Genistein decreases basal hepatic cytochrome P450 1A1 protein expression and activity in Swiss Webster mice

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18 **Abbreviations**

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20 AhR; aryl hydrocarbon receptor

21 ARNT; aryl hydrocarbon receptor nuclear translocator

22 ChIP; chromatin immunoprecipitation

23 Cyp1a1; cytochrome P450 1A1

24 ER α ; estrogen receptor-alpha

25 EROD; ethoxyresorufin-O-deethylase

26 HAH; halogenated aromatic hydrocarbons

27 HCA; heterocyclic amines

28 HSP90; heat shock protein 90

29 MROD; methoxyresorufin-O-deethylase

30 NF-1; nuclear factor-1

31 PAH; polycyclic aromatic hydrocarbons

32 PCR; polymerase chain reaction

33 TCDD; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

34 XRE; xenobiotic response element

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Abstract

Soy consumption has been associated with risk reduction for chronic diseases such as cancer. One proposed mechanism for cancer prevention by soy is through decreasing cytochrome P450 1A1 (Cyp1a1) activity. However, it is not known with certainty which soy components modulate Cyp1a1, nor the characteristics or mechanisms involved in the responses following short-term (<20 d) dietary treatment without concomitant carcinogen-mediated induction. Therefore, the objective was to test the hypothesis that physiologic concentrations of dietary genistein and/or daidzein will decrease basal hepatic Cyp1a1 protein expression and activity in male and female Swiss Webster mice via inhibiting the bindings of aryl hydrocarbon receptor (AhR)-AhR nuclear translocator (ARNT) and estrogen receptor-alpha (ER α) to the *Cyp1a1* promoter region xenobiotic response element (XRE). The mice were fed the AIN-93G diet supplemented with 1500 mg/kg of genistein or daidzein for up to 1 wk. Genistein, but not daidzein, significantly decreased basal hepatic microsomal Cyp1a1 protein expression and activity. AhR protein expression was not altered. Molecular mechanisms were investigated in Hepa-1c1c7 cells treated with 5 μ mol/L purified aglycones genistein, daidzein, or equol. Cells treated with genistein exhibited inhibitions in ARNT and ER α bindings to the *Cyp1a1* promoter region. This study demonstrated that genistein consumption reduced constitutive hepatic Cyp1a1 protein expression and activity, thereby contributing to the understanding of how soy isoflavone aglycones modulate cytochrome P450 biotransformation enzymes.

Key words: aryl hydrocarbon receptor nuclear translocator; cytochrome P450 1A1; estrogen receptor-alpha; genistein; mouse; xenobiotic response element.

1. Introduction

Soy consumption has been associated with decreasing the risk for cancer [1]. A protective profile is suggested to be represented by a decrease in the bioactivation of procarcinogens via phase I metabolism [2], such as cytochrome P450 1A1 (Cyp1a1) which is the predominate enzyme responsible for converting procarcinogens into mutagenic forms that produce DNA adducts, potentially leading to carcinogenesis [3, 4].

Cyp1a1 induction is mediated by the aryl hydrocarbon receptor (AhR)-aryl hydrocarbon receptor nuclear translocator (ARNT) heterodimer which binds to the dioxin or xenobiotic response element (XRE) promoter region upstream of *Cyp1a1* [5]. Estrogen receptor-alpha (ER α) has been demonstrated to bind to the enhancer and TATA box regions of *Cyp1a1* in MCF-7 cells treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) + estradiol, thereby increasing gene expression [6]. Yet the role of ER α as a co-regulator with AhR of *Cyp1a1* target gene activation is not clear. It has also been reported that ER α is a transcription co-repressor [7]. Genistein, daidzein, and equol (a daidzein intestinal metabolite) interact with ER α [8] and it has been demonstrated that sera from rats fed soy protein isolate (with isoflavones) inhibit the binding of the AhR to the *Cyp1a1* XRE [9]. Additional studies using cells [10-13], rats [2, 14, 15], and mice [16] determined that soy and/or isoflavone treatments inhibit Cyp1a1. However, it is not

known with certainty which soy bioactive compound, genistein, daidzein, or other factors, are the components which modulate the Cyp1a1 enzyme, the time course and extent of response with short-term (<20 d) dietary exposure under basal conditions (i.e. not following carcinogen-mediated induction), and the mechanisms involved.

The aim of the current study was to examine hepatic Cyp1a1 activity and regulation in response to isoflavone aglycones genistein and daidzein. The main objective was to determine the differential effects of genistein and daidzein consumption on constitutive hepatic microsomal Cyp1a1 protein expression and activity in young male and female mice. We also sought to investigate the effects of the soy isoflavone aglycones on molecular mediators of Cyp1a1 induction or repression in a whole animal model and in a hepatic cell culture model. Specifically, we hypothesized that consumption of physiologic concentrations of genistein and/or daidzein aglycones will decrease hepatic Cyp1a1 protein expression and activity in young male and female mice under short-term (<20 d) feeding and basal conditions via inhibiting the bindings of AhR-ARNT and ER α to the *Cyp1a1* XRE promoter. To achieve this, young Swiss Webster mice were used as a model to study dietary phytochemical modulation of hepatic biotransformation enzymes under optimal health conditions of young adulthood. The molecular mechanisms modulating *Cyp1a1* induction were investigated in a murine liver cell line commonly used to investigate phase I metabolism. The dietary soy phytochemical treatment concentration of 1500 mg/kg diet has been demonstrated to produce average serum genistein concentrations of 1.12 μ mol/L in mice, which is within the range of serum genistein concentrations observed in humans following consumption of soy foods

($<1 \mu\text{mol/L}$ to a maximum of approximately $5 \mu\text{mol/L}$) [17-21]. The in vitro concentration of $5 \mu\text{mol/L}$ of genistein, daidzein, or the daidzein metabolite equol (**Figure 1**) is within the upper limit of physiological relevance.

2. Methods and Materials

2.1. Chemicals and reagents

Genistein, daidzein, equol, and resveratrol (all aglycones) were purchased from Indofine Chemical Company (Hillsborough, NJ). Flavone (2-Phenyl-4*H*-1-benzopyran-4-one), the backbone of the flavone class of flavonoids, was purchased from Sigma-Aldrich (St. Louis, MO), and was used as a positive control for the induction of Cyp1a1 in the mice. β -naphthoflavone, positive control for cell culture experiments, was also purchased from Sigma-Aldrich. Western blot gels and buffers were purchased from Bio-Rad Laboratories (Hercules, CA). Cell culture reagents, Cyp1a1 primers, probe, and real-time PCR reagents were purchased from Life Technologies (Carlsbad, CA). Cyp1a1, AhR, actin, and horseradish peroxidase-linked donkey anti-goat IgG antibodies, used for western immunoblotting, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ARNT, ER α , and nuclear factor-1 (NF-1) antibodies, used for ChIP, were purchased from Santa Cruz Biotechnology, whereas AhR antibody was obtained from Enzo Life Sciences (Farmingdale, New York). Unless noted otherwise, all other

chemicals were purchased from Sigma-Aldrich or Thermo Fisher Scientific (Waltham, MA).

2.2. Animals and diets

Male and female Swiss Webster mice (Charles River Laboratories, Wilmington, MA) were 8-10 weeks old, weighed 18-30 g, and were housed as sex-matched pairs in wire-bottom cages. The room temperature was maintained at 25°C with a 12 h on and 12 h off light cycle. The animal study protocol was approved by the University of California, Davis Animal Use and Care Committee. The Association for Assessment and Accreditation of Laboratory Animal Care guidelines were followed.

The control AIN-93G purified rodent diet (casein-based and phytoestrogen-free) met full needs for rodent growth and was purchased from Dyets (Bethlehem, PA). The mice were fed the control diet for 1 wk prior to feeding trials, and were allowed free access to food and water. The mice (n = 5 per group) were randomly assigned to one of the four treatment groups: control (AIN-93G) or control supplemented with 1500 mg/kg diet of flavone, genistein, or daidzein aglycones (**Table 1**). The mice were killed via CO₂ asphyxiation on day 1, 3, 5, or 7 after consumption of their assigned diet.

2.3. Tissue preparation and collection

Whole liver samples were perfused with phosphate-buffered saline (PBS) (0.14 mol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L KH₂PO₄, and 8.0 mmol/L Na₂HPO₄, pH 7.4), and

excised. They were frozen in liquid nitrogen and stored at -80°C until further analysis. Cytosolic (containing AhR) and microsomal (containing Cyp1a1) fractions were obtained using standard differential centrifugation as previously described [22]. In summary, whole liver samples were homogenized in 4 mL buffer (50 mmol/L Tris-HCl, 0.154 mol/L KCl, 1 mmol/L EDTA, pH 7.4) per g tissue with a motor-driven pestle. The liver homogenates were centrifuged at 10,000 x g for 20 min at 4°C. The supernatants were centrifuged at 100,000 x g for 60 min at 4°C. The cytosolic supernatants were stored at -80°C until further analysis. The microsomal pellets were removed and homogenized by hand with a pestle in 1 mL buffer [0.1 mol/L Sodium phosphate, 1 mmol/L EDTA, and 20% (v:v) glycerol, pH 7.4] per g tissue. The microsomal fractions were centrifuged at 100,000 x g for 60 min at 4°C, the supernatants removed, followed by homogenizing the pellets in 0.25 mL buffer per g fresh tissue. The homogenates were stored at -80°C until further analysis.

2.4. Enzyme activity assays

Assays measuring enzyme activities were performed using the HTS 7000 Plus Bio Assay Reader (PerkinElmer Life Sciences, Waltham, MA). Microsomal ethoxyresorufin-O-deethylase (EROD) and methoxyresorufin-O-deethylase (MROD) assays (pmol of resorufin formed per minute per mg of liver microsomal protein) were measured in 96 well plates. EROD assays were used to detect Cyp1a1 activity, whereas MROD assays measured Cyp1a2 activity. The formation of resorufin was detected spectrofluorimetrically at 540 nm (excitation) and 590 nm (emission) after 10 min at

37°C [23]. A resorufin standard curve was used to determine the pmol of resorufin formed, and these values were used to determine percent of control. Lack of EROD activity in cytosolic fractions confirmed effective microsomal separation. Cytosolic and microsomal proteins were measured using the Bio-Rad protein assay, based on the Bradford method (Bio-Rad Laboratories).

2.5. Western immunoblot analysis

Western immunoblots were performed on liver microsomal and cytosolic proteins obtained from whole liver homogenates at day 3. This time point was chosen due to the significant decreases in hepatic Cyp1a1 activity for both sexes. Protein (20 μ g microsomal protein/well or 75 μ g cytosolic protein/well) was loaded and separated on 12% SDS-PAGE gels (Bio-Rad Laboratories). The separated proteins were blotted onto PVDF membranes and incubated with blocking reagent [TBS (Tris Buffered Saline, with 5% milk and 0.05% Tween 20) Blotto A, Santa Cruz Biotechnology] overnight at 4°C. The proteins were probed with antibodies directed against microsomal Cyp1a1 (sc-9828; Santa Cruz Biotechnology), cytosolic AhR (sc-8089; Santa Cruz Biotechnology), and actin (sc-1616; Santa Cruz Biotechnology), which was used as a control, at a concentration of 1:200 for 1 h. The membranes were washed with TBS [10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% (v:v) Tween 20, pH 8.0]. The membranes were subsequently exposed to horseradish peroxidase-linked donkey anti-goat IgG antibodies (sc-2020; Santa Cruz Biotechnology) at a concentration of 1:5000 for 1 h, washed with TBS, followed by a TBS wash without Tween 20. Western blot signals

were detected using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Santa Cruz Biotechnology). Immunoquantitation was determined using the ChemiDoc XRS densitometric scanning imager (Bio-Rad Laboratories).

2.6. Cell culture and treatments

Hepa-1c1c7 cells were purchased from American Type Culture Collection, Manassas, VA. The cells were grown in 75 cm² flasks in an incubator at 37°C and 5% CO₂, with Minimum Essential Medium (MEM α), 10% fetal bovine serum, and 1% penicillin/streptomycin. The medium was changed every 3 days, while splitting the cells with trypsin when confluency was observed.

Cells were treated with control [0.1% (v:v) dimethylsulfoxide (DMSO)], 1 μ mol/L β -naphthoflavone (positive control), or 5 μ mol/L genistein, daidzein, or equol for 24 h, followed by RNA isolation and real-time PCR analysis. For the ChIP assays, Hepa-1c1c7 cells were exposed to control [0.1% (v:v) DMSO], 1 μ mol/L β -naphthoflavone, or 5 μ mol/L genistein, daidzein, equol, or resveratrol (positive control for ER α binding) for 1 h.

2.7. Chromatin immunoprecipitation assays

Hepa-1c1c7 cells were grown in three 100 mm culture dishes for each treatment until confluent. The medium was replaced with fresh medium containing the appropriate

phytochemical concentrations. The cells were treated with control [0.1% (v:v) DMSO], 1 μ mol/L β -naphthoflavone, or 5 μ mol/L genistein, daidzein, equol, or resveratrol for 1 h. The cells were processed using the ChIP-IT Enzymatic kit (Active Motif, Carlsbad, CA). Briefly, the procedure was initiated by cross-linking the proteins to DNA via the addition of 1% formaldehyde to the culture dishes. The cross-linking was stopped with glycine solution. The cells were scraped with a rubber policeman using 2 mL of scraping solution [PBS supplemented with 0.5 mmol/L phenylmethanesulfonyl fluoride (PMSF)], followed by centrifugation for 10 min at 720 x g. The protocol was continued according to the manufacturer's instructions. During the ChIP procedure, 2 μ g of the following antibodies were used: AhR (BML-SA210-0025; Enzo Life Sciences), ARNT (sc-8078 X; Santa Cruz Biotechnology), ER α (sc-542 X; Santa Cruz Biotechnology), NF-1 (sc-8986 X; Santa Cruz Biotechnology), RNA pol (supplied with ChIP-IT Control Kit; Active Motif), or IgG (supplied with ChIP-IT Control Kit; Active Motif). The antibodies were added to the pre-cleared chromatin in 0.65 mL siliconized tubes, and incubated overnight on a rotator at 4°C. The *Cyp1a1* XRE and promoter DNA (obtained from the ChIP protocol) were analyzed by PCR using primers stated previously [24] and Platinum Taq High Fidelity (Life Technologies). The cycle parameters were as follows: first cycle at 94°C for 2 min, 35 cycles at 94°C for 20 s, 55°C for 30 s, and 68°C for 30 s, and a final extension at 68°C for 7 min. PCR products were analyzed on a 1% agarose gel containing ethidium bromide, and visualized under UV illumination using the Bio-Rad imaging system.

2.8. RNA isolation and cDNA synthesis

Hepa-1c1c7 cells were seeded in six-well plates (10^6 cells per well) and grown in an incubator at 37°C and 5% CO₂ for 24 h. The medium was replaced with fresh medium containing the designated phytochemical concentration, followed by a 24 h treatment period. The mRNA was isolated by following the manufacturer's instructions for the RNeasy Plus mini kit (Qiagen, Valencia, CA). The concentrations and purity of RNA were detected by measuring the absorbance at 260 and 280 nm utilizing the HTS 7000 Plus Bio Assay Reader. Single-stranded cDNA was synthesized using oligo (dT) primers and SuperScript II Reverse Transcriptase (Life Technologies) by following the supplied instructions.

2.9. Quantitative real-time PCR analysis

Real-time PCR was utilized to quantify the amount of Cyp1a1 mRNA [National Center for Biotechnology Information (NCBI) Reference Sequence NM001136059.1] following the various treatments using primers and probe from Life Technologies. The cycle parameters were as follows: first cycle at 50°C for 2 min, followed by 95°C for 10 min, and 40 cycles at 95°C for 15 s, followed by 60°C for 1 min. The comparative Ct method was used to determine the fold changes for each treatment, with actin used as the internal standard.

2.10. Statistical analyses

All data were examined using SigmaStat version 3.5 for Windows (Systat Software, Chicago, IL). Data were analyzed using ANOVA, followed by Student-Newman-Keuls test for all pairwise comparison of means to detect differences compared to control. An α -level of 0.05 was used to determine significance. The data are represented as means $\pm$ standard deviation (SD). The graphs were prepared using GraphPad Prism version 5.02 for Windows (GraphPad Software, La Jolla, CA).

3. Results

3.1. Body and liver weights

The initial and final body weights, in addition to the liver/body weight ratios for each treatment group, did not differ compared to controls, as previously described [22].

3.2. Genistein decreased basal hepatic Cyp1a1 activity in male and female mice

Microsomal fractions derived from whole liver preparations were used to determine Cyp1a1 and Cyp1a2 activities from male and female mice fed 1500 mg/kg diet of flavone, genistein, or daidzein, for 1, 3, 5, or 7 d. Genistein significantly decreased basal hepatic Cyp1a1 (EROD) activity at each time point in the male mice by 64-79% of control ($P<.05$ -.001), and in the female mice by 50-63% of control ($P<.05$ -.001)

(average liver EROD specific activity for the control male mice was 58.8, whereas the females exhibited 49.3 pmol of resorufin/minute/mg of liver microsomal protein) (**Figures 2A & 2B**). In contrast, flavone increased Cyp1a1 activity in the males (except day 1) by 170-220% of control ($P<.001$), and in the females (at each time point) by 300-430% of control ($P<.001$). The only effect observed with daidzein treatment was an increase in Cyp1a1 activity in the male mice by 140% of control ($P<.05$) at day 7. Cyp1a2 activity was measured to compare with Cyp1a1 activity. There were no consistent Cyp1a2 activity changes for any treatment group compared to controls (**Figure 3**), as Cyp1a2 activation can occur through AhR-independent signal transduction pathways [25]. However, flavone decreased Cyp1a2 activity by 49% of control at day 1 ($P<.001$), whereas daidzein increased activity by 144% of control at day 3 ($P<.05$), in males. In contrast, daidzein decreased Cyp1a2 activity by 74% of control at day 1 ($P<.05$), and genistein decreased activity by 68% of control at day 5 ($P<.05$), in females.

3.3. Genistein decreased basal hepatic Cyp1a1 protein expression in male and female mice

Whole liver preparations were used to obtain microsomal fractions that were utilized to detect Cyp1a1 protein expression. Western immunoblots were performed to determine if the decreases in Cyp1a1 activity are concomitant with decreases in Cyp1a1 protein expression following genistein exposure. Whole liver microsomal fractions were analyzed from male and female mice fed 1500 mg/kg diet of flavone, genistein, or

daidzein, for 3 d. The male and female mice treated with genistein had significantly lower basal liver Cyp1a1 protein expressions by approximately 50% of control ($P<.001$) (**Figures 2C & 2D**). In contrast, there were no significant differences for the daidzein treated mice compared to controls. Despite increases found in liver Cyp1a1 activity following flavone treatment, western immunoblot results did not show an increase in Cyp1a1 protein expression.

3.4. Genistein and daidzein do not alter hepatic cytosolic AhR protein expression

Liver cytosolic fractions were analyzed from whole liver homogenates to determine if the isoflavones affect the expression of AhR. Western immunoblots were performed on whole liver cytosolic fractions from male and female mice fed 1500 mg/kg diet of flavone, genistein, or daidzein, for 3 d. There were no significant differences in hepatic AhR protein expression for any of the treatments compared to the control mice (**Figure 4**).

3.5. Genistein inhibited the bindings of ARNT and ER α to the Cyp1a1 xenobiotic response element

ChIP assays were utilized in Hepa-1c1c7 cells to determine a mechanism regarding the decreases in Cyp1a1 protein expression and activity in the male and female mice treated with genistein. The cells were exposed to control [0.1% (v:v) DMSO], 1 μ mol/L β -naphthoflavone, or 5 μ mol/L genistein, daidzein, equol, or resveratrol (positive control

for ER α binding) for 1 h. The ChIP results indicated that genistein inhibited the bindings of ARNT and ER α to the *Cyp1a1* XRE promoter region (**Figure 5**). There were no significant differences regarding AhR binding for the isoflavone treated cells compared to control. All other treatments increased the binding of ER α compared to control.

3.6. Daidzein and equol increased the bindings of NF-1, AhR, and RNA polymerase to the *Cyp1a1* promoter

ChIP assays were utilized in Hepa-1c1c7 cells to investigate if genistein decreases *Cyp1a1* protein expression and activity by inhibiting NF-1 and RNA polymerase bindings to the *Cyp1a1* promoter region. The cells were exposed to control [0.1% (v:v) DMSO], 1 μ mol/L β -naphthoflavone, or 5 μ mol/L genistein, daidzein, or equol for 1 h. The ChIP results demonstrated that genistein does not inhibit NF-1, RNA polymerase, and AhR bindings compared to control. In contrast, daidzein and equol treatments increased the bindings of NF-1, AhR, and RNA polymerase to the *Cyp1a1* promoter region (**Figure 6**).

3.7. Genistein, daidzein, and equol do not affect *Cyp1a1* mRNA abundance

Quantitative real-time PCR assays were utilized to determine if the decreases in *Cyp1a1* protein expression and activity observed in the male and female mice treated with genistein correspond to mRNA decreases in Hepa-1c1c7 cells exposed to the aglycone form of genistein. This analysis helps to provide insight into whether the decreases in

ARNT and ER α bindings to the *Cyp1a1* XRE by genistein affect transcription. Hepa-1c1c7 cells were treated with control [0.1% (v:v) DMSO], 1 μ mol/L β -naphthoflavone (positive control), or 5 μ mol/L genistein, daidzein, or equol for 24 h. Surprisingly, there were no significant changes detected in *Cyp1a1* mRNA abundance following genistein, daidzein, or equol treatments (**Figure 7**). In contrast, β -naphthoflavone increased *Cyp1a1* mRNA expression by 6-fold of control ($P<.001$).

4. Discussion

This study demonstrated that isoflavone-specific responses in basal activity of the *Cyp1a1* enzyme can occur after short-term treatments of bioactive soy phytochemicals in mice. The consumption of genistein decreased liver *Cyp1a1* protein expression and enzyme activity below the basal constitutive amounts observed with the control casein diet in male and female Swiss Webster mice. This provides additional evidence that genistein modulates hepatic metabolizing enzymes and has implications for disease prevention by dietary components such as soy. Singhal et al demonstrated that rats fed soy protein isolate (430 mg/kg diet of total isoflavones) for >20 d displayed significantly reduced constitutive *Cyp1a1* (EROD) activity [15]. The observation of a decrease in EROD activity is in agreement with the current study; however, our study design differs as we implemented short-term (<20 d) feeding trials consisting of casein + purified individual isoflavones (genistein or daidzein) to identify if these compounds affect constitutive EROD activity. The results from this current study suggest that genistein is a soy component that is associated with decreasing EROD activity in a rodent model.

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386 AhR expression has been shown to be downregulated in rats following long-term (>20
387 d) consumption of soy protein isolate (with isoflavones), thereby reducing AhR-driven
388 genes [14]. In our study, the AhR western immunoblots from the genistein and daidzein
389 treated mice were not significantly different compared to controls, consistent with
390 previous results using purified genistein or daidzein showing long-term consumption
391 does not affect AhR protein levels [9], whereas long-term feeding of soy protein isolate
392 (with isoflavones) does [9, 15]. These findings suggest that yet to be identified soy
393 protein associated phytochemicals are responsible for reduced AhR signaling by a
394 mechanism which interferes with AhR-ARNT heterodimerization and XRE binding, with
395 resultant targeting of AhR for ubiquitination and subsequent degradation [9, 15].

396

397 A potential explanation for the down regulation of Cyp1a1 protein expression and
398 enzyme activity by genistein in this study is tyrosine kinase inhibition [26]. There is
399 support for tyrosine kinase inhibition, given that signal transduction pathways involving
400 protein kinases have the ability to mediate activation of the AhR, particularly when low-
401 affinity AhR ligands are involved in the process of xenobiotic induction [27]. These
402 effects would impair nuclear translocation of the AhR, and therefore decrease binding to
403 the XRE which could result in a decrease in *Cyp1a1* transcription. In human primary
404 keratinocytes, genistein has been demonstrated to decrease the binding of the AhR to
405 the XRE by preventing the dissociation of heat shock protein 90 (HSP90) from the AhR
406 in a tyrosine kinase inhibitory manner [28].

407

However, based on our ChIP results, genistein did not decrease the binding of the AhR to the XRE compared to control. Interestingly, genistein inhibited ARNT and ER α bindings to the XRE. To our knowledge, this is the first study to demonstrate that genistein inhibits the bindings of ARNT and ER α to the *Cyp1a1* promoter region. It has been shown that there is interference in AhR-ARNT heterodimerization from rats fed soy protein isolate (with isoflavones) [9, 15]. Our results suggest that genistein is a component of soy that may interfere with AhR-ARNT heterodimerization, as it inhibited the binding of ARNT to the XRE; however, it did not inhibit the binding of AhR. It has also been demonstrated that sera from the rats fed soy protein isolate decreased the binding of the AhR to the *Cyp1a1* XRE compared to control [9]. This result differs from our study in which purified genistein, daidzein, and equol did not affect constitutive AhR binding. These observations suggest that other soy phytochemicals or their metabolites, not the aglycone forms of genistein, daidzein, or equol, are responsible for decreasing AhR binding to the *Cyp1a1* XRE. In our study, treatment with β -naphthoflavone surprisingly did not result in a concomitant binding of the AhR (as well as ARNT) to the *Cyp1a1* XRE, yet mRNA expression was increased as expected. These results may be due to cell type, treatment concentration, treatment duration, or other experimental conditions.

ER α has been demonstrated to increase *Cyp1a1* expression [6], and this is in agreement with our study as every treatment (except genistein) increased ER α binding to the *Cyp1a1* XRE compared to control. The ER has also been shown to increase AhR responsiveness by “squenching” HSP90 [29]. Possible explanations for genistein

inhibiting the binding of ER α include directing ER α away from the *Cyp1a1* promoter to estrogen-responsive genes, which decreases transcription of *Cyp1a1* [30], and/or degradation of ER α via a proteasome-dependent mechanism [29]. Interestingly, ER α down-regulation has also been recently observed in MCF-7 cells treated with indole-3-carbinol, which is a phytochemical found in cruciferous vegetables [31].

NF-1 is involved in both ER- and AhR-mediated transcription. The ER has also been indicated to “sequester” available NF-1, which reduces the binding of the AhR complex to *Cyp1a1*, and directs NF-1 away from the *Cyp1a1* promoter to estrogen-responsive genes [30]; however, in this study, genistein did not differ compared to control regarding NF-1 and AhR bindings to the *Cyp1a1* promoter. In contrast, the increased bindings of NF-1, AhR, and RNA polymerase to the *Cyp1a1* promoter following daidzein and equol treatments, might explain the increased *Cyp1a1* activity observed in the male mice treated with daidzein after day 7. The fine-tuning by transcription factors is complex and depends on many factors, such as cell-type, growth conditions, genes, species, sex, and age [29]. More research is needed to further explain the AhR-ER α cross-talk mechanisms.

The observation of genistein not causing a change in constitutive *Cyp1a1* mRNA abundance is in agreement with studies involving genistein or soy treatments in human intestinal Caco-2 cells [32], monkeys [33], and rats [15, 34]. In contrast, genistein (10 μ mol/L) increased *Cyp1a1* mRNA in MCF-7 cells [35], as well as in rats fed a standardized soy extract (37% isoflavones expressed as genistein) 100 mg/kg p.o for

up to 21 days [36]. Caco-2 cells [32] and V79-MZ cells (transfected with human CYP enzymes) [33] treated with less than 10 $\mu\text{mol/L}$ of genistein, did not significantly differ compared to controls regarding Cyp1a1 activity. Furthermore, Hepa-1c1c7 cells transfected with the *Cyp1a1* dioxin/xenobiotic response element and treated with 1 $\mu\text{mol/L}$ of genistein or daidzein, did not influence relative luciferase activity. In contrast, treatment with non-physiologic concentrations (10 $\mu\text{mol/L}$) of genistein and daidzein significantly increased luciferase activity [37]. These results underscore the importance of in vitro treatments at physiologic concentrations, as used in this study.

This current investigation provides insights regarding the in vivo and in vitro effects of purified soy isoflavones on Cyp1a1 modulation. We have shown that consumption of genistein decreased basal hepatic Cyp1a1 protein expression and activity in male and female Swiss Webster mice. To help explain the mechanisms associated with these decreases, ChIP assays demonstrated that genistein inhibited the bindings of ARNT and ER α to the *Cyp1a1* XRE promoter region. However, these decreased bindings did not correspond with reduced Cyp1a1 mRNA expression in Hepa-1c1c7 cells treated with genistein compared to control. The in vitro results suggest that the decreases in Cyp1a1 protein expression and activity observed in the mice are not solely caused by the aglycone form of genistein. It has been suggested that soy phytochemicals (other than genistein and daidzein aglycones) or metabolites are responsible for interfering with AhR binding to the XRE, and subsequently decreasing *Cyp1a1* induction [9]. Our investigation further adds to the suggestion that genistein metabolites or conjugates (e.g., glucuronides and/or sulfates), in part, may be responsible for decreasing Cyp1a1

protein expression and activity in mice. In a previous study, rats fed soy protein isolate, with genistein at approximately 13 mg/kg bodyweight, exhibited a genistein serum profile of 50.4% glucuronides, 45.9% sulfates, and 3.6% aglycones. Female humans displayed a genistein serum profile of 78.4% glucuronides, 20.4% sulfates, and 1.2% aglycones following consumption of a soy beverage containing 1 mg/kg bodyweight of genistein. It is not known if these sulfate and glucuronide conjugates are bioactive in vivo; however, in vitro experiments have demonstrated that genistein and daidzein sulfoconjugates exhibit biological activity [38]. Cell-type and/or experimental conditions may also account for contrasting outcomes. For example, in MCF-7 and Hepa-1c1c7 cells, ER α has been shown to exert differing effects on *Cyp1a1* induction following treatments of estradiol + TCDD [6, 7, 29]. It is acknowledged that the in vitro effects of the aglycone form of genistein observed in this study may differ from the effects that occur in vivo. Additional studies need to be performed regarding the impact of genistein metabolites on *Cyp1a1* expression and activity, as this current study utilized isoflavone aglycones that were commercially available at the time of the experimental process.

The results of this investigation have an interesting application to human nutrition. Carcinogens activated by CYP1A1 include polycyclic aromatic hydrocarbons (PAH), halogenated aromatic hydrocarbons (HAH), and heterocyclic amines (HCA) [39, 40]. In the United States, a major source of PAH [41] and HCA [42] is grilled or barbecued meat. Significant amounts of PAH and HAH have also been detected in automobile exhaust, tobacco smoke, industrial wastes, and air pollution [4, 40]. Additionally, human *CYP1A1* polymorphisms have been identified which increase the risk of certain cancers,

due to increased CYP1A1 activity [3, 39, 43, 44]. Individuals exposed to potential environmental procarcinogens and/or possess *CYP1A1* polymorphisms may increase the production of carcinogens in the human body. Therefore, consumption of soy can mitigate detrimental gene-environment interactions by reducing CYP1A1 activity - decreasing the risk factors for cancer.

This study is not without limitations. The serum isoflavone concentrations were not measured due to technical restrictions. Additionally, the ChIP results were not confirmed with real-time PCR. Liver mRNA was not isolated from the animal study, preventing direct comparison between the cell and animal model data with regard to mRNA response to phytochemical treatment. The inherent difficulties of extrapolating experimental data using aglycone bioactives from in vitro work to whole animal metabolism and human physiologic concentrations of dietary phytochemicals are acknowledged.

In conclusion, we accept the research hypothesis that dietary genistein at a concentration within the expected physiologic range decreases hepatic *Cyp1a1* protein expression and activity in young male and female mice under short-term feeding and basal conditions and one mechanism may be via inhibiting the bindings of AhR-ARNT (specifically ARNT) and ER α to the *Cyp1a1* promoter region, based on our data from an in vitro hepatic cell model (Hepa-1c1c7). These findings extend previous knowledge regarding effects of physiologic concentrations of dietary isoflavones on *Cyp1a1* by demonstrating modulation of constitutive enzyme levels. This has implications for

metabolism of the bioactive compounds themselves, in addition to chemoprevention against carcinogenic dietary and environmental compounds. Further studies are needed to add additional insights into the molecular mechanisms of phase I modulation by isoflavones, their metabolites or conjugates, and other soy protein associated phytochemicals.

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Figure captions

Figure 1: The chemical structures for genistein (A), daidzein (B), and equol (C). The chemical structures were created using ChemDraw Pro 13.0 Suite for Windows (PerkinElmer Informatics, Waltham, MA).

Figure 2: Hepatic microsomal Cyp1a1 activity and protein expression from Swiss Webster mice (n = 5 per group) fed control (AIN-93G), or control supplemented with 1500 mg/kg diet of flavone, genistein, or daidzein for 1, 3, 5, or 7 d. Microsomal Cyp1a1 enzyme activity was measured by the ethoxyresorufin-O-deethylase (EROD) assay for male (A) and female (B) Swiss Webster mice. Genistein significantly decreased basal hepatic Cyp1a1 (EROD) activity at each time point in the male mice by 64-79% of control ($P < .05$ -.001), and in the female mice by 50-63% of control ($P < .05$ -.001). Microsomal Cyp1a1 protein expression was detected via western immunoblots for the males (C) and females (D) at day 3 using 20 μ g microsomal protein, with actin used as a control (data not shown). Male and female mice treated with genistein had significantly lower basal liver Cyp1a1 protein expressions by approximately 50% of control ($P < .001$). The data are represented as means $\pm$ SD. Treatment groups with a * ($P < .05$) or ** ($P < .001$) indicates a difference compared to control. An α -level of 0.05 was used to determine significance, followed by ANOVA and Student-Newman-Keuls test.

Figure 3: Hepatic microsomal Cyp1a2 activity from Swiss Webster mice (n = 5 per group) fed control (AIN-93G), or control supplemented with 1500 mg/kg diet of flavone, genistein, or daidzein for 1, 3, 5, or 7 d. Microsomal Cyp1a2 enzyme activity was

measured by the methoxyresorufin-O-deethylase (MROD) assay for male (A) and female (B) Swiss Webster mice. There were no consistent changes in Cyp1a2 (MROD) activity for any of the treatment groups compared to controls. However, flavone treatment decreased Cyp1a2 activity by 49% of control at day 1 ($P<.001$), whereas daidzein treatment increased activity by 144% of control at day 3 ($P<.05$), in males. In contrast, daidzein treatment decreased Cyp1a2 activity by 74% of control at day 1 ($P<.05$), and genistein treatment decreased activity by 68% of control at day 5 ($P<.05$), in females. The data are represented as means $\pm$ SD. Treatment groups with a * ($P<.05$) or ** ($P<.001$) indicates a difference compared to control. An α -level of 0.05 was used to determine significance, followed by ANOVA and Student-Newman-Keuls test.

Figure 4: Representative hepatic cytosolic AhR protein expression from Swiss Webster mice (n = 5 per group) fed control (AIN-93G), or control supplemented with 1500 mg/kg diet of flavone, genistein, or daidzein for 3 d. Western immunoblots were used to detect AhR protein expression from male (A) and female (B) mice using liver cytosolic fractions from day 3 using 75 μ g cytosolic protein, with actin used as a control (data not shown). There were no significant differences in AhR protein expression for any of the treatment groups compared to the control mice. An α -level of 0.05 was used to determine significance, followed by ANOVA and Student-Newman-Keuls test.

Figure 5: Representative chromatin immunoprecipitation (ChIP) assay results from Hepa-1c1c7 cells treated with control (C) [0.1% (v:v) DMSO], 1 μ mol/L β -naphthoflavone (NF), or 5 μ mol/L genistein (G), daidzein (D), equol (E), or resveratrol

(R) for 1 h. Aryl hydrocarbon receptor (AhR) (A), AhR nuclear translocator (ARNT) (B), estrogen receptor- α (ER α) (C), and negative IgG (D) antibodies (2 μ g each) were used to pull-down the appropriate protein-DNA complexes. Input DNA (E) was used as the loading control. PCR reactions were conducted on the ChIP DNA using primers specific to the *Cyp1a1* upstream region encompassing the xenobiotic response element (XRE). Genistein inhibited the bindings of ARNT and ER α to the *Cyp1a1* promoter region. The other treatments increased the binding of ER α compared to control ($P < .001$). There were no significant differences regarding AhR binding for the isoflavone treated cells compared to control. The band densities were measured, with the data represented as means $\pm$ SD from three separate experiments. The treatment groups with a ** ($P < .001$) indicates a difference compared to control. An α -level of 0.05 was used to determine significance, followed by ANOVA and Student-Newman-Keuls test. (ND = not detected).

Figure 6: Representative chromatin immunoprecipitation (ChIP) assay results from Hepa-1c1c7 cells treated with control (C) [0.1% (v:v) DMSO], 1 μ mol/L β -naphthoflavone (NF), or 5 μ mol/L genistein (G), daidzein (D), or equol (E) for 1 h. Aryl hydrocarbon receptor (AhR), nuclear factor-1 (NF-1), RNA polymerase, and negative IgG antibodies (2 μ g each) were used to pull-down the appropriate protein-DNA complexes. Input DNA was used as the loading control. PCR reactions were conducted on the ChIP DNA using primers specific to the *Cyp1a1* promoter region. Daidzein and equol treatments increased the bindings of NF-1, AhR, and RNA polymerase to the

Cyp1a1 promoter region, whereas genistein treatment did not differ compared to control. The data are a representation from three separate experiments.

Figure 7: Quantitative real-time PCR assay results from Hepa-1c1c7 cells treated with control [0.1% (v:v) DMSO], 1 μ mol/L β -naphthoflavone (NF), or 5 μ mol/L genistein, daidzein, or equol for 24 h. Real-time PCR primers and probe were specific for the mouse *Cyp1a1* gene. There were no significant changes detected in *Cyp1a1* mRNA following genistein, daidzein, or equol treatments. In contrast, β -naphthoflavone increased *Cyp1a1* mRNA expression by 6-fold of control ($P<.001$). The data are represented as means $\pm$ SD from three experiments repeated in triplicate. The treatment group with a ** ($P<.001$) indicates a difference compared to control. An α -level of 0.05 was used to determine significance, followed by ANOVA and Student-Newman-Keuls.

Table 1.

Ingredient composition of the diets administered to mice (g/kg)

	AIN-93G	AIN-93G + phytochemical
Phytochemical	-	1.5 (Flavone, genistein, or daidzein)
Casein	200	200
Dyetrose	132	132
Corn starch	397.5	397.5
Sucrose	100	100
Cellulose	50	50
Soybean Oil	70	70
t-Butylhydroquinone	0.014	0.014
Mineral mix	35	35
Vitamin mix	10	10
L-Cystine	3	3
<u>Choline Bitartrate</u>	<u>2.5</u>	<u>2.5</u>

The AIN-93G purified rodent diet was purchased from Dyets (Bethlehem, PA). Flavone (2-Phenyl-4*H*-1-benzopyran-4-one) was purchased from Sigma-Aldrich (St. Louis, MO). Purified isoflavone aglycones, genistein and daidzein, were obtained from Indofine Chemical Company (Hillsborough, NJ).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7