP3A apoprotein expression, immunoquantified Western blots were compared using 2-way ANOVA followed by Student-Newman-Keuls test for specific comparisons between means. A $P < 0.05$ was used as the cutoff value for significant difference using SigmaStat for Windows (Jandal Scientific Software). For comparison of diet and inducer effects on CYP3A-dependent monooxygenase activities and mRNA expression, the data were not normally distributed. Therefore, *P*-values were calculated using the Kruskal-Wallace nonparametric rank test using SPSS 12.0. In this case, data are presented as medians with interquartile ratios. The litter was used as the unit of measure.

RESULTS

Effects of feeding SPI on CYP3A expression and activities in weanling male rats. CYP3A apoprotein was readily detectable in liver microsomes from 25-d-old male rats fed AIN diets containing SPI as the sole protein source throughout development. In contrast, there was no detectable CYP3A apoprotein in 25-d-old male rats fed AIN-93G diets containing CAS ($P < 0.001$) (**Figs. 1** and **2**). Although CYP3A18 was the only readily detectible mRNA species in CAS-fed rats at age 25 d, increased CYP3A apoprotein expression in SPI-fed rats appeared to be due to variable increases in the mRNA concentrations of CYP3A1(CYP3A23) and CYP3A2 ($P < 0.05$), whereas CYP3A18 mRNAs levels were unchanged (**Table 1**, **Fig. 3**). When oxidation of the 2 CYP3A substrates, testosterone and midazolam, was examined in hepatic micro-

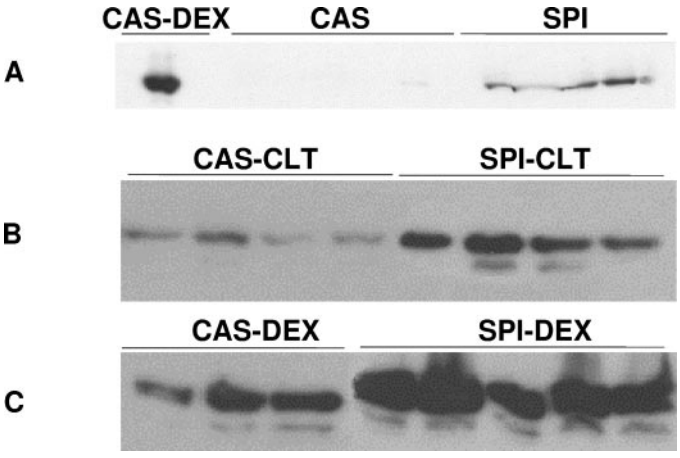

FIGURE 1 Representative Western immunoblots demonstrating effects of diet and inducers on hepatic CYP3A apoprotein expression in 25-d-old male rats exposed to CAS and SPI diets. *Panel A*: representative blots containing 10 μg of microsomal protein from DEX-treated CAS-fed rats or 50 μg of microsomal protein from CAS-fed rats ($n = 5$) or SPI-fed rats ($n = 4$). *Panel B*: representative blots containing 10 μg of microsomal protein from CLT-treated CAS-fed rats or SPI-fed rats ($n = 4$). *Panel C*: representative blots containing 10 μg of microsomal protein from DEX-treated CAS-fed rats ($n = 3$) or DEX-treated SPI-fed rats ($n = 5$).

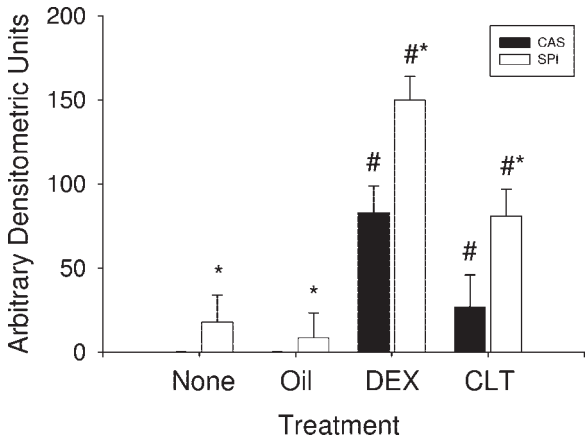

FIGURE 2 Immunoquantification of hepatic microsomal CYP3A apoprotein expression in 25-d-old male rats exposed to CAS and SPI diets demonstrating effects of feeding SPI diets with or without treatment with the known CYP3A-inducers dexamethasone (DEX) or clotrimazole (CLT). Data are means ± SEM, $n = 7$–9. *Different from CAS, $P < 0.05$; #different from control, $P < 0.05$ (2-way ANOVA followed by Student Newman-Keuls test).

TABLE 1

CYP3A-dependent activities and apoprotein and mRNA expression in liver of 25-d-old male rats exposed to CAS and SPI diets[1]

Substrate	CAS	SPI
	nmol/(mg microsomal protein · min)	
Testosterone (6β-Hydroxylation)	3.5 (3.0, 6.8)	9.7 (4.3, 17.8)
Midazolam		
Total	0.4 (0.3, 1.5)	1.6 (0.9, 2.5)*
4-Hydroxylation	0.2 (0.2, 0.9)	0.9 (0.5, 1.4)
1-Hydroxylation	0.04 (0.0, 0.4)	0.5 (0.3, 0.8)*
1,4-Dihydroxylation	0.2 (0.1, 0.2)	0.2 (0.2, 0.3)
	Arbitrary densitometric units	
Apoprotein		
CYP3A	ND[2]	11 (2, 21)*
	Arbitrary densitometric unit ratio[3]	
mRNA		
CYP3A1	76 (44, 168)	452 (59, 1100)
CYP3A2	35 (26, 221)	305 (208, 984)*
CYP3A9	ND	ND
CYP3A18	102 (68, 134)	118 (75, 150)

[1] Data are medians (interquartile ratios); $n = 7$ rats from separate litters. * Different from CAS, $P < 0.05$ (Kruskal-Willis nonparametric rank test.)
[2] ND, not detected.
[3] Relative to mean CAS = 100.

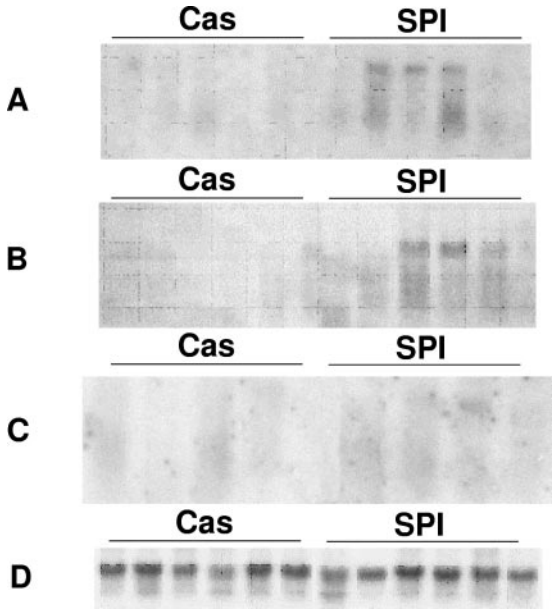

FIGURE 3 Representative Northern blots demonstrating the effects of feeding SPI-containing diet on expression of hepatic CYP3A mRNAs in 25-d-old male rats exposed to CAS and SPI diets relative to expression in rats fed casein (CAS). Representative blots containing 20 μg of total mRNA from CAS-fed rats (n = 4–6) or SPI-fed rats (n = 4–6). *Panel A*: Oligonucleotide specific for CYP3A(CYP3A23). *Panel B*: Oligonucleotide specific for CYP3A2. *Panel C*: cDNA specific for CYP3A9. *Panel D*: Oligonucleotide specific for CYP3A18.

somes from CAS- and SPI-fed rats, SPI-feeding resulted in a 1.5-fold increase in stereoselective testosterone 6β-hydroxylation ($P = 0.13$) and a 3-fold increase in midazolam turnover ($P < 0.05$), associated particularly with highly increased formation of 1-hydroxymidazolam ($P < 0.05$) (Table 1).

Effects of the CYP3A inducers, dexamethasone and clotrimazole, on CYP3A expression and activity in weanling rats fed CAS or SPI. After either DEX or CLT treatment, CYP3A apoprotein was substantially elevated in liver microsomes from both CAS- and SPI-fed rats compared with oil vehicle–treated controls ($P < 0.05$) (Fig. 2). However, expression was higher in SPI-fed than CAS-fed rats after treatment with the same inducer ($P < 0.05$). The effects of SPI and inducer on CYP3A apoprotein expression appeared to be additive; 2-way ANOVA revealed no significant diet × inducer interactions (Fig. 1 and 2). Consistent with the apoprotein data, CYP3A-dependent midazolam 4-hydroxylation was induced to a greater extent ($P < 0.05$) after DEX treatment in SPI-fed rats compared with CAS-fed rats (**Table 2**). In contrast, testosterone 6β-hydroxylation, which is more specific for CYP3A2, was induced by DEX to the same extent in rats fed both diets. Although CYP3A mRNA and apoprotein expression were induced after CLT treatment, no increases in CYP3A activities were observed (Table 2). Northern analysis of CYP3A mRNA levels demonstrated that 18 h after treatment with the corn oil vehicle, CYP3A1 and CYP3A2 mRNA expressions were reduced in SPI-fed rats (**Table 3** compared with Table 1). Treatment with DEX induced CYP3A1, CYP3A2, and CYP3A9 mRNA expression to a similar extent in rats fed either diet ($P < 0.05$). In contrast to DEX treatment, treatment with CLT induced CYP3A2 mRNA expression to the same extent in rats fed both diets, but CYP3A1 and CYP3A18 were significantly increased by CLT only in livers of SPI-fed rats (Table 3).

Diet and dexamethasone effects on expression of CYP3A1 nuclear transcripts in weanling rats. To examine the effects of SPI-feeding on CYP3A1 transcription rates, heterologous nuclear RNA expression in livers from rats fed CAS or SPI was examined by RT-PCR using a primer set specific for an exon-intron boundary in the CYP3A1 transcript. As a positive control, total RNA derived from livers of DEX-treated, CAS-fed rats was also probed. Hepatic Hn RNA transcript levels were greater in DEX-treated rats fed CAS than in CAS-fed rats ($P < 0.05$), but not in SPI-fed rats relative to CAS-fed rats (**Fig. 4**).

DISCUSSION

We previously demonstrated increased expression and glucocorticoid-induction of CYP3A enzymes in the livers of adult male rats fed diets containing SPI throughout development (17). Because SPI is the sole protein source utilized in soy infant formula, these data raised questions about the potential for increased clearance and reduced efficacy of pediatric medications in soy-fed infants and the potential for increased activation of dietary and endogenous procarcinogens. In the

TABLE 2

Effects of DEX and CLT on CYP3A-dependent testosterone and midazolam metabolism in liver microsomes from 25-d-old male rats exposed to CAS and SPI diets[1]

	CAS			SPI		
Substrate	Vehicle	DEX	CLT	Vehicle	DEX	CLT
	nmol product formed/(mg microsomal protein · min)					
Testosterone						
6β-hydroxylation	8.2 (4.0, 11.3)	20.0 (10.4, 22.5)[a]	5.7 (2.7, 7.5)	8.4 (4.4, 10.1)	20.6 (6.5, 21.5)[a]	9.2 (3.9, 13.8)
Midazolam						
Total	1.6 (1.2, 3.2)	3.2 (1.5, 5.1)	1.5 (0.8, 2.6)	1.2 (0.7, 2.3)	3.9 (1.7, 5.8)[a]	1.8 (1.3, 3.3)
4-Hydroxylation	0.8 (0.6, 1.9)	1.3 (0.8, 2.7)	0.7 (0.4, 1.3)	0.8 (0.3, 1.3)	2.1 (0.8, 3.3)[a]	0.9 (0.5, 2.5)
1-Hydroxylation	0.2 (0.2, 0.6)	1.0 (0.3, 2.3)	0.2 (0.1, 0.9)	0.4 (0.2, 0.8)	1.2 (0.1, 1.7)	0.9 (0.2, 1.4)
1,4-Dihydroxylation	0.5 (0.4, 0.7)	0.6 (0.3, 0.8)	0.5 (0.3, 0.6)	0.2 (0.0, 0.4)[b]	0.8 (0.7, 1.1)[a]	0.1 (0.0, 0.5)

[1] Data are medians (interquartile ratios) for duplicate assays conducted on hepatic microsomes from 7–9 rats from separate litters. [a] Different from vehicle within a diet, $P < 0.05$. [b] Different from CAS within a treatment, $P < 0.05$ (Kruskal-Wallace nonparametric rank test).

[2] The vehicle for all gavage treatments was 600 μL of corn oil, DEX, 50 mg/kg dexamethasone, and CLT, 50 mg/kg clotrimazole.

TABLE 3

Effects of inducers on CYP3A mRNA expression in liver from 25-d-old male rats fed CAS or SPI during development[1]

	CAS			SPI		
	Vehicle[2]	DEX	CLT	Vehicle	DEX	CLT
	Arbitrary densitometric unit ratio					
CYP3A1[3]	51 (3, 145)	1095 (178, 1153)[a]	22 (14, 66)	17 (9, 28)	813 (520, 1480)[a]	152 (83, 61)[ab]
CYP3A2[3]	101 (51, 143)	1183 (149, 1795)[a]	382 (197, 1322)[a]	123 (89, 230)	366 (237, 733)[a]	332 (101, 1541)[a]
CYP3A9[4]	ND	100 (22, 207)[a]	11 (8, 13)[a]	ND	53 (33, 112)[a]	13 (11, 21)[a]
CYP3A18[3]	93 (80, 104)	84 (66, 94)	70 (63, 76)[a]	72 (57, 79)[b]	61 (58, 80)	109 (78, 157)[ab]

[1] Data are medians (interquartile ratios) for duplicate assays conducted on livers from 7–9 rats from separate litters. [a] Different from vehicle within a diet, $P < 0.05$. [b] Different from CAS within a treatment, $P < 0.05$ (Kruskal-Wallace nonparametric rank test). ND, not detected.
[2] Vehicle for all gavage treatments was 600 μL of corn oil, DEX, 50 mg/kg dexamethasone, and CLT, 50 mg/kg clotrimazole.
[3] Expressed relative to CAS vehicle mean = 100.
[4] The CYP3A9/18S ratio is expressed relative to the CAS-DEX group mean = 100.

current study, we extended our original observations to demonstrate that increased expression of hepatic CYP3A also occurs in soy-fed rats as early as postnatal d 25 and that different CYP3A enzymes are induced in a diet-dependent fashion by DEX and CLT at this age.

Interestingly, basal CYP3A apoprotein and mRNA expression in liver from CAS-fed male pups at age 25 d was almost nonexistent except for readily detectible CYP3A18 mRNA. Feeding SPI-based diets in the absence of any other treatment resulted in the appearance of readily detectible CYP3A apoprotein in Western blots, increased CYP3A-dependent microsomal monooxygenase activities and, although there was large interindividual variability, median expression of CYP3A1 (23) and CYP3A2 mRNA was elevated in SPI-fed rats (Table 1). The large variability in CYP3A response to the soy diet may be due to large interindividual variability in isoflavone metabolite patterns and tissue concentrations we observed previously in rats after soy feeding (32). Some of this may be due to the fact that soy-associated isoflavones require hydrolysis from glucoside sugars (genistin, daidzin) to aglycones (genistein, daidzein) by intestinal microflora, and a number of unique bioactive isoflavone metabolites such as equol (derived from daidzein) are also derived from bacterial metabolism (33,34).

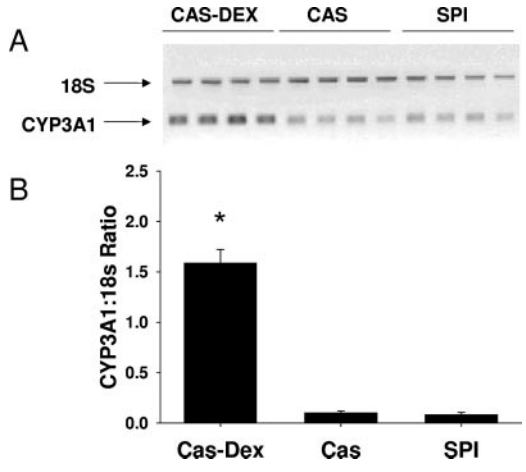

FIGURE 4 Expression of CYP3A1 Hn RNA in livers of 25-d-old male rats fed CAS, SPI, or CAS and induced with DEX. *Panel A*: Representative amplicon bands for CYP3A1 HnRNA, and 18S rRNA. *Panel B*: Quantification of RT-PCR. Data are presented as means ± SEM, $n = 4$. *Different from CAS, $P < 0.05$ (Student's t test).

The basal expression data for CYP3A mRNAs presented here differs from the data on the development of CYP3A mRNA expression published previously for Sprague-Dawley rats by Mahnke et al. (19). In that study, CYP3A23 and CYP3A2 mRNAs were expressed at high levels in male rats from 1 to 20 wk of age, CYP3A18 mRNA was expressed at a low level, and CYP3A9 was not expressed until postnatal d 49. However, it is important to note that in that study, the rats were fed a commercial grain-based diet rather than semipurified artificial diets as in the current study. Because soy is a major component of commercial rodent diets, which also contain many phytochemicals derived from other grain sources, at least part of the differences between the present data in rats fed CAS-based AIN-93G diets and those of Mahnke et al. (19) may be attributable to the soy content of grain-based diets. It is also noteworthy that CYP3A6 expression in rabbits is dramatically increased during weaning to commercial grain-based diets at age 3–4 wk, and this increase in CYP3A expression could be accelerated by early weaning at wk 2 (35).

At present, due to the difficulties of conducting drug pharmacokinetic studies in infants, it is not known whether feeding SPI as the sole protein component of soy infant formula results in increased CYP3A expression and activity in formula-fed infants. However, CYP3A enzymes are the major CYP forms expressed in neonatal human liver (3). In the fetus, the major enzyme expressed is CYP3A7, which has low catalytic activity toward many pediatric medications (36,37). In the weeks after birth, CYP3A7 expression declines to the very low level seen in the adult liver, whereas the major adult enzyme, CYP3A4, begins to rise (21). In a recent in vitro study, soy infant formula addition to culture medium was shown to significantly increase expression of a CYP3A4 reporter construct in HepG2 cells, whereas addition of breast milk had no effect (38). Moreover, in a second recent in vitro study, Li and Shay (39) demonstrated significant increases in endogenous CYP3A4 expression and inducibility by the drug atorvastatin in HepG2 cells exposed to an isoflavone-containing soy extract and the pure isoflavones, genistein and equol.

The molecular mechanisms underlying the effects of SPI on CYP3A expression are not yet known. Our data demonstrating lack of effects of SPI feeding on expression of CYP3A1 HnRNA despite 4-fold increases in mRNA content suggest that the inductive effects of soy on CYP3A1 are post-transcriptional. The effects could be due to mRNA stabilization or altered RNA processing, perhaps as a result of changes in

mRNA binding proteins (40,41). This is in contrast to the transcriptional regulation of CYP3A1 (23) by DEX which is mediated via a newly described orphan receptor, pregnane-X-receptor (PXR) (42). The lack of a significant diet × DEX interaction on CYP3A apoprotein expression suggests that SPI and glucocorticoids induce CYP3A expression via different mechanisms at this stage of rat development. This is in contrast to our previous data on the effects of SPI feeding throughout life on CYP3A-inducibility by DEX in adult male rats (17) in which we observed a significant synergism between SPI and DEX, presumably as the result of soy effects on PXR-mediated signaling. However, CYP3A induction characteristics differ between young and old rats (22) and the mechanisms underlying this difference remain to be resolved.

In the current study, induction of CYP3A enzymes was both inducer- and diet-dependent. DEX treatment induced CYP3A1 (CYP3A23), CYP3A2, and CYP3A9 mRNAs in both CAS- and SPI-fed rats, but had no effect on the expression of CYP3A18 mRNA in rats fed either diet. In contrast, although CLT was a weaker inducer overall, in CAS-fed rats, CLT significantly induced CYP3A2 and CYP3A9 mRNA expression, whereas in SPI-fed rats, CLT induced expression of all 4 CYP3A mRNAs. Some of these differences likely reflect differences in the mode of action of the 2 inducers. DEX is good ligand for rat PXR and works via the PXR-retinoid X receptor transcription factor pathway (42–44). In contrast, CLT is a much weaker PXR ligand and is a mixed inducer with potent inhibitory actions toward CYP3A that result in biphasic inhibition/induction responses as the result of feedback via disruption of endogenous CYP3A-dependent metabolic pathways (45–47). Thus, the lack of increase in CYP3A-dependent monooxygenase activities 17 h after CLT treatment despite induced CYP3A mRNA and apoprotein expression reported in Table 3 is consistent with the aforementioned biphasic effect of this compound.

Another important issue concerns which component of SPI is responsible for the increased expression of CYP3A enzymes. The effect may arise as the result of the soy protein itself or from soy-associated phytochemicals such as the isoflavones. Li and Shay (39) recently demonstrated CYP3A4 induction in hepatoma cells in vitro after treatment with isoflavone extracts, genistein and equol. Genistein was reported to be a CYP3A substrate (48,49), and it is common for CYP substrates to induce their own metabolism (50). However, this occurred at concentrations in excess of 10 μmol genistein/L, a concentration considerably higher then the genistein concentration attained in rats in vivo after consumption of SPI-containing diets (32,33,51). Over 90% of genistein found in vivo after SPI consumption is present as inactive glucuronide conjugates and sulfates as the result of first-pass conjugation during absorption from the gut (33,51). We found <1 μmol/L active genistein aglycone in rat plasma after SPI consumption (32). Therefore, it is unclear whether isoflavones are the bioactive soy component responsible for CYP3A induction. Moreover, CYP3A induction appears not to be associated with the estrogenic effects of soy isoflavones. Estradiol does not induce CYP3A in hepatocytes in vitro (52) and SPI feeding appears not to result in estrogenic effects such as the induction of uterine hyperplasia in vivo in intact rats (53).

Thus, we demonstrated a significant increase in CYP3A expression and activity in rat liver in weanling rats fed SPI during early development. In addition, SPI and CYP3A inducers interact to elevate CYP3A expression in a diet- and inducer-specific fashion. The molecular mechanism for SPI induction of CYP3A1 appears to be post-transcriptional. It is unclear which component of SPI is responsible for this induction effect. It also remains to be determined whether such induction occurs in infants consuming soy infant formula or whether maternal consumption of vegetarian, soy-based diets during pregnancy and/or lactation increases the metabolism and/or clearance of pediatric medications that are CYP3A substrates. Because CYP3A is implicated in the activation of dietary procarcinogens and endogenous estrogens, sustained CYP3A increases might also increase cancer risk. These issues will be the focus of future studies.

ACKNOWLEDGMENTS

The authors would like to acknowledge the technical assistance of Matt Ferguson, Tammi Dallari, Kim Hale, Holly Hardy, Chris Curtis, Jamie Badeuax, and Mark Robinette in the conduct of these studies. In addition they wish to thank Henry Strobel, University of Texas, Houston, TX for the gift of the CYP3A9 cDNA and DuPont Solace Inc. (St Louis, MO) for providing the SPI utilized in this study.

LITERATURE CITED

1. Ronis, M.J.J. & Ingelman-Sundberg, M. (1998) Induction of human P450 enzymes: mechanisms and implications. In: A Handbook of Human Toxicology (Woolf, T., ed.), pp. 239–262. Marcel Decker, New York, NY.

2. Westlind-Johansson, A., Malmenbo, S., Johansson, A., Otter, C., Andersson, T. B., Johansson, I., Edwards, R. J., Boobis, A. R. & Ingelman-Sundberg, M. (2003) Comparative analysis of CYP3A expression in human liver suggest only a minor role for CYP3A5 in drug metabolism. Drug Metab. Disp. 31: 755–761.

3. Scheutz, E. G., Beach, D. L. & Guzelian, P. S. (1994) Selective expression of cytochrome P450 CYP3A mRNAs in embryonic and adult human liver. Pharmacogenetics 4: 11–20.

4. Wilkinson, G. R. (1996) Cytochrome P4503A (CYP3A) metabolism: prediction of in vivo activity in humans. J. Pharmacokinet. Biopharm. 24: 475–490.

5. Kanamori, M., Takahashi, H. & Echizen, H. (2002) Developmental changes in the liver weight and body weight-normalized clearance of theophylline, phenytoin and cyclosporine in children. Int. J. Clin. Pharmacol. Ther. 40: 485–489.

6. Stevens, J. C., Hines, R. N., Gu, C., Koukouritaki, S. B., Manro, J. R., Tandler, P. J. & Zaya, M. J. (2003) Developmental expression of the major human hepatic CYP3A enzymes. J. Pharmcol. Exp. Ther. 307: 573–582.

7. Xie, W., Radominska-Pandya, A., Shi, Y., Simon, C. M., Nelson, M. C., Ong, E. S., Waxman, D. J. & Evans, R. M. (2001) An essential role for nuclear receptors SXR/RXR in detoxification of cholestatic bile acids. Proc. Natl. Acad. Sci. U.S.A. 98: 3375–3380.

8. Badawi, A. F., Cavalieri, E. L. & Rogan, E. G. (2001) Role of human cytochrome P450 1A1, 1A2, 1B1 and 3A4 in the 2-, 4- and 16α-hydroxylation of 17β-estradiol. Metabolism 50: 1001–1003.

9. Lee, A. J., Cai, M. X., Thomas, P. E., Conney, A. H. & Zhu, B. T. (2003) Characterization of the oxidative metabolites of 17β-estradiol and estrone formed by 15 selectively expressed human cytochrome P450 isoforms. Endocrinology 144: 3382–3398.

10. Van Vleet, T. R., Kleain, P. J. & Coulombe, R. A., Jr. (2002) Metabolism and cytotoxicity of aflatoxin B1 in cytochrome P450-expressing human lung cells. J. Toxicol Environ. Health 65A: 853–867.

11. Yamazaki, H., Inui, Y., Wrighton, S. A., Guengerich, F. P & Shimada, T. (1995) Procarcinogen activation by cytochrome P450 3A4 and 3A5 expressed in *Escherichia coli* and by human liver microsomes. Carcinogenesis 16: 2167–2170.

12. Zheng, W., Jin, F., Dunning, L. A., Shu X. O., Dai, Q., Wen, W. Q., Gao, Y. T. & Holtzman, J. L. (2001) Epidemiological study of urinary 6β-hydroxycortisol to cortisol ratios and breast cancer risk. Cancer Epidemiol. Biomark. Prev. 10: 237–242.

13. Dorne, J. L., Walton, K. & Renwick, A. G. (2003) Human variability in CYP3A4 metabolism and CYP3A4-related uncertainty factors for risk assessment. Food Chem. Toxicol. 41: 201–224.

14. Matsumura, K., Saito, T., Takahashi, Y., Ozeki, T., Kiyotani, K., Fujieda, M., Yamazaki, H., Kunitoh, H. & Kamataki, T. (2004) Identification of a novel polymorphic enhancer of the human CYP3A4 gene. Mol. Pharmacol. 65: 326–334.

15. Harris, R. Z., Jang, G. R. and Tsunoda, S. (2003) Dietary effects on drug metabolism and transport. Clin. Pharmacokinet. 42: 1071–1088.

16. Bailey, D. G., Malcolm, J., Arnold, O. & Spence, J. D. (1998) Grapefruit juice-drug interactions. Br. J. Clin. Pharmacol. 46: 101–110.

17. Ronis, M.J.J., Rowlands, J. C., Hakkak, H. & Badger, T. M. (1999) Altered enzyme expression and glucocorticoid-inducibility of hepatic CYP3A and CYP2B enzymes in male rats fed diets containing soy protein isolate. J. Nutr. 129: 1958–1965.

18. Setchell, K. D., Zimmer-Nechemias, L., Cai, J. & Heubi, J. E. (1997) Exposure of infants to phytoestrogens from soy-based infant formula. Lancet 350: 23–27.

19. Mahnke, A., Strotkamp, D., Roos, P. H., Hanstein, W. G., Chabot, G. G.

& Nef, P. (1997) Expression and inducibility of cytochrome P450 3A9 (CYP3A9) and other members of the CYP3A subfamily in rat liver. Arch. Biochem. Biophys. 337: 62–68.

20. Wright, M. C., Edwards, R. J., Pimenta, M., Ribeiro, V., Lechner, M. C. & Paine, A. J. (1997) Developmental changes in the constitutive and inducible expression of cytochrome P450 3A2. Biochem. Pharmacol. 54: 841–846.

21. Lacroix, D., Sonnier, M., Moncion, A., Cheron, G. & Cresteil, T. (1997) Expression of CYP3A in human liver—evidence that the shift between CYP3A7 and CYP3A4 occurs immediately after birth. Eur. J. Biochem. 247: 625–634.

22. Pereira, T. M. & Lechner, M.C. (1995) Differential regulation of the cytochrome P450 CYP3A1 gene transcription by dexamethasone in immature and adult rat liver. Eur. J. Biochem. 229: 171–177.

23. Eliasson, E., Mkrtchian, S., Halpert, J. & Ingelman-Sundberg, M. (1994) Substrate-regulated, cAMP-dependent phosphorylation, denaturation and degradation of glucocorticoid-inducible rat liver cytochrome P450 3A1. J. Biol. Chem. 269: 18378–18383.

24. Wang, H., Kawashima, H. & Strobel, H. W. (1997) cDNA cloning of a novel CP3A from rat brain. Biochem. Biophys. Res. Commun. 221: 157–162.

25. Reeves, P. G., Nielson, F. H. & Fahey, G. C., Jr. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. 123: 1939–1951.

26. Chipman, J. K., Kuruckgy, N. & Walker, C. H. (1978) The metabolism of dieldrin and two of its analogues: the relationship between rates of microsomal metabolism and rates of excretion of metabolites in the male rat. Biochem. Pharmacol. 28: 1337–1345.

27. Desjardins, J. P. & Iversen, P. L. (1995) Inhibition of rat cytochrome P450 3A2 by an antisense phosphorothioate oligodeoxynucleotide in vivo. J. Pharmacol. Exp. Ther. 275: 1608–1613.

28. Ma, F. & Lau, C. E. (1996) Determination of midazolam and its metabolites in serum microsamples by high-performance liquid chromatography and its application to pharmacokinetics in rats. J. Chromatogr. B. Biomed. Appl. 682: 109–113.

29. Ronis, M.J.J., Lumpkin, C. K., Ingelman-Sundberg, M. & Badger, T. M. (1991) Effects of short term ethanol and nutrition on the hepatic microsomal system in a model utilizing total enteral nutrition in the rat. AlcoholClin. Exp. Res. 15: 693–699.

30. Fang, N., Ronis, M.J.J., Hardy, H., Irby, D., Rowlands, J. C., Hakkak, R., Badger, T. M. (2002) Characterization of new medazolam metabolites by hepatic microsomes from dexamethasone-induced male rats by LC-MS/MS. FASEB J. 16: A244 (abs.).

31. Rowlands, J. C., Wang, H., Hakkak, R., Ronis, M.J.J., Strobel, H. W. & Badger, T. M. (2000) Chronic intragastric infusion of ethanol-containing diets induces CYP3a9 while decreasing CYP3A2 in male rats. J. Pharmacol. Exp. Ther. 295: 747–752.

32. Cimino, C., Ronis, M.J.J. Rowlands, J. C., Zhang, Z. & Badger, T. (2001) Analysis of plasma and urine isoflavone conjugates: Sequential enzymic digestion. FASEB J. 15: A985 (abs.).

33. Badger, T. M., Ronis, M.J.J. & Fang, N. (2003) The health effects of dietary isoflavones. In: Molecular Nutrition (Zemplini, J. & Daniel, H., eds.), pp. 201–217. CAB International, Wallingford, UK.

34. Hoey L, Rowland I. R., Lloyd, A. S., Clarke, D. B. & Wiseman, H. (2004) Influence of soya-based infant formula consumption on isoflavone and gut microflora metabolite concentrations in urine and on faecal microflora composition and metabolic activity in infants and children. Br. J. Nutr. 91: 607–616.

35. Pineau, T., Daujat, M., Pichard, L., Girard, F., Angevain, J., Bonfils, C. & Maurel, P. (1991) Developmental expression of rabbit cytochrome P450 CYP1A1, CYP1A2 and CYP3A6 genes. Effects of weaning and rifampicin. Eur. J. Biochem. 197: 145–153.

36. Treluyer, J. M., Bowers, G., Cazali, N., Sonnier, M., Rey, E., Pons, G. & Cresteil, T. (2003) Oxidative metabolism of amprenavir in the human liver. Effect of CYP3A maturation. Drug Metab. Disp. 31: 275–281.

37. Treluyer, J. M., Rey, E., Sonnier, M., Pons, G. & Cresteil, T. (2001) Evidence of impaired cisapride metabolism in neonates. Br. J. Clin. Pharmacol. 52: 419–425.

38. Xu, H., Harper, P. A., Kim, R. B., Kliewer, S. A., Lonnerdal, B. L. & Ito, S. (2004) Effects of human milk and formula on transcriptional regulation of cytochrome P450 3A4. FASEB J. 18: A1202 (abs.).

39. Li, Y. & Shay, N. F. (2004) Isoflavone-drug interactions in HepG2 cells and human hepatocytes. FASEB J. 18: A851 (abs.).

40. Tenenbaum, S. A., Carson, C. C., Larger, P. J. & Keene, J. D. (2000) Identifying mRNA subsets in messenger ribonucleoprotein complexes by using cDNA arrays. Proc. Natl. Acad. Sci. U.S.A. 97: 14085–14090.

41. Zhang, Y., Lu, Z., Ku, L., Chen, Y. & Feng, Y. (2003) Tyrosine physphorylation of QKI mediates developmental signals to regulate mRNA metabolism. EMBO J. 22: 1801–1810.

42. Goodwin, B., Redinbo, M. R. & Kliewer, S. A. (2002) Regulation of CYP3A gene transcription by the pregnane-X-receptor. Annu. Rev. Pharmacol. Toxicol. 42: 1–23.

43. Pascussi, J. M., Drocourt, L., Gerbal-Chaloin, S., Fabre, J. M., Maurel, P. & Vilarem, M. J. (2001) Dual effect of dexamethasone on CYP3a4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor. Eur. J. Biochem. 268: 6346–6358.

44. Zhang, W., Purchio, A., Chen, K., Burns, S. M., Contag, C. H. & Contag, P. R. (2003) In vivo activation of the human CYP3A4 promoter in mouse liver and regulation by pregnane X receptors. Biochem. Pharmacol. 65: 1889–18896.

45. Ronis, M.J.J., Ingelman-Sundberg, M. & Badger, T. M. (1994) Induction, inhibition and suppression of multiple hepatic cytochrome P450 isozymes in the male rat and bobwhite quail (*Colinus virginianus*) by ergosterol biosynthesis inhibiting fungicides (EIBFs). Biochem. Pharmacol. 48: 1953–1965.

46. Moore, L. B., Parks, D. J., Jones, S. A. Bledsoe, R. K., Consler, T. G., Stimmel, J. B., Goodwin, B., Liddle, C., Blanchard, S. G., Wilson, T. M., Collins, J. L. & Kliewer, S. A. (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. J. Biol. Chem. 275: 15122–15127.

47. Turan, V. K., Mishin, V. M. & Thomas, P. E. (2001) Clotrimazole is a selective and potent inhibitor of rat cytochrome P450 3A subfamily related testosterone metabolism. Drug Metab. Disp. 29: 837–842.

48. Jager, W., Sartori, M., Herzog, W. & Thalhammer, T. (1998) Genistein metabolism in liver microsomes of Wistar and mutant TR(−) rats. Res. Commun. Mol. Pathol. Pharmacol. 100: 105–116.

49. Roberts-Kirchoff, E. S., Crowley, J. R., Hollenberg, P. F. & Kim, H. (1999) Metabolism of genistein by rat and human cytochrome P450s. Chem. Res. Toxicol. 12: 610–616.

50. Ruckpaul, K. & Rein, H., eds. (1990) Principles, Mechanisms and Biological Consequences of Induction. Taylor & Francis, London, UK.

51. Badger, T. M., Ronis, M.J.J., Hakkak, R., Rowlands, J. C. & Korourian, S. (2002) The health consequences of early soy consumption. J. Nutr. 132: 559S–565S.

52. Olsen, A. K., Hansen, K. T. & Friis, C. (1997) Pig hepatocytes as an in vitro model to study the regulation of human CYP3A4: prediction of drug-drug interactions with 17-alpha ethinylestradiol. Chem.-Biol. Interact. 107: 93–108.

53. Badger, T. M., Ronis, M.J.J. & Hakkak, R. (2001) Developmental effects and health aspects of soy protein isolate, casein and whey in male and female rats. Int. J. Toxicol. 20: 165–174.