Hepatic gene expression following consumption of soy protein isolate in female Sprague–Dawley rats differs from that produced by 17β-estradiol treatment

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Abstract

Although soy foods have been recognized as an excellent source of protein, there have been recent concerns regarding potential adverse effects of isoflavone phytochemicals found in soy products, which are known to bind and activate estrogen receptors. Here, we used global hepatic gene expression profiles in ovariectomized female Sprague–Dawley rats treated with 17β-estradiol (E_2) or fed with soy protein isolate (SPI) as a means of estimating potential estrogenicity of SPI. Female Sprague–Dawley rats were fed AIN-93G diets containing casein (CAS) or SPI starting at postnatal day (PND) 30. Rats were ovariectomized on PND 50 and infused with E_2 or vehicle in osmotic pumps for 14 d. Microarray analysis was performed on liver using Affymetrix GeneChip Rat 230 2.0. Serum E_2 levels were within normal ranges for the rat and SPI feeding did not increase uterine wet weight in the absence or presence of E_2. SPI feeding altered ($P<0{\cdot}05$, $\geq \pm 1{\cdot}5$-fold) the expression of 82 genes, while E_2 treatment altered 892 genes. Moreover, only 4% of E_2-affected genes were also modulated by SPI, including some whose expression was reversed by SPI feeding. The interaction between E_2 and SPI uniquely modulated the expression profile of 225 genes including the reduction of those involved in fatty acid biosynthesis or glucocorticoid signaling and an induction of those involved in cholesterol metabolism. The different hepatic gene signatures produced by SPI feeding compared with E_2 and the lack of increase in uterine wet weight in rats fed with SPI suggest that SPI is not estrogenic in these tissues.

Journal of Endocrinology (2009) **202,** 141–152

Introduction

Soy is a popular dietary constituent in Asian countries such as Japan, Korea, and China. Lately, there has been an increasing use of soy products in western diets as the result of purported health benefits. There is epidemiological evidence suggesting that soy consumption reduces the risk of hormonal cancers, such as breast, ovarian, and prostate cancers, in addition to chronic diseases such as obesity, atherosclerosis, diabetes, and renal disease (Clair & Anthony 2005, Goetzl *et al.* 2007, Velasquez & Bhathena 2007). Mechanistic laboratory studies suggest that soy-associated phytochemicals (>137; Fang *et al.* 2004), and soy protein, independently or synergistically, may be responsible for the plethora of beneficial effects associated with soy consumption. Some of the phytochemicals associated with soy such as isoflavones, saponins, and phytosterols have endocrine actions (Munro *et al.* 2003). The isoflavones – genistein and daidzein, as well as the daidzein metabolite equol – have molecular structures similar to endogenous estrogens and bind to estrogen receptors (ER) α and β (Setchell 2001). They have been shown to be estrogenic, anti-estrogenic, or partial agonists depending upon the tissue, cell type, isoflavone concentration, and other conditions, such as age and hormonal status (Barkhem *et al.* 1998, Patisaul *et al.* 2001, Hwang *et al.* 2006). Due to this property, they are often referred to as 'selective ER modulators (SERMs)' (Setchell 2001). There has been considerable interest in the estrogenic activity of diets that contain soy isoflavones with regard to potential for alleviation of post-menopausal symptoms. However, the same property has raised legitimate concerns regarding estrogenization in infants fed soy formula, males, and pre-pubertal females (Munro *et al.* 2003, McLachlan *et al.* 2006).

Isoflavones occur in soy protein isolate (SPI) and other soy products as polar β-glycoside conjugates, such as genistin and daidzin. Bacterial hydrolysis of these isoflavones, in the GI tract, releases the principal bioactive aglycones, genistein, and daidzein. These aglycones and bacterial metabolites such as the daidzein metabolite equol are absorbed from the intestinal tract and conjugated in both the intestinal mucosa and the

DOI: 10.1677/JOE-09-0059
0022–0795/09/0202–141
Online version via http://www.endocrinology-journals.org

liver mainly to glucuronides and sulfates (Ronis *et al.* 2006); only 1–5% of the aglycones remain unconjugated. Fermented soy products such as miso and tempeh, may contain a higher concentration of aglycones (Barrett 2006, Rozman *et al.* 2006). The route and concentration of exposure to isoflavones, the animal model, soy processing, gut microflora, and endogenous estrogenic status are among many factors that play crucial roles in determining health outcomes of soy consumption. Soy foods have a long history in Asia of being safe, and evidence continues to mount regarding the health benefits of soy consumption. By contrast, data generated from purified isoflavones, used at pharmaceutical concentrations and/or injected into rodents, suggest a risk of reproductive dysfunctions including endometrial and uterine hyperplasia in females and reduced sperm count in males (Delclos *et al.* 2001, Nagao *et al.* 2001, Unfer *et al.* 2004).

Previous observations from our laboratory suggested reduced carcinogenicity and atherosclerosis in animals fed soy diets (Badger *et al.* 2005, Singhal *et al.* 2008*a*). Moreover, we have reported no compromise of cognitive function in infants fed soy formula (Jing *et al.* 2008). Nonetheless, the safety concerns regarding potential estrogenicity of soy foods cannot be ignored. The present study examines the question – is SPI estrogenic in the liver? The objective was to compare the effect of SPI diets with actions of the endogenous estrogen, 17β-estradiol (E_2). Mature ovariectomized female Sprague–Dawley rats were fed with a diet containing SPI and followed by E_2 infusion in half of the rats. The effects of feeding SPI in the presence or absence of E_2 were compared with E_2 using uterine wet weight and hepatic gene expression profile.

Materials and Methods

Animal care and experiment design

The experiment received prior approval from the Institutional Animal Care and Use Committee at UAMS. Adult female Sprague–Dawley rats were purchased from Charles River Laboratories and were fed diets made with casein (CAS) or SPI. Semi-purified diets were made according to the AIN-93G formula (Reeves *et al.* 1993) except that corn oil replaced soybean oil and the protein source was either CAS or SPI (Singhal *et al.* 2007). The SPI diet had 430 mg total isoflavones/kg containing 276 mg/kg genistein and 132 mg/kg daidzein. Average daily consumption of genistein and daidzein were 19·3 mg/kg per day and 9·2 mg/kg per day respectively. Total isoflavone concentrations in 24-h urine pools was ∼40 μmol/l (Ronis *et al.* 2001).

Rats were fed SPI or CAS diets ($N=12$ in each group) from postnatal day (PND) 30 until PND 64. On PND 50, rats were ovariectomized. Half of each group was subcutaneously infused with E_2 and half with polyethylene glycol vehicle (Sigma) using Alzet 2002 mini-osmotic pumps (Alza Corp., Mountain View, CA, USA) that were calibrated to release 0·5 μl/h for 14 d to produce an E_2 dose of 5 μg/kg per day. On PND 64, all the rats were anesthetized with Nembutal (100 mg/kg, i.p.) followed by decapitation. Blood, liver, and uterus were collected and frozen at −80 °C until analysis. Treatment groups were designated as: 1) Control, CAS diet; 2) E_2, E_2 treatment on CAS background; 3) SPI, SPI diet; and 4) SPI + E_2, E_2 treatment on SPI background.

Isoflavone and E_2 analyses

Serum isoflavones (total serum isoflavones) were measured by LC–MS or electrochemical detection after conjugate hydrolysis, as described in detail by Gu *et al.* (2006). Serum E_2 levels were determined by ultra-sensitive RIA, using DSL-4800 kit (Diagnostic Systems Laboratories, Webster, TX, USA) following the manufacturer's protocol. The kit has a lower detection limit of 2·2 pg/ml of E_2.

Microarray preparation, normalization, and data analysis

Microarray preparation and data analysis were performed following minimum information about microarray experiments – supportive relational database (Brazma *et al.* 2001). Total RNA was isolated and cleaned using ∼100 mg hepatic tissue using TRI reagent (Molecular Research Center Inc, Cincinnati, OH, USA) from three rats in each treatment group, as described previously (Singhal *et al.* 2008*b*). First- and second-strand cDNA synthesis, biotin-labeled cRNA synthesis, fragmentation of cRNA, and hybridization reactions were performed using one-cycle cDNA synthesis kit (Affymetrix Inc, Santa Clara, CA, USA). Briefly, 8 μg purified RNA was used to synthesize cDNA. Labeled cRNA was synthesized from cDNA using a GeneChip IVT labeling kit (Affymetrix) according to the manufacturer's instructions. Twenty micrograms cRNA was then fragmented in a solution of 5× fragmentation buffer and RNase for 35 min. Complementary RNAs from each rat liver ($n=3$) were hybridized to an individual Affymetrix GeneChip Rat genome 230 2.0. for 16 h at 45 °C in the hybridization oven set at 60 r.p.m. The probe array was washed and stained using Affymetrix kit in GeneChip fluidics station 450 and scanned using GeneChip Scanner 3000. For each of the 31 099 genes on the Affymetrix Rat genome 230 2.0 array, the data on induction or repression values were analyzed using GeneChip Operating Software obtained from Affymetrix.

The data files (.CEL files) containing the probe-level intensities were processed using the robust multiarray analysis algorithm (GeneSpring 7.3×, Agilent Technologies Inc., Wilmington, MA, USA) for background correction, normalization, and log2 transformation (Irizarry *et al.* 2003). Subsequently, the data were subjected to per-chip and per-gene normalization using GeneSpring normalization algorithms. A list of differentially expressed genes was generated by performing one-way ANOVA ($P<0·05$; Welch) analysis followed by Benjamini and Hochberg false discovery rate and Student–Newman–Keuls multiple testing correction. This list was used to make comparisons between various treatments: SPI versus control; E_2 versus control; and

SPI+E_2 versus control, criteria: $>+1{\cdot}5$ or $<-1{\cdot}5$ (i.e. $\pm 1{\cdot}5$), and $P<0{\cdot}05$. This list of differentially expressed genes was used to evaluate the pattern of gene expression profile by hierarchical clustering, using GeneSpring software or Cluster 2.1.1 and 'Tree View' version 1.60 software supplied by Eisen Lab, Stanford University (http://rana.lbl.gov/EisenSoftware.htm). The top networking pathways, canonical functions, and top molecular functions were determined by Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc, Redwood City, CA, USA).

Microarray validation by real-time PCR

Total RNA (1 μg) was reverse-transcribed using iSCRIPT cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. cDNA samples were amplified using previously described conditions (Singhal *et al.* 2008*a*). RT-PCR was performed on nine randomly chosen genes, some of them were of interest and discussed in the results and discussion section. Expression levels of genes were normalized to *Gapdh* gene levels.

Statistical analysis

Statistical analysis was performed using Sigma Stat software package (Systat Software Inc, San Jose, CA, USA). Data were analyzed by two-way ANOVA followed by Student–Newman *post hoc* test and were considered significant if $P<0{\cdot}05$. Differences between treatment groups in microarray data were analyzed by 'fold changes in volcano plot' (GeneSpring Software) and changes were considered significant at $\pm 1{\cdot}5$-fold and $P<0{\cdot}05$ followed by Benjamini and Hochberg multiple testing correction for false discovery rate.

Results

Organ weight and serum E_2 levels

E_2 infusion in ovariectomized rats for 14 d resulted in a reduction ($P<0{\cdot}05$) in body weight (BW) while the relative liver and wet uterus weights (organ weight normalized to the BW) were increased ($P<0{\cdot}05$), as compared with the non-E_2 infused groups fed with either CAS or SPI diets, $n=6$. SPI feeding, in the presence or absence of E_2 infusion, did not have any effect on total BW or relative liver and uterus weights. Serum E_2 levels were higher ($P<0{\cdot}05$) in the group infused with E_2 compared with non-E_2 groups; no effect of SPI feeding was observed on the serum E_2 levels. Moreover, no interactions, on either parameter mentioned here, were observed between SPI and E_2, statistically determined by two-way ANOVA (Table 1).

Serum isoflavones

When fed with SPI the total isoflavone concentrations (aglycone+conjugates) of genistein, daidzein, equol, and glycetin were $0{\cdot}4\pm0{\cdot}081$, $0{\cdot}26\pm0{\cdot}053$, $0{\cdot}24\pm0{\cdot}7$, $0{\cdot}04\pm0{\cdot}005$ μg/ml respectively. SPI feeding in the presence of E_2 increased ($P<0{\cdot}05$, $n=6$) the levels of equol to $0{\cdot}57\pm0{\cdot}07$ μg/ml while genistein, daidzein, and glycetin were $0{\cdot}26\pm0{\cdot}063$, $0{\cdot}16\pm0{\cdot}025$, and $0{\cdot}02\pm0{\cdot}002$ μg/ml respectively. Two-way ANOVA suggested an interaction between SPI and E_2 resulting in an increase in equol levels. No effect of E_2 on the summed total serum isoflavone levels, $1{\cdot}27\pm0{\cdot}19$ μg/ml by SPI feeding in the absence of E_2 and $1{\cdot}35\pm0{\cdot}24$ μg/ml by SPI feeding in the presence of E_2, was observed (Table 2).

Hierarchical clustering

The list of 'differentially expressed genes' contained 1160 genes with known biological functions. Correlation-based unsupervised hierarchical clustering analysis was performed on the treatment (*x*-axis) and gene expression type (*y*-axis). The E_2 treated groups – E_2 (CAS+E_2) and SPI+E_2 – clustered together, while the control (CAS) and SPI clustered together, suggesting greater common effects of E_2 treatment on hepatic genes irrespective of the diets. Using pseudogene lines, the heat-map was divided into four sub-clusters: I – genes induced by E_2; II – genes induced by SPI; III – genes repressed by E_2; and IV – genes repressed by SPI (Fig. 1A).

Global hepatic gene signature of SPI versus E_2

E_2 treatment resulted in significant modulation (fold change – induction or repression by more than 1·5-fold; and $P<0{\cdot}05$) of the expression profile of 892 genes; 188 upregulated and 704 downregulated. The top three networks with maximum number of genes affected, as identified by GeneSpring and IPA, were: metabolism and transport; cellular

Table 1 Relative organ weights and serum estradiol (E_2) levels of rats with different treatments

	Body weight	% Liver weight	% Uterus weight	E_2 (pg/ml)
Groups				
CAS	$291\pm9{\cdot}1^a$	$4{\cdot}3\pm0{\cdot}14^a$	$0{\cdot}07\pm0{\cdot}009^a$	$5{\cdot}0\pm1{\cdot}01^a$
CAS+E_2	$244\pm6{\cdot}6^b$	$5{\cdot}0\pm0{\cdot}23^b$	$0{\cdot}26\pm0{\cdot}017^b$	$27{\cdot}9\pm4{\cdot}9^b$
SPI	$289\pm6{\cdot}4^a$	$4{\cdot}0\pm0{\cdot}11^a$	$0{\cdot}07\pm0{\cdot}003^a$	$3{\cdot}2\pm0{\cdot}26^a$
SPI+E_2	$227\pm3{\cdot}6^b$	$4{\cdot}6\pm0{\cdot}05^b$	$0{\cdot}25\pm0{\cdot}016^b$	$30{\cdot}4\pm6{\cdot}2^b$

% Liver and uterus weight are relative to body weight. Serum estradiol (E_2) levels were determined by RIA as described in Materials and Methods. Means ($n=6$) with different letters differ significantly, $P<0{\cdot}05$.

Table 2 Serum isoflavone levels. Total isoflavone and metabolites in the sera of OVX-female rats ($n=6$) fed with diets containing soy protein isolate (SPI) or casein (CAS) as sole protein source with or without estradiol (E_2) supplementation. Data are mean ± S.E.M.

	Genistein	Daidzein	Equol	Glycetin	Total isoflavone
Groups					
CAS	0	0	0	0	0
E_2	0	0	0	0	0
SPI	0·40±0·08	0·26±0·05	0·24±0·07	0·04±0·01	1·27±0·19
SPI+E_2	0·26±0·06	0·16±0·03	0·57±0·07*,†,‡	0·03±0·0	1·35±0·24

Since no isoflavones were detected in treatment groups CAS and E_2, comparisons were made only between SPI and SPI+E_2 treatment groups. *,†,‡represent significant effect of – SPI diet, E_2 supplementation, and interaction between SPI and E_2 respectively, $P<0·05$.

growth and proliferation; and cell cycle. Early genes responsive to stress – leukemia inhibitory factor receptor (*Lifr*, 43-fold), nephroblastoma overexpressed (*Nov*, 10·6-fold), and early growth response-1 (*Egr1*, fivefold) genes expression – were highly induced. The E_2 treatment has been associated with altered hepatic glucose homeostasis, improved insulin sensitivity, reduction in obesity, and lipid metabolism (Boverhof *et al.* 2004, Gao *et al.* 2006). Similar to other investigators, we observed a modulation in genes involved in glucose homeostasis and insulin sensitivity, including an upregulation in insulin-like growth factor-binding protein 1 (*Igfbp-1* and *-2*) and insulin receptor-related receptor (*Insrr*) by 4- and 1·6-fold, while insulin receptor substrate 3 (*Irs3*) and insulin induced gene 2 (*Insig2*) were downregulated by 3·7- and 1·8-fold respectively. Genes involved in fatty acid metabolism and transport such as carnitine acetyltransferase (*Crat*), fatty acid desaturase3 (*Fad3*), fatty acid-binding protein 2 (*Fabp2*), *Cd36*, carnitine palmitoyltransferase 1 (*Cpt1*), and hydroxysteroid 17β dehydrogenase 3 (*Hsd7β3*), were upregulated. A list of genes altered by E_2 treatment is included in online supplementary material (OSM Table 1, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol202/issue1/).

Functional role of estrogens in protection of premenopausal women against inflammatory and fibrogenic hepatic diseases has been established (Shimizu & Ito 2007). The mechanisms, however, are not well known. Transforming growth factor-β (*Tgfβ*) plays a crucial role in hepatic fibrogenesis (Parsons *et al.* 2007) and estrogens have been known to reduce CCl_4-induced liver fibrosis in rats (Xu *et al.* 2002). Here, we found that E_2 supplementation resulted in reduced *Tgfβ1* gene expression. IPA identified an enrichment in downregulation of genes encoding the entire TGF1 network – TGF-receptor, TGF-inducible homeobox-1, matrix metallopeptidase 2 (*Mmp2*), tissue inhibitor of metalloproteinase-3 (*Timp3*), and *Smad-2/3* were downregulated (OSM Figure 1A, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol202/issue1/).

SPI feeding to the mature female rats in the absence of endogenous estrogens resulted in the modification in the expression profile of 82 genes (Venn diagram Fig. 1B). Among the 46 upregulated genes, many belong to metabolic processes. For example, glucose metabolism included phosphatidyl inositol 3-kinase (*Pi3ka*) and early hepatic insulin-responsive gene -*Eiih* genes; xenobiotic metabolism included glutaredoxin1 (*Glrx1*) and glutathione-*s*-transferase (*Gst*) genes; and fatty acid metabolism including the gene coding for CD36. The downregulated genes included genes coding for estrogen metabolism, sulfotransferase (*Estsul/Ste*) and transcriptional regulator, Cbp/p300-interacting transactivator (*Cited2*) and hairy and enhancer of split 1 (*Hes1*). A complete list of genes altered by SPI treatment is provided in OSM Table 2, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol202/issue1/. Networking of genes altered by SPI feeding is represented in OSM Figure 1B.

Analysis of genes altered by both SPI and E_2 revealed 39 genes in common (Venn diagram Fig. 1B). Correlation-based hierarchical cluster analysis (OSM Figure 2, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol202/issue1/) of these 39 genes suggested that not all of the genes altered by SPI were in the same direction as E_2. Expression of four genes downregulated by ≥1·5-fold by E_2 treatment viz. cysteine- and glycine-rich protein 2 (*Csrp2*), cathepsin C (*Ctsc*), inositol (myo)-1 monophosphatase 2 (*Impa2*), and carnitine *O*-octanoyltransferase (*Crot*), were upregulated by SPI feeding by ≥1·5-fold, while E_2 upregulated arrestin domain-containing 2 (*Arrdc2*) gene was downregulated by SPI, suggesting whether SPI has estrogen-like actions or anti-estrogenic actions is dependent upon the individual gene and cannot be generalized. Some genes highly upregulated by E_2 treatment – *Lifr* (43-fold) and *Cd36* (9-fold) – were increased following SPI feeding only by 2·5- and 2-fold respectively; while, HLA-B-associated transcript 1A (*Bat1*) and *Cyp2C13*, genes highly downregulated by E_2 treatment by 8·1- and 5·5-fold respectively, were repressed by SPI feeding by only 5·3- and 3·8-fold respectively (Table 3).

Genes changed by the interaction between E_2 and SPI

Expression profile of 225 genes was modulated ($P<0·05$, 1·5-fold) by the interaction between SPI and E_2, not by either treatment alone (Venn diagram Fig. 1B). IPA performed

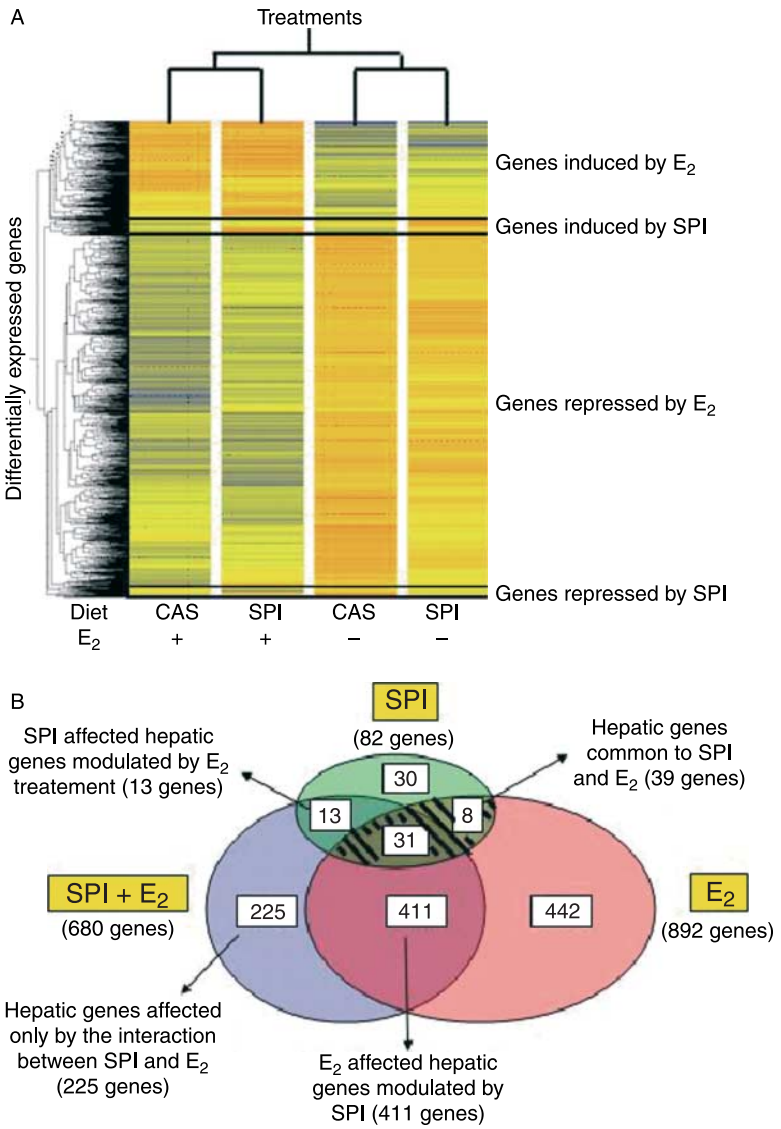

Figure 1 Differential signatures of SPI, E_2, and SPI+E_2 on hepatic gene expression. (A) Hierarchical cluster analysis of SPI altered (±1·5-fold, $P<0·05$) hepatic genes in the presence or absence of E_2. The heat map was generated from the differentially expressed gene list in GeneSpring software. Using pseudolines, the heat map was divided into four clusters – 1) genes induced by E_2; 2) genes induced by SPI; 3) genes repressed by E_2; and 4) genes repressed by SPI. Colors – orange, yellow, and blue represent upregulation, no relative effect, and downregulation of hepatic genes respectively. (B) Venn diagram on the differentially expressed genes (described in Materials and Methods).

on these genes identified lipid metabolism, carbohydrate metabolism, and cellular proliferation and development as the highly influenced biological functions. The common key regulators of fatty acid metabolism, carbohydrate metabolism, and glucocorticoid signaling were down-regulated. For example, sterol regulatory-binding protein factor 1 gene, *Srepf1/Srep1*, a transcription factor involved in sterol biosynthesis and fatty acid metabolism and glucocorticoid receptor (Nr3c1/GR) were downregulated by 1·9- and 1·5-fold. Consequently, the SREBP-1-responsive genes, fatty acid synthase (*Fasn*, 2·5-fold), steroyl CoA desaturase (*Scd*, 2·5-fold), acetyl CoA carboxylase-α, (*Acaca*, 1·8-fold), ATP citrate lyase (*Acly*, 1·9-fold), and fatty acid-binding protein 5 (*Fabp5*, 2·1-fold) involved in fatty acid metabolism; and glucokinase (*Gck*, 1·94-fold), glucose-6-phosphatase (*G6pc*, 1·6-fold), pyruvate kinase (*Pklr*, 1·6-fold) involved in carbohydrate metabolism were downregulated (values in bracket indicate fold reduction).

Table 3 Functional characterization and fold changes of hepatic genes common to estradiol (E_2) and soy protein isolate (SPI)

	Gene symbol	Gene title	GO biological process term	E_2-fold change	SPI-fold change
RefSeq					
NM_031048	*Lifr*	Leukemia inhibitory factor receptor	Stress response	43·27	2·41
NM_031561	Cd36	cd36 antigen	Fatty acid transport	8·90	1·99
NM_031649	*Klrg1*	Killer cell lectin-like receptor subfamily G, member 1	Cell surface receptor linked signal transduction	3·71	1·50
NM_022258	*C44*	α-1-B glycoprotein	–	2·36	1·54
NM_024129	*Dcn*	Decorin	Extracellular matrix organization	2·26	2·03
NM_001013083	*Cpa2*	Carboxypeptidase A2	Proteolysis	2·05	1·76
NM_017094	*Ghr*	GH receptor	Growth	1·98	1·60
NM_053781	*Akr1b7*	Aldo-keto reductase family 1, member B7	Cellular lipid metabolic process	1·80	1·82
XM_224720	*Arrdc2*	Arrestin domain-containing 2	–	1·74	0·63
NM_053549	*Vegfb*	Vascular endothelial growth factor B	Angiogenesis	1·73	1·63
NM_130408	*Cyp26a1*	Cytochrome P450, family 26, subfamily A, polypeptide 1	Electron transport	1·59	1·88
XM_227117	*Pcdh18*	Protocadherin 18	Homophilic cell adhesion	1·53	1·55
NM_053019	*Avpr1a*	Arginine vasopressin receptor 1A	Regulation of blood pressure by vasopressin	1·51	1·52
XM_343169	*Cfd*	Complement factor D	Innate immune response	1·58	1·55
NM_053322	*Pom210*	Nuclear pore membrane glycoprotein 210	Protein targeting	1·62	1·56
NM_001025137	*Ier5*	Immediate early response 5	–	1·62	1·56
NM_024360	*Hes1*	Hairy and enhancer of split 1	Transcription	1·69	1·88
NM_177425	*Csrp2*	Cysteine- and glycine-rich protein 2	Multicellular organismal development	1·70	1·71
NM_017097	*Ctsc*	Cathepsin C	–	1·72	1·50
NM_016991	*Adra1b*	Adrenergic receptor, α-1b	–	1·81	1·59
NM_133560	*Trak2*	Trafficking protein, kinesin-binding 2	Protein targeting	1·86	1·79
NM_012883	*Estsul*	Sulfotransferase, estrogen preferring	Estrogen metabolic process	1·87	1·70
NM_031154	*Gstm3*	Glutathione S-transferase, μ-type 3	Metabolic process	1·87	1·51
XM_214217	*Tdh*	L-threonine dehydrogenase	Cellular metabolic process	1·88	2·23
NM_001009536	*Coil*	Tripartite motif protein 25	–	1·88	1·68
NM_145089	*Asrgl1*	Asparaginase-like 1	Glycoprotein catabolic process	2·01	1·57
NM_053698	*Cited2*	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	Transcription	2·06	1·99
NM_012533	*Cpb1*	Carboxypeptidase B1	Proteolysis	2·07	1·69
NM_031005	*Actn1*	Actinin, α-1	Regulation of apoptosis	2·10	1·56
NM_172224	*Impa2*	Inositol (myo)-1(or 4)-monophosphatase 2	Signal transduction	2·22	1·55
NM_212507	*Ltb*	Lymphotoxin B	Immune response	2·28	2·34
NM_023965	*Gp91*-phox	Cytochrome b-245, β-polypeptide	Electron transport	2·35	1·52
XM_341700	*Cotl1*	Coactosin-like 1	–	2·35	1·50
NM_031987	*Crot*	Carnitine *O*-octanoyltransferase	Fatty acid transport	2·36	1·73
NM_138510	*Akr1c18*	Aldo-keto reductase family 1, member C18	Progesterone metabolic process	2·75	2·30
NM_001003711	*Jph4*	Junctophilin-4	–	2·91	2·04
NM_001008831	*Rt1*-Ba	RT1 class II, locus Ba	Immune response	3·57	3·08
NM_138514	*Cyp2c13*	Cytochrome P450 2c13	Electron transport	5·49	3·72
NM_133300	*Bat1*	HLA-B-associated transcript 1A	–	8·20	5·32

The GCK-regulatory protein, *Gckr*, was reduced by 1·54-fold. Transcription factors – CCAAT/enhancer-binding protein (C/EBPα and -β) – co-regulated by Srebp and GR were downregulated by 1·5-fold. Gene coding for farensyl-X-receptor, *Fxr*, a transcription factor involved in the cholesterol metabolism, and regulated by CEBPα was downregulated by 1·6-fold, resulting in the increased expression of the FXR-repressed gene – *Cyp7a1*, involved in the catabolism of cholesterol to bile acids, by 2·8-fold (Fig. 2). A more detailed classification of gene expression changes as based on known biological functions, derived from NetAffx and GeneSpring programs, is represented in Table 4.

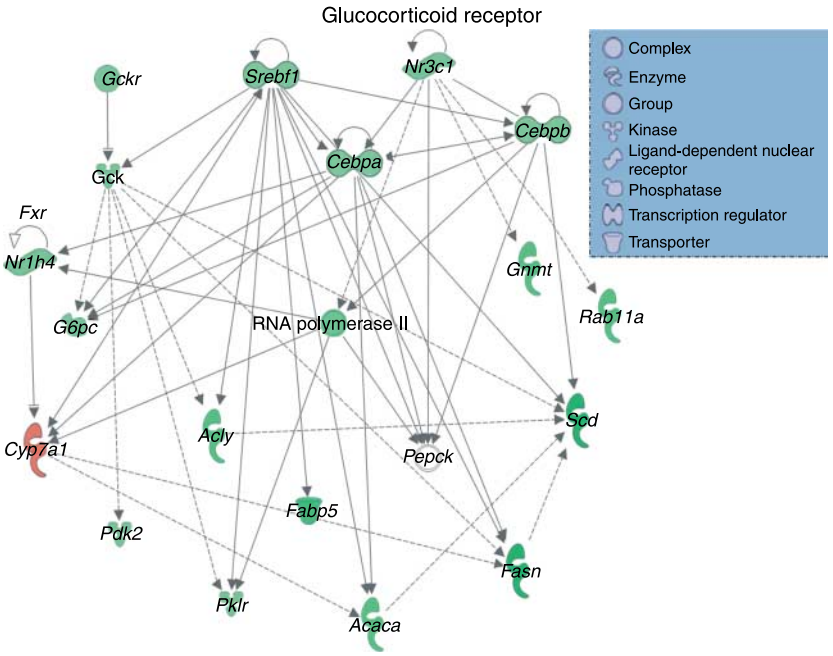

Figure 2 Ingenuity Pathway Analysis (IPA) gene network. The highest significant gene network identified in the Ingenuity Pathway Analysis of the gene changed by the interaction between SPI feeding and E_2 treatments. Genes are colored according to the log ratio gene expression values. Colors green and red represent downregulation and upregulation respectively. The solid and dotted arrows indicate direct and indirect interaction respectively, between the two genes as known in the literature and detected by IPA.

Corroboration of the microarray results by QRTPCR for a selected group of genes is shown in (Fig. 3). Statistics performed on nine selected genes expression values from the real-time PCR suggest similar interactions to those observed in our microarray analysis.

Discussion

Several *in vivo* and *in vitro* studies suggest that consumption of soy foods, such as soy infant formula, may have adverse effects on development, breast cancer, and reproduction, as the result of estrogenic actions of soy isoflavones (Duffy *et al.* 2007, Bhatia & Greer 2008, Boucher *et al.* 2008). These purported estrogenic effects have been attributed to soy isoflavones binding to and activating ERα and/or ERβ (Casanova *et al.* 1999, Beck *et al.* 2003). If consumption of soy foods activates estrogen signaling pathways, one would expect that animals treated with estrogens or fed diets with SPI would have similar gene expression profiles. We used two 'bioassays' of estrogen actions: a) uterine wet weight is a traditional and widely employed bioassay of classical estrogenicity; and b) hepatic genomic profiling in ovariectomized rats fed SPI- or CAS-containing diets, with or without restoration of E_2 to physiologically relevant plasma concentrations. Our results clearly demonstrate that SPI does not elicit responses characteristic of E_2, the major estrogen in women, in these tissues. On the other hand, and in agreement with findings made by others (Adlercreutz *et al.* 1993, Welshons *et al.* 2006), we observed that estrogenic status is a key factor in determining the SPI-mediated response.

Our rat studies were designed to closely model soy consumption by pre- and post-menopausal women, conditions of 'normal' and low E_2 levels respectively. Our results clearly demonstrate that irrespective of the estrogenic status, feeding SPI-containing diets to rats does not restore OVX-mediated loss in uterine wet weight whereas E_2 supplementation does. Although normally performed in immature rodents, the uterotrophic response is used as a classic bioassay for determining ERα agonists (OECD 2001, Padilla-Banks *et al.* 2001). Thus, current findings and our previous data (Badger *et al.* 2001) demonstrate that SPI feeding does not elicit classical estrogenic responses on ERα-responsive tissues such as the uterus, even when utilized as the sole protein source. By contrast, mature OVX rats fed purified equol (400 mg/kg) or injected with purified genistein (54 mg/kg) displayed mild estrogen-like uterine stimulation (Rachon *et al.* 2007*a*, Rimoldi *et al.* 2007). Studies utilizing purified soy isoflavones were shown to regulate gene expression similar to estrogens in female (Naciff *et al.* 2002) and male reproductive tissues (Naciff *et al.* 2004, 2005). However, other studies comparing SPI with genistein at the gene expression level in

Table 4 Functional characterization and fold changes of genes altered only in the presence of both soy protein isolate (SPI) and estradiol (E_2)[a]

	Gene symbol	Gene title	GO biological process term	Fold change
RefSeq				
XM_343823	*Serpina7*	Serine (or cysteine) peptidase inhibitor, clade A, member 7	Response to drug	7·20
XM_215985	*Rapgef4*	Rap guanine nucleotide exchange factor (GEF) 4	Insulin secretion	3·55
XM_221074	*Abca8b*	ATP-binding cassette, subfamily A (ABC1), member 8b	Transport	3·24
NM_012942	*Cyp7a1*	Cytochrome P450, family 7, subfamily a, polypeptide 1	Cholesterol metabolic process	2·78
NM_138826	*Mt1a*	Metallothionein 1a	Cellular metal ion homeostasis	2·76
NM_017136	*Sqle*	Squalene epoxidase	Cholesterol metabolic process	2.61
NM_134329	*Adh7*	Alcohol dehydrogenase 7 (class IV), μ- or σ-polypeptide	Retinoid metabolic process	2·08
NM_031594	*P2rx4*	Purinergic receptor P2X, ligand-gated ion channel 4	Transport	2·03
XM_342340	*Pla2g12a*	Phospholipase A2, group XIIA	Lipid catabolic process	1·99
NM_080886	*Sc4mol*	Sterol-C4-methyl oxidase-like	Fatty acid metabolic process	1·96
NM_013058	*Id3*	Inhibitor of DNA-binding 3, dominant negative helix-loop-helix protein	Negative regulation of transcription from RNA polymerase II promoter	1·94
NM_012847	*Fnta*	Farnesyltransferase, CAAX box, α	Transforming growth factor β-receptor signaling pathway	1·91
NM_019256	*P2rx7*	Purinergic receptor P2X, ligand-gated ion channel, 7	Transport	1·90
NM_172033	*Plekhb1*	Pleckstrin homology domain-containing, family B, member 1	Signal transduction	1·87
NM_001008773	*Eif1a*	Eukaryotic translation initiation factor 1A	Translation	1·85
NM_012503	*Asgr1*	Asialoglycoprotein receptor 1	Endocytosis	1·84
NM_017235	*Hsd17b7*	Hydroxysteroid (17-β) dehydrogenase 7	Steroid biosynthetic process	1·80
NM_001013212	*Snapc3*	Small nuclear RNA activating complex, polypeptide 3	Transcription	1·79
NM_001013179	*Hes6*	Hairy and enhancer of split 6	Regulation of transcription, DNA-dependent	1·61
NM_012576	*Gr/Nr3c1*	Glucocorticoid receptor	Glucocorticoid receptor signaling pathway	−1·50
NM_017084	*Gnmt*	Glycine *N*-methyltransferase	*S*-adenosylhomocysteine metabolic process	−1·53
NM_012524	*Cebpa*	CCAAT/enhancer-binding protein (C/EBP), α	Signal transduction	−1·53
NM_013120	*Gckr*	Glucokinase regulatory protein	Carbohydrate metabolic process	−1·55
NM_024125	*Cebpb*	CCAAT/enhancer-binding protein (C/EBP), β	Signal transduction	−1·57
NM_021745	*Fxr/Nr1h4*	Nuclear receptor subfamily 1, group H, member 4	Cholesterol metabolic process	−1·59
NM_013098	*G6pc*	Glucose-6-phosphatase, catalytic	Glycogen biosynthetic process	−1·61
NM_012624	*Pklr*	Pyruvate kinase, liver and red blood cell	Carbohydrate metabolic process	−1·61
NM_012753	*Cyp17a1*	Cytochrome P450, family 17, subfamily a, polypeptide 1	Steroid biosynthetic process	−1·85
XM_213329	*Srebf1/Srebp*	Sterol regulatory element-binding factor 1	Signal transduction	−1·88
NM_016987	*Acly*	ATP citrate lyase	Lipid metabolic process	−1·90
NM_012565	*Gck*	Glucokinase	Response to glucose stimulus	−1·95
NM_145878	*Fabp5*	Fatty acid-binding protein 5, epidermal	Lipid metabolic process	−2·16
NM_033234	*Hbb*	Hemoglobin-β chain complex	Transport	−2·17
NM_012651	*Slc4a1*	Solute carrier family 4, member 1	Transport	−2·26
NM_017206	*Slc6a6*	Solute carrier family 6, member 6	Transport	−2·35
NM_017272	*Aldh1a7*	Aldehyde dehydrogenase family 1, subfamily A7	Metabolic process	−2·40
NM_017332	*Fasn*	Fatty acid synthase	Fatty acid biosynthetic process	−2·45
NM_001007722; NM_001013853	*Hba-a1*	Hemoglobin-α 2 chain	Transport	−2·47

(continued)

Table 4 *Continued*

	Gene symbol	Gene title	GO biological process term	Fold change
NM_139192	*Scd1*	Stearoyl-Coenzyme A desaturase 1	Fatty acid biosynthetic process	−2·51
XM_214935	*Sult2b1*	Sulfotransferase family, cytosolic, 2B, member 1	Lipid metabolic process	−2·58
NM_012886	*Timp3*	Tissue inhibitor of metalloproteinase 3	Response to estrogen stimulus	−2·59
XM_216194	*Dguok*	Deoxyguanosine kinase	Nucleic acid metabolic process	−2·64
XM_213590	*Hrasls*	HRAS-like suppressor	Regulation of cell growth	−2·70
NM_198776	*Hbb*	β-Glo	Transport	−3·30
NM_013197	*Alas2*	Aminolevulinic acid synthase 2	Response to hypoxia	−3·31
NM_012531	*Comt*	Catechol-*O*-methyltransferase	Catecholamine metabolic process	−4·33

[a]For complete listing of genes refer to Online Supporting Material.

female reproductive tissues revealed a completely different set of genes expressed following SPI feeding compared with feeding purified genistein at the same levels present in SPI (Eason *et al.* 2005, Su *et al.* 2007). Thus, when the results of these published studies are considered in combination with our results, it is clear that the effects of purified soy isoflavones differ from that of SPI feeding, even when the dose of isoflavones are approximately the same.

Analysis of total serum isoflavone concentrations showed that while feeding diets in which SPI represented the sole protein source resulted in substantial concentrations of genistein, daidzein, and equol (levels of genistein equaling those of infants fed soy formula), these concentrations were not sufficient to exhibit classical estrogenic responses mediated through ERα. One interesting finding was that serum equol concentrations in SPI-fed rats treated with E_2 were greater than those of vehicle-treated SPI-fed rats. Equol is the most potent isoflavone metabolite and is produced in all or most rodents and monkeys, but in only about 25% of human subjects, and is not produced in human infants (Gu *et al.* 2006). We have no explanation of the elevated serum equol concentrations, but since equol is formed in the gut by bacterial metabolism of daidzein, E_2 may promote daidzein metabolism. This may have functional significance for women who are equol producers and who also take birth control pills or estrogen replacement therapy.

Our current weight gain data support those of others showing an attenuation of BW following E_2 supplementation in OVX rats (Cooke & Naaz 2004, Rachon *et al.* 2007*a*,*b*). However, no such effects were observed with SPI feeding. Additionally, SPI feeding did not alter serum E_2 levels. Treatment with purified genistein, however, reduces the activity of aromatase, the enzyme that metabolizes androgen to estrogens, resulting in reduced serum E_2 concentrations (Adlercreutz *et al.* 1993). This is another example of differing actions of SPI and purified isoflavones.

It has been noted that making conclusions based on the estrogen-dependent changes in the whole genomic profile is more sensitive than relying on single bioassays such as uterine hyperplasia (Naciff *et al.* 2004). Previously, it was demonstrated that the gene expression profile in uterus and ovary of immature Sprague–Dawley rats fed with Purina 5001 diet (total genistein + daidzein = 0·49 mg/g was completely different from low doses of ethinyl E_2 (0·1 μg/kg per day for 4 d). Additionally, similar to our current data, no uterine stimulation was observed, suggesting no estrogenic effect of laboratory animal feed on the reproductive system in the immature female rat (Naciff *et al.* 2004). In the present study, liver was selected to compare the gene expression profile of SPI with E_2. Although not involved in reproduction, liver is a highly estrogen-responsive tissue as evident from the role of estrogens in the regulation of liver regeneration, inflammation and injury, and hepatic insulin sensitivity, as well as lipid- and sterol-mediated signaling (Hall *et al.* 2001, Murphy & Korach 2006). The liver also predominately expresses ERα (Kuiper *et al.* 1997), although low levels of ERβ have been reported (Petersen *et al.* 1998) and serves as a relatively clean tissue to study the effect of exogenous and endogenous estrogens on classical ERα signaling. We have identified a significant alteration in the gene expression profile of 892 genes, which itself suggests that estrogens are robustly involved in hepatic signaling. Genes involved in physiological processes including lipid and cholesterol metabolism, fibrogenesis, xenobiotic metabolism, transport, inflammation, cellular proliferation, and differentiation were altered by E_2 supplementation, as previously reported by other laboratories (Gao *et al.* 2006, 2008).

The relevance of the current study is in the comparison of gene expression signatures of SPI fed with E_2-treated ovariectomized rats. SPI feeding resulted in significant alterations in the expression of only 82 genes. IPA identified SPI-altered genes that are also regulated by estrogens and progesterone, such as aquaporin1 (*Aqp1*), Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (*Cited2*), and phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (*Pik3r1*), revealing that some E_2-regulated genes are also regulated by SPI (OSM Table 2). Naciff *et al.* (2004) reported a downregulation of *Aqp1*, a gene encoding a protein that is a member of a family of membrane channel proteins which facilitate bulk water

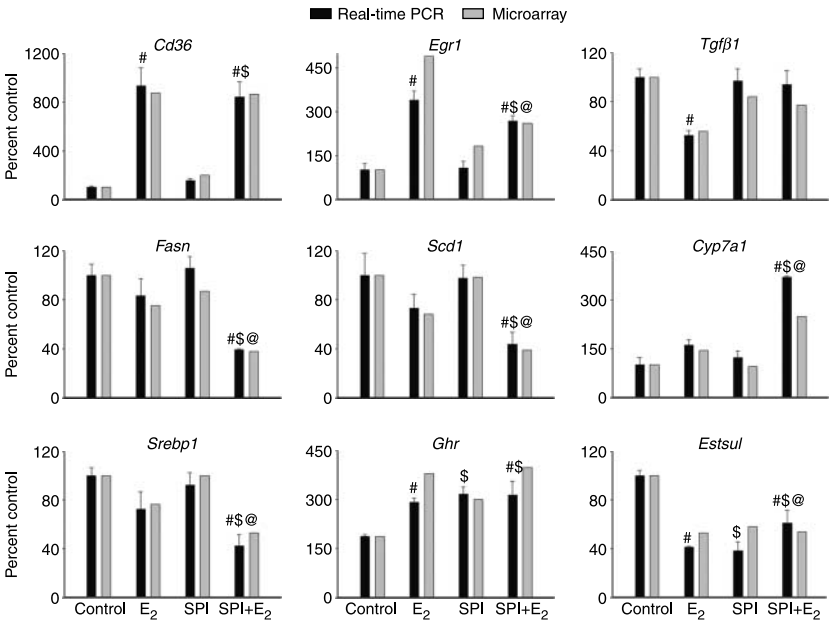

Figure 3 Pattern of change in mRNA expression level, $N=6$; in nine selected genes, normalized with the expression level of GAPDH (internal control), determined by real-time PCR. Both real-time PCR and microarray data are represented as percent of control. Real-time PCR were analyzed by two-way ANOVA followed by Student–Newman *post hoc* test ($P<0{\cdot}05$). #, $ and @ represent effects of E_2, SPI, and interaction between SPI and E_2 respectively.

transport and aquaporins, by soy/alfalfa-based diet in immature rat uterus, which was opposite to the effect of estrogenic stimulation by a high E_2 dose (Li *et al.* 1997). Interestingly, we also found a suppression of this gene by SPI feeding; however, no estrogenic stimulation was observed.

Further comparative analysis determined that genes common to SPI feeding and E_2 treatment, 39 genes, were only 4% of the genes changed by E_2, suggesting an involvement of different biologic pathways in SPI-mediated signaling. Among these 39 genes, very few were identified by PROMOSER (Halees *et al.* 2003), a large-scale mammalian promoter and transcription start site identification service available online. The promoter analysis performed on the selected genes identified by PROMOSER revealed that only a few of these genes possessed estrogen-responsive element (ERE), binding sites for ER, as identified by DRAGON ERE version 2.0 program (Bajic *et al.* 2003). Immediate early genes – *Lifr* (E_2: 43-fold; and SPI: 2·5-fold induction) and *Egr1* (E_2: fivefold; and SPI: no induction) – were identified with ERE-like consensus sequences (GGTCAnnnTGACC) and half EREs (GGTCA) in the promoter element upstream -5000 of transcription start site, suggesting a direct E_2-mediated transcriptional increase in these genes, while Cd36 gene (E_2: ninefold; and SPI: twofold induction) lacked any ERE-like sequence. This suggests that E_2- or SPI-mediated hepatic signaling does not necessarily involve classical ER/ERE pathways. Genome-wide identification of ERα-binding sites in mouse liver, using ChIP on Chip molecular technique, suggested that besides ERE a number of motifs including forkhead sites, AP-1, Sp1, bHLH, and ETS sites are important for E_2-mediated hepatic gene expression (Gao *et al.* 2008). Our data suggest that SPI-feeding results in a unique signature in hepatic gene expression. Phytoestrogens present in SPI may change expression of a few genes by ERα recruitment to EREs or by modulating other transcription factors that mediate estrogenic responses. Hierarchical clustering revealed that SPI feeding altered the expression of five genes in the direction opposite to E_2 treatment, demonstrating a SERM-like action of SPI on selective genes. Whether the effect of SPI would be the same or opposite to E_2 depends solely upon the gene type and cannot be generalized. Interestingly, 225 genes were uniquely modulated by the combination of E_2 plus SPI and not by either treatment alone. The biochemical and physiological effects of SPI are known to be affected by the estrogenic status (Adlercreutz *et al.* 1993, Welshons *et al.* 2006). For example, both SPI and E_2 enhanced DMBA-mediated *Cyp1a1* induction in ovariectomized females but this was repressed by SPI feeding in the presence of E_2 (Singhal *et al.* 2008*b*). Interaction between SPI-associated phytochemicals and peptides, and E_2 may alter the expression profile and binding pattern of transcription factors, co-activators, and co-repressors resulting in the modulation of gene expression. Here, we observed changes in the expression profile of many

transcription factors, including RNA polymerase II (*Pol II*), responsible for transcriptional elongation of genes. Repression in the expression of Pol II was linked with reduced expression of many transcription factors such as *Cebp-a* and *-b*, *Dbp*, glucocorticoid receptor, *Fxr*, and *Srebp1*, suggesting altered metabolic profile by the combination of SPI + E_2, as compared with SPI or E_2 alone. This also strengthens the point that the SPI gene expression signature in: children prior to puberty, men and women, and post-menopausal women would all be predicted to differ.

Conclusion

The present data demonstrate that SPI, the sole protein source of infant formula, is not uterotrophic and it has a unique pattern of hepatic gene expression that differs substantially from that of E_2. These data call into question concerns about the potential estrogenic effects of soy formulas in infants (5, 6, 12–15, 59, 60). It should be noted that the rats with access to SPI *ad libitum* consumed 19·3 mg/kg per day of genistein (Ronis *et al*. 2001), which is much higher than the average intake of genistein in US (0·014–0·14 mg/kg per day) or in Japan (0·21–0·43 mg/kg per day; Rozman *et al*. 2006) or infants on soy formula 4–6 mg/kg per day (Setchell *et al*. 1997). It is unlikely that the general population would consume sufficient amounts of phytoestrogens in the diet to cause any adverse effects. Our conclusion in liver is also supported by a series of studies performed by Naciff *et al*. in reproductive tissues in immature rats where soy-containing diets were not observed to be estrogenic. However, further validation of the current data by examining the SPI gene signature in estrogen-responsive reproductive tissues containing both ERα and ERβ in neonatal animals such as a piglet, which have a more similar pattern of isoflavone metabolites to humans than rodents (Gu *et al*. 2006) is required to completely rule out the possibility of estrogenic effects of soy infant formula.

Declaration of interest

R Singhal, K Shankar, T M Badger, and M J Ronis declare that there are no conflicts of interest.

Funding

The work presented here is supported in part by USDA (CRIS# 6251-51000-005-03S), a CAGSRF award from UAMS (R S) and a Technology Transfer Award from SCC-SOT (R S).

Acknowledgements

The authors are thankful to Drs Shanmugam Nagarajan and John C Marecki for their critical suggestions in compiling the manuscript. Online Supporting Material (OSM) is available with the online posting of this paper.

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Received in final form 8 April 2009
Accepted 15 April 2009
Made available online as an Accepted Preprint
16 April 2009