Reduction in 7,12-Dimethylbenz[a]anthracene-Induced Hepatic Cytochrome-P450 1A1 Expression Following Soy Consumption in Female Rats Is Mediated by Degradation of the Aryl Hydrocarbon Receptor¹

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Abstract

Consumption of a soy diet has been found to reduce cancer incidence in animals and is associated with reduced cancer risk in humans. In this study, the effect of consuming soy protein isolate (SPI) on the aryl hydrocarbon receptor (AhR)-mediated signaling pathway was investigated. Female Sprague-Dawley rats were fed AIN-93G diets with (+) or without (−) SPI-bound phytochemicals or casein (CAS) protein and gavaged orally with 7,12-dimethylbenz[a]anthracene (DMBA) or sesame oil. We found reduced (p < 0.05) DMBA-induced hepatic cytochrome-P450 1A1 (CYP1A1) activity, apoprotein, and mRNA expression along with the reduced binding of AhR-AhR nuclear translocator complex to CYP1A1 gene promoter in SPI⁻¹-fed rats compared with CAS- or SPI⁻¹-fed rats. Basal AhR protein expression was lower (p < 0.05) in SPI⁻¹-fed rats compared with CAS- or SPI⁻¹-fed groups. AhR levels were reduced (p < 0.05) after rats were fed SPI⁻¹ for >20 d. Experiments in which SPI⁻¹-fed rats were weaned to CAS diets demonstrated that AhR reduction by SPI⁻¹ is not imprinted metabolically. To determine the molecular mechanisms of SPI⁻¹-mediated AhR reduction, an ex vivo model was developed using FGC-4 cells treated with serum from CAS- or SPI⁻¹-fed rats. SPI⁻¹ serum treatment of FGC-4 cells reduced AhR expression and DMBA-induced CYP1A1 expression (p < 0.05). The reduction in AhR expression was in part due to the shorter half-life of AhR protein. Our findings suggest that the cancer preventive effect of soy-based diets is mediated in part by reduction in AhR protein level posttranslationally, which reduces procarcinogen-induced CYP1A1 induction and metabolic activation. J. Nutr. 137: 19–24, 2007.

Introduction

Nutritional status and dietary factors can influence the development of cancer and may play crucial roles in cancer prevention. Epidemiological studies suggest lower risk for certain cancers in populations that consume Asian diets rich in soy (1,2). A number of studies have shown that animals fed diets containing soy protein have reduced incidence of chemically induced mammary, prostate, and colon cancers (3–5), but the mechanism(s) is still unknown. Environmental pollutants are potential sources of cancer initiation and promotion. Polycyclic aromatic hydrocarbons (PAH) and halogenated aromatic hydrocarbons, such as dioxins, anthracenes, and cholanthrene, are commonly found environmental carcinogens. PAH have been isolated from diesel exhaust, barbecued meat, tobacco smoke, overheated cooking oil, etc. (6). These procarcinogens are bioactivated into mutagenic carcinogens (7,8) by phase I enzymes such as cytochrome-P450 1A1 (CYP1A1), which results in DNA adduct formation and ultimately leads to carcinogenesis (9,10).

CYP1A1 is activated by the binding of these chemicals to aryl hydrocarbon receptor (AhR), a cytosolic ligand-activated basic helix-loop-helix transcription factor. In the cytoplasm, AhR remains in the inactivated state bound with chaperone proteins, a dimer of heat shock protein 90 and the immunophilin-like X-associated protein 2, which collectively influence AhR ligand binding, stabilization, cellular localization, and transcriptional activity (11,12). Binding of the ligand (such as procarcinogen) to AhR is followed by the nuclear translocation and heterodimerization with AhR nuclear translocator (ARNT). AhR-ARNT heterodimer subsequently binds to the consensus DNA site, xenobiotic response element (XRE), present in the promoter region of CYP1A1.

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² Abbreviations used: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CAS, casein; CYP1A1, cytochrome P-450 1A1; DMBA, 7,12-dimethylbenz[a]anthracene; EMSA, electrophoretic mobility shift assay; EROD, 7-ethoxyresorufin O-deethylase; GD, gestational day; PAH, polycyclic aromatic hydrocarbons; PND, postnatal day; SPI, soy protein isolate; SPI⁻¹, soy protein isolate with associated phytochemicals; SPI⁺, soy protein isolate without phytochemicals; XRE, xenobiotic response element.
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Any intervention that leads to decreased CYP1A1 activity is expected to reduce PAH-mediated cancer incidence. Soy-associated phytochemicals, such as genistein (13), have been reported to inhibit benzo[a]pyrene-induced DNA adduct formation in vitro by decreasing CYP1A1 activity. We showed a reduction in 7,12-dimethylnaphthalene-induced CYP1A1 activity in male rats (15). Neither the identity of the bioactive factors in SPI nor the mechanisms by which they regulate AhR expression have been identified. In this study, female Sprague-Dawley rats were fed AIN-93G diets made with SPI associated with phytochemicals (SPI−) or without phytochemicals (SPI+) and the mechanism of reduction in hepatic CYP1A1 induction was investigated.

Material and Methods
Materials. All the chemicals, unless otherwise noted, were purchased from Sigma Aldrich. Rabbit anti-AhR was a generous gift from Dr. Richard Pollenz, University of South Florida. Goat anti-CYP1A1 was purchased from Gentest. Goat anti-GAPDH and horseradish peroxidase conjugated-bovine anti-goat and goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology. SPI− and SPI+ proteins were supplied by Solae Company.

Animals and diets. The experiment received prior approval from the Institutional Animal Care and Use Committee at UAMS. Female Sprague-Dawley rats were obtained from Charles Rivers Laboratories and were housed in polycarbonate cages in an environmentally controlled room with a 12-h-light/12-h dark cycle and were provided with pelleted diets made of casein (CAS) or SPI. The specialized semipurified diets were made according to the AIN-93G diet formula (16), except corn oil replaced soybean oil and the proteins were supplied by Solae Company.

Experiment 1. Female rats were fed CAS, SPI+, or SPI− diets starting at postnatal day 21 (PND21). To determine the effect of the semipurified diets on DMBA-mediated CYP1A1 induction, rats (n = 6 per group, PND50) were orally gavaged with 65 mg/kg DMBA or sesame oil and were killed after 24 h.

Experiment 2. To evaluate the time course of soy effects on basal AhR expression, female rats between age PND21 and PND46 were fed a diet made with CAS. Rats (n = 6 per group) were switched to SPI− diet on PND46, PND56, PND62, PND64, or PND65, corresponding to SPI− diet exposure for 20, 10, 4, 2, or 1 d, respectively, and killed on PND66. An additional group (n = 2) was fed the CAS-based diet as a reference.

Experiment 3. Pregnant rats (gestation d 4) were randomly assigned to 1 of the 2 groups (n = 6 per group) to either a CAS or SPI− diet throughout gestation and lactation. At birth, litters were culled to 6 male and 6 female pups per litter. Female offspring (n = 6 per group, PND21) were weaned to either CAS or SPI− diets until PND33. The effect of diet switching at weaning on AhR expression was evaluated.

Ex vivo model. Rat hepatoma FGC-4 cells (obtained from Mary C. Weiss, Pasteur Institute) were cultured in Dulbecco’s Modified Eagle’s Medium and fetal bovine serum (Invitrogen), as previously described (18). All the experiments were performed at 80% confluency from cell passages 9 to 14. After 24 h incubation in serum-free Dulbecco’s Modified Eagle’s Medium, cells were treated with serum from rats at the final concentration of 5%. Sera were pooled from rats fed with CAS (n = 6) or SPI− (n = 6) from the groups used in Experiment 1 and filter sterilized before use. For CYP1A1 induction, DMBA (1 μmol/L) was used after 4 h of serum treatment and cells were harvested after 20 h. For AhR half-life, cells were treated with cycloheximide (10 μg/mL) before 1 h of serum treatment and harvested at 0, 12, and 24 h.

RNA isolation and quantitative real-time PCR. Total RNA was isolated from nearly 100 mg of hepatic tissue using TRI reagent (Molecular Research Center). Procedures for RNA purification, cDNA synthesis, and primer design were as described by Eason et al. (19). Corresponding primer sequences are as follows: AhR (U04860): 5′-TTG AGT TGC TTC TTG GA-3′; R 5′-1170-AGT GAT GAT GTA ATC TGG TCT T-1146 3′; CYP1A1 (NM_012540): 5′-520-TCA GTA GCC TCA GAC ACA C-541 3′; R 5′-620-GCC ATC AGC TTC TGG AAC TTG-6003′; and GAPDH (AF106860): 5′-800-TGG GAC CGC ATC TTC TGG-820 3′. R 5′-901-TGG TAA CCA GGC GTC CGA TA-882 3′. mRNA levels were normalized to that of the GAPDH mRNA to control for input RNA.

Western blotting. Microsomes and tissue lysate were prepared from liver (300 mg) as described by Chippman et al. (20) and Pollenz et al. (21), respectively. Cell lysate was prepared as described by Song et al. (22). Proteins were solubilized in 2× solubilization buffer (21). Proteins (hepatic lysate AhR, 30 μg, microsomal CYP1A1, 10 μg, and cell lysate AhR and CYP1A1, 30 μg) were loaded on 8% and 16% nondenaturing gels, respectively, for AhR and CYP1A1, and transferred to nitrocellulose membrane. Blots were probed with rabbit anti-AhR (1:1 mg/L) or goat anti-CYP1A1 (1:1000) in Tris buffered saline with 0.1% Tween 20 containing nonfat milk powder and subsequently with saturating concentrations of corresponding HRP-conjugated secondary antibodies for 1 h. To normalize protein loading, the immunoblots were stripped by buffer consisting of 0.25 mol/L Tris, pH 6.8, 10% SDS, and β-mercaptoethanol and reprobed with goat anti-GAPDH (1:200) followed by HRP-conjugated bovine anti-goat (1:3000).

7-Ethoxyresorufin O-deethylase activity. CYP1A1-dependent 7-ethoxyresorufin O-deethylase (EROD) activity was determined using 7-ethoxyresorufin as a substrate. Hepatic microsomal protein (3 mg) was incubated with assay buffer consisting of 50 mmol/L potassium phosphate (pH 7.4) and 0.1 mmol/L EDTA followed by the addition of 20 μL of 1 g/L 7-ethoxyresorufin and 100 μmol/L NADPH. Resorufin formation was calculated from the area under the curve (AUC) of the fluorescence decay curve obtained using an RF-5301PC scanning spectrophotometer (Shimadzu Scientific Instruments) under the conditions of linearity for incubation time and protein.

Electrophoretic mobility shift assay. Nuclear extracts were prepared using Cell Lycik kit from Sigma. Double-stranded oligonucleotides were prepared by combining and heating equimolar amounts of the XRE complementary single stranded oligonucleotides (5′-GAGCTCGAGGGTGCGTTGAGAGGCC-3′ and 5′-GGCTCTTCTTCCAGCGGAACTCCC-3′) to 95°C for 5 min in nuclease-free water and cooling at room temperature overnight. Electrophoretic mobility shift assay (EMSA) was carried out in 20 μL of reaction volume using 40 μg of nuclear extract in conditions previously described by Backlund et al. (23). To determine the specific binding to XRE, a 200-fold excess of unlabeled XRE oligonucleotide was used.

Statistical analysis. Data presented here are expressed as means ± SEM. All data were analyzed using the Sigma Stat for Windows program (Jandel Scientific Software). Student’s t test was used to compare data from 2 groups. For multiple group comparisons, data were analyzed by 1-way or 2-way ANOVA with post hoc Student-Newman-Keuls tests. Differences were considered significant if P < 0.05.

Results
Reduced CYP1A1 activity, mRNA, and apoprotein induction in rats fed SPI− diet. Both constitutive and DMBA-induced...
CYP1A1 activities were reduced ($P < 0.05$, Fig. 1A) in livers of rats fed the SPI$_1$-containing diet compared with CAS-fed or SPI$_2$-fed rats. The decrease in activity in SPI$_1$-fed rats was associated with reductions ($P < 0.05$) in DMBA-induced CYP1A1 apoprotein (Fig. 1B) and mRNA levels (Fig. 1C), but the diets did not affect the constitutive expression of CYP1A1 apoprotein or mRNA.

SPI$_1$ inhibited interaction of AhR-ARNT complex with XRE. DMBA treatment resulted in the translocation of cytosolic AhR to the nucleus followed by an increased interaction between AhR-ARNT and the XRE of CYP1A1. The DMBA-induced AhR-ARNT protein/XRE interaction was reduced in the rats fed SPI$_1$ compared with the groups fed CAS. However, DMBA-induced AhR-ARNT binding to XRE was not affected in SPI$_2$-fed rats. Use of 200-fold higher unlabeled XRE completely abolished the binding of labeled XRE with the activated AhR-ARNT complex, indicating the specificity of the AhR-ARNT interaction with XRE double-stranded DNA (Fig. 2).

$\text{SPI}_1$ diet reduced basal AhR protein expression. We evaluated the effect of SPI$_1$ on the basal AhR expression in rats that were not gavaged with DMBA (Expt. 1). The basal AhR protein level in the total liver lysate was lower ($P < 0.05$) in the SPI$_1$-fed rats compared with CAS- or SPI$_2$-fed rats (Fig. 3).

Time course effect of $\text{SPI}_1$ on AhR expression. To evaluate the duration required for SPI$_1$-mediated AhR reduction, rats were fed the SPI$_1$ diet for different periods of time starting at...
PND46 (Expt. 2). Short-term SPI\(^+\) feeding of rats (from 1–10 d) did not affect AhR expression. Feeding rats the SPI\(^+\) diet for 20 d reduced (\(P < 0.05\)) AhR protein levels compared with SPI\(^+\)-feeding for fewer days (Fig. 4).

**Reduction in AhR expression by SPI\(^+\) is not due to metabolic imprinting.** Pups of CAS-fed dams that were weaned to SPI\(^+\) diets from PND21–PND33 had a 31% reduction (\(P < 0.05\)) in AhR protein expression compared with pups exposed to CAS throughout development [gestational day 4-postnatal day 3 (GD4-PND33)]. Rats fed SPI\(^+\) from GD4 to PND21 (in utero through weaning) and subsequently switched to CAS tended to have lower AhR expression than pups exposed to CAS throughout development (\(P = 0.065\), Fig. 5).

SPI\(^+\) mediated reduction in CYP1A1 induction and AhR expression in ex vivo model. A 25 and 50% reduction in DMBA-induced CYP1A1 protein (\(P < 0.05\), Fig. 6A) and CYP1A1 mRNA (\(P < 0.05\), Fig. 6A) occurred in the SPI\(^+\) serum-treated FGC-4 cells relative to the CAS serum-treated group.
AhR protein was reduced 24% (P < 0.05, Fig. 6B) by the SPI+ serum, but AhR mRNA was not affected. (Fig. 6B).

**SPI+ mediated temporal degradation of AhR in ex vivo model.** Cycloheximide treatment of FGC-4 cells followed by treatment with serum from SPI+ -fed rats reduced (P < 0.05) AhR protein levels compared with cells treated with CAS-fed rat serum at 12 and 24 h (Fig. 7).

**Discussion**

Soy has been shown to reduce xenobiotic-induced carcinogenesis in various in vivo and in vitro models (3,4,24–26). However, little is known about the mechanisms by which soy-based diets regulate the expression of CYP1A1 and essentially no data are available on the AhR-mediated pathway that has been implicated in carcinogenesis. In this study, we showed that feeding SPI+ reduces DMBA-induced CYP1A1 expression by reducing the basal AhR protein levels. This reduction in basal AhR protein levels is not imprinted metabolically.

Both soy protein (27,28) and soy-associated phytochemicals have been found to be anticarcinogenic (3); therefore, it is imperative to address which of these soy components contributes to the cancer protective effects. In a previous study, we demonstrated that rats fed SPI+ have a reduced CYP1A1 activity, but whether that reduction was due to bound phytochemicals or protein itself was not addressed (14,15). Therefore, in this study, AIN-93G diets made with SPI associated with (SPI+) or without (SPI-) phytochemicals were used to evaluate the involvement of phytochemicals in soy-mediated actions. The effects of 2 primary phytochemicals in soy, genistein and daidzein, on CYP1A1 expression in an in vitro model are somewhat controversial. Chan et al. reported a reduction in DMBA-mediated genotoxicity by inhibiting CYP1A1 and CYP1B1 in a mammary MCF-7 cell line upon treatment with genistein (29). However, a study performed with fermented soy extract showed no effect of soy on CYP1A1 abundance (30). We found lower DMBA induction of hepatic CYP1A1 activity, measured by EROD assay, in rats fed SPI+, but not SPI-, and treated with a single oral dose of DMBA. In the absence of an inducer, CYP1A1 is not expressed. Therefore, a reduction in constitutive EROD activity by SPI+, as observed here, could be explained by reduced expression of other cytochrome P450 enzymes affected by soy treatment in control female livers. Significant reductions in the DMBA-induced CYP1A1 apoprotein and mRNA occurred in the SPI+ -fed groups as compared with groups fed diets made with SPI- or CAS, whereas the diets did not affect the constitutive level of the enzyme. The findings from our study clearly suggest the involvement of soy phytochemicals, but not the protein itself, in regulating DMBA-induced CYP1A1 expression. However, which SPI+ phytochemicals or phytochemical metabolites are responsible for these effects remain to be determined.

CYP1A1 gene is transactivated by the binding of ligand-activated AhR-ARNT complex to its promoter region. However, benzimidazoles have also been shown to induce CYP1A1 by activating Src tyrosine kinase (31). Studies by Backlund et al. suggested that in hepatic H4IE cell line, genistein and daidzein efficiently inhibited OME-mediated but not AhR ligand-mediated induction of CYP1A1 (23). Results from EMSA suggest a less intense band in DMBA-gavaged SPI+ -fed rats as compared with that of SPI- or CAS-treated rats, which indicated reduced interaction of activated AhR-ARNT with CYP1A1 promoter in SPI+ -fed rats. This could be attributed to reduced AhR mRNA synthesis or stimulated AhR protein turnover. We found no differences at the basal AhR mRNA level (data not shown), whereas basal AhR protein was lower in SPI+ -fed rats compared with CAS and SPI- fed rats. This suggests that the SPI+ -rich diet-mediated reductions in basal AhR levels are at the posttranscriptional level. The lower basal level of the protein corresponds to a reduced DMBA-activated AhR-ARNT/XRE interaction and thus reduced induction of CYP1A1. To our knowledge, this is the first time that SPI+ has been shown to reduce AhR-mediated CYP1A1 induction by reducing AhR-ARNT binding to the XRE.

Soy has a number of protein-associated isoflavone glycosides that are hydrolyzed by the gut bacteria during digestion (32). Therefore, an in vitro model cannot mimic the in vivo SPI+ feeding model, because the soy extracts will not be metabolized appropriately. To overcome this drawback, we developed an ex vivo model using the FGC-4 rat hepatoma cell line. Because we wanted to replicate our in vivo findings in rats, we used a rat liver-based cell line. The FGC-4 cell line is derived from H4IEC3 hepatoma and expresses a wide spectrum of liver specific functions (33), especially cytochrome P450 enzymes (18). Similar to the in vivo findings, serum from SPI+ -fed rats reduced DMBA-mediated CYP1A1 induction at the mRNA and basal AhR protein expression without affecting the constitutive mRNA levels of AhR compared with CAS serum treated cells. Both in vivo and ex vivo data suggest SPI+ -mediated a posttranscriptional effect on AhR. To evaluate if the reduction was mediated posttranslationally, cells were treated with a protein synthesis inhibitor, cycloheximide. A significant time-dependent reduction in AhR protein levels occurred in cells treated with both sera and the reduction due to SPI+ serum treatment was greater at 12 and 24 h compared with CAS serum treatment. This suggests there is something bound to SPI that triggers a faster degradation of AhR protein.

We conducted further studies to evaluate how long it takes to observe SPI+ -mediated hepatic AhR degradation and we found that AhR is degraded rats fed with SPI+ for >20 d. Epidemiologic evidence suggests soy-mediated protection against breast cancer in adults even when exposure only occurred earlier in life (34). Moreover, early exposure to genistein had been shown to exert an imprinting effect on EGFr receptor expression (35). Also, we previously demonstrated a reduction in CYP1A1 activity by life-time consumption of SPI+ (14). Therefore, we hypothesized that SPI+ -mediated AhR reduction is imprinted metabolically over the generations. A significant reduction in AhR protein expression occurred in the group that was exposed to a SPI+ -rich diet after weaning from dams fed CAS compared with the group with developmental exposure to SPI+ and switched to CAS at weaning, suggesting no role of metabolic imprinting in SPI+ effects on AhR signaling.

AhR has been implicated in PAH-mediated DNA adduct formation (36), oxidative stress (37), and activation of protooncogenes such as c-myc, fos, and jun (38), demonstrating the oncogenic potential of AhR. Studies performed with transgenic mice suggest that both constitutive and activated AhR expression promotes hepatocarcinogenesis (39) and stomach tumors (40). Altogether, this suggests that AhR is a promising molecular target and a reduction in basal AhR levels by soy feeding, as suggested here, would be potentially therapeutic for the prevention of human diseases, such as cancer. Though the effect we observed on soy inhibition of AhR-mediated signaling is not a complete block, this appears to be 1 of the several mechanisms by which soy reduces carcinogenesis.

From this study, we conclude that consumption of SPI+ reduces AhR transactivation and CYP1A1 inducibility as a result of a reduction in AhR levels. These effects appear to require the
phytochemicals associated with the soy protein. This mechanism in part explains the anticarcinogenic actions of soy-based diets.

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Literature Cited