Dietary Soy Protein Isolate Modifies Hepatic Retinoic Acid Receptor-β Proteins and Inhibits Their DNA Binding Activity in Rats\textsuperscript{1,2}

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Abstract

Retinoic acid receptors (RAR) belong to the same nuclear receptor superfamily as thyroid hormone receptors (TR) that were previously shown to be modulated by dietary soy protein isolate (SPI). This study has examined the effect of dietary SPI and isoflavones (ISF) on hepatic RARE gene expression and DNA binding activity. In Expt. 1, Sprague-Dawley rats were fed diets containing 20% casein or 20% alcohol-washed SPI in the absence or presence of increasing amounts of ISF (5–1250 mg/kg diet) for 70, 190, or 310 d. In Expt. 2, weanling Sprague-Dawley rats were fed diets containing 20% casein with or without supplemental ISF (50 mg/kg diet) or increasing amounts of alcohol-washed SPI (5, 10, and 20%) for 90 d. Intake of soy proteins significantly elevated hepatic RARβ2 protein content dose-dependently compared with a casein diet, whereas supplemental ISF had no consistent effect. Neither RARβ protein in the other tissues measured nor the other RAR (RARα and RARγ) in the liver were affected by dietary SPI, indicating a tissue and isoform-specific effect of SPI. RARβ2 mRNA abundances were not different between dietary groups except that its expression was markedly suppressed in male rats fed SPI for 310 d. DNA binding activity of nuclear RARβ was significantly attenuated and the isoelectric points of RARβ2 were shifted by dietary SPI. Overall, these results show for the first time, to our knowledge, that dietary soy proteins affect hepatic RARβ2 protein content and RARβ DNA binding activity, which may contribute to the suppression of retinoid-induced hypertriglyceridemia by SPI as reported. J. Nutr. 137: 1–6, 2007.

Introduction

Soy intake has been shown to be hypotriglyceridemic in both animals (1–3) and hyperlipidemic human subjects (4). This action of soy components has been further confirmed in our recent rat study showing dietary alcohol-washed soy protein isolate (SPI)\textsuperscript{6} containing minimal isoflavones (ISF) remarkably decreased plasma triacylglycerol levels (5). Furthermore, we previously reported that diets containing SPI significantly increased hepatic thyroid hormone receptor (TR) β1 protein content and suppressed the DNA binding activity of nuclear TR proteins (6,7). Retinoic acid receptors (RAR) are also members of the nuclear receptor superfamily and can form heterodimers with TR to interact with the target genes (8). However, whether soy intake affects RAR gene expression and function is unknown.

RA is a metabolite of vitamin A and plays important roles in controlling immune function, reproduction, cell growth, differentiation (9), and lipid metabolism (10,11). RA is important in the prevention and treatment of various cancers (12,13). For instance, loss of hepatic RA function leads to development of steatohepatitis and liver tumors (14). Most of the physiological functions of RA are mediated through RAR and altered RAR activity or RAR-mediated pathway is associated with many types of carcinogenesis (15–18). Three types of RAR (RARα, RARβ, and RARγ) have been characterized (19,20) and are encoded by independent genes. Several isoforms are generated from each gene by alternative splicing or usage of distinct promoters (21–23). For example, RARβ has 4 isoforms (RARβ1, RARβ2, RARβ3, and RARβ4) that are generated by alternative gene splicing of primary transcripts initiated from 2 promoters, P1 and P2 (23,24).

Retinoids have been extensively used in the treatment of various cancers (25–27). However, one of the major adverse effects of retinoid treatment is the induction of hypertriglyceridemia, which has been observed in both rats (28) and humans (12,29–31). Interestingly, replacement of dietary casein with SPI markedly reduced the severity of RA-induced hypertriglyceridemia in rats (10,32). Nevertheless, the underlying mechanism(s) is not well understood. It has been shown that dietary SPI had no effect on serum retinoid level in RA-treated rats compared with the casein

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\textsuperscript{3} Abbreviations used: HRP, horseradish peroxidase; ISF, isoflavone; pI, isoelectric point; RAR, retinoic acid receptor; SPI, soy protein isolate; TR, thyroid hormone receptor; 2D, 2-dimensional.

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control (32) and that retinoid-induced hypertriglyceridemia is mediated through retinoid receptors (10,33). This suggests that SPI may exert its suppressive actions on the retinoid-induced hypertriglyceridemia via modulation of RAR or RAR-regulated gene expression. The objective of this study was to examine if dietary SPI and soy-derived ISF affect hepatic RAR gene expression and DNA binding ability in rats.

Materials and Methods

Chemicals and reagents. Alcohol-washed SPI (Pro Fam 930 containing 90% protein) and Novasoy (soy ISF concentrate) were purchased from Archer Daniels Midland Company. Casein protein (90% total protein) was from ICN and Harlan Teklad. Acrylamide, N,N'-methylene-bis-acrylamide, ammonium persulfate, dithiothreitol, Tris, phenylmethylsulfonyl fluoride, maleic acid, boric acid, Nonidet P-40, and EDTA were from Sigma Chemical. Western blotting detection kits and Hybond-N™ Membrane were obtained from Amersham. Goat anti-rabbit and anti-mouse immunoglobulin G (H+L)-horseradish peroxidase (HRP) conjugated antibodies, and Bio-Rad protein assay kits were purchased from Bio-Rad Laboratories. X-ray film was from MJS Biolynx. Affinity purified rabbit polyclonal antibodies against human RARα, RARβ, and mouse monoclonal antibody against human RARγ were from Santa Cruz Biotechnology and all tested to cross-react with rat antigens. RARγ antibody was shown to detect both RARβ1 and RARβ2 isoforms. Biotin 3'-End DNA Labeling kit and LightShift Chemiluminescent electrophoretic mobility shift assay kit were from Pierce Biotechnology.

Animals, diets, and tissue samples. The animal experimental protocol was approved by the Health Canada Animal Care Committee and all animal handling and care followed the guidelines of the Canadian Council for Animal Care. Expt. 1 was designed to examine the effect of alcohol-washed SPI (containing minimal amount of ISF) and increasing amounts of added soy ISF using casein as a control. Briefly, Sprague-Dawley male and female rats (Charles River) at age of 50 d were randomly divided into 6 groups and fed one of the 6 diets as previously described (6) (Diet 1: 20% casein; Diet 2: 20% alcohol-washed SPI; Diets 3–6: 20% SPI supplemented with 5, 50, 250, or 1250 mg ISF/kg diet) for 70, 190, or 310 d. At the end of each feeding period, 10 male and 10 female rats per dietary group were killed for collection of tissues. Expt. 2 was to determine the effect of increasing amounts of alcohol-washed SPI and the residual ISF contained in the alcohol-washed SPI. Weanling Sprague-Dawley rats were randomly divided into 5 groups (8 males and 8 females per group) and fed 1 of the 5 diets for 90 d. All 5 diets were formulated according to the specifications for the AIN93G diet (34) except that cysteine was replaced by 1-methionine and in Diets 3–5, casein by equal amounts of alcohol-washed SPI (5, 10, and 20%). To determine the potential effect of remaining ISF in alcohol-washed SPI (31.7 mg/kg diet of 20% SPI), Diet 2 was supplemented with 50 mg/kg diet of ISF from Novasoy. The detailed dietary composition was reported elsewhere (7). At the end of feeding periods, rats were necropsied and the plasma and tissue samples were collected and stored at −80°C until analysis. The actual ISF content in each diet was determined by Waters HPLC linear gradient with UV detection monitored at 254 nm (35) and reported previously (6,7).

Protein extraction and western-blotted analysis. Total protein extraction and western-blot analysis were carried out as previously described (6) with minor modifications. Briefly, total proteins (80 µg) were resolved by 12% SDS-PAGE and electrotransferred (30 V, 4°C, overnight) onto nitrocellulose membranes. After blocking, membranes were incubated overnight at 4°C with primary antibodies and subsequently with HRP-conjugated secondary antibodies (1:5000) at room temperature for 45 min. Immuno-reactivity was detected by chemiluminescence radiography in accordance with the manufacturer’s instructions and the images were scanned. The intensities of the protein bands of interest and the Ponceau-stained proteins were determined densitometrically using Scion Image software. The intensities of the target proteins were normalized by the respective Ponceau-stained total protein (36).

RARβ mRNA quantification. Total RNA was isolated from rat liver samples with TRIzol reagent (Life Technologies). Five hundred nanograms of total RNA were reverse transcribed for cDNA synthesis using random primer oligonucleotides. One-tenth of the cDNA synthesized was then amplified with the following primers: rat RARβ2 (forward: 5'-CTCTCAGAGCCCTGGCTCATG-3' [292–311], reverse: 5'-CTGTG-GACTCCTCGTTTGA-3' [702–683]) [GenBank accession number A002942] and universal 18S RNA primers and competimers (Ambion) in a ratio of 2:8. PCR cycle conditions were 94°C for 5 min, 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min for 30 cycles, 72°C for 10 min. Samples were resolved on 2% agarose gels and visualized with ethidium bromide. The images were taken using BioDoc-It Imaging System (UVI Inc.) and analyzed with Scion Image software. RARβ2 mRNA levels were normalized against their respective 18S RNA content.

Two-dimensional western-blotted analysis of RARβ. Two-dimensional (2D) gel electrophoresis was carried out according to the method of O’Farrell (37). Briefly, liver total proteins (200 µg) pooled from 3 rats fed the same diets were subjected to isoelectric focusing in 2% glass tube gels of pH 3.5–10 (Amersham Pharmacia Biotech). After equilibration, the tube gels were sealed to the top of stacking gels on top of 10% acrylamide slab gels and run for ~4 h. The gel was blotted transversely onto polyvinylidene difluoride membrane overnight and stained with Coomassie Blue. The membranes were immunostained with rabbit anti-human RARβ polyclonal antibody (1:500 dilution) and detected using an emission of chemiluminescence kit.

Nuclear protein preparation and electrophoretic mobility shift assay. Hepatic nuclear protein extracts were prepared as previously described (7). Double-stranded DNA oligonucleotides containing consensus sequences (5'-TCAGAGGTTGCCACCGAAAAATCTCG-3') for RAR-specific binding was labeled at the 3'-end with biotin-N4-CTP and terminal deoxynucleotidyl transferase. Nuclear protein extracts (2 µg) were incubated with biotin-labeled DNA probes in the binding buffer for 15 min at room temperature. DNA-protein complexes were resolved on a native 6% polyacrylamide gel in Tris-buffered EDTA (pH 8.0) and electrotransferred (80 V for 1.5 h) onto positively charged nylon membranes. The biotin-labeled DNA probes bound to the nuclear proteins were immunostained with streptavidin-HRP conjugate and detected by chemiluminescence autoradiography. The binding specificity of RARβ to DNA probe was determined by cold probe (100×) competition and supershifting with 1 µg rabbit anti-human RARβ antibody. The intensity of the specific nuclear protein band was densitometrically determined and normalized by the amount of total probe (bound + free).

Statistical analyses. Results are expressed as means ± SEM. Effects of treatment on hepatic RARβ protein content and mRNA steady-state abundances were analyzed by 2-way ANOVA, which included the main effects of diet and feeding duration as well as interaction of diet × feeding duration. All the other data presented were analyzed by 1-way ANOVA. The males and females were analyzed separately. Differences between individual means were determined by Fisher’s least significant difference test. A probability of P < 0.05 was considered significant. Data were analyzed using STATISTICA Version 7.1 (StatSoft).

Results

Hepatic total and nuclear RARβ protein content. Consumption of 20% alcohol-washed SPI in the absence of supplemental ISF for 70 d markedly increased hepatic RARβ protein content in both female and male rats compared with a casein-based diet (Fig. 1A,B; P < 0.01). Supplementation of increasing amounts of ISF to 20% alcohol-washed SPI-based diet had no additional effect on RARβ content compared with SPI alone (P > 0.05; Fig. 1A,B) except that addition of 5 or 1250 mg ISF/kg diet slightly elevated RARβ levels in female rats (Fig. 1A). Dietary SPI significantly increased hepatic RARβ protein content at all 3 time points (for 70, 190, and 310 d) examined in both female
and male rats compared with a casein-based diet (Fig. 1C, D). However, neither SPI nor ISF had any effect on RARα or RARγ (data not shown).

Intake of increasing amounts of alcohol-washed SPI (5, 10, and 20%) for 90 d markedly elevated hepatic nuclear RARβ protein content in a dose-dependent manner in both sexes (Fig. 2A, B). Addition of ISF (50 mg ISF/kg diet) to the casein-based diet had no effect on hepatic nuclear RARβ protein content.

**Hepatic RARβ mRNA abundance.** Hepatic RARβ mRNA steady-state levels measured by relative semiquantitative reverse transcription-PCR did not differ between dietary groups in female rats (data not shown). Consumption of 20% alcohol-washed SPI decreased RARβ mRNA abundance in the male rats fed for 310 d (P < 0.01; Fig. 3B) but had no effect at earlier time points measured (70 and 190 d).

**Isoelectric points of the hepatic RARβ proteins.** Intensities of most protein spots in the 3 Coomassie Blue-stained 2D images (Fig. 4A, C, E) were similar, indicating even loading and transfer. Two spots with a molecular weight of 51 kDa were immunostained with RARβ antibody in the liver proteins of the casein-fed male rats. Their isoelectric points (pIs) were 7.20 and 7.48, respectively (Fig. 4B). Interestingly, in addition to these spots, 2 more spots with the same molecular weight but different pIs (6.59 and 6.88) were detected in the liver samples from SPI-fed rats (Fig. 4D, F). Similar results were obtained in the female rats (data not shown).

**Hepatic RARβ DNA binding activity.** Three bands were detected in the liver samples, which may represent different forms of RAR complexes (i.e. homodimers and heterodimers). Addition of a 100-fold excess of unlabeled DNA oligonucleotide probe (cold probe) eliminated all the binding activity of the 2 lower bands and reduced the abundance of the top band (lanes 6 and 12 vs. lanes 2 and 8, respectively; Fig. 5), indicating the 2 lower bands and part of the top band are RAR-specific binding complex. Supershifting analysis showed that the 2 lower bands but not the top one were shifted by RARβ antibody (lanes 5 and 11). Nuclear RARβ DNA binding abilities in both male and female rats fed diets containing either 5% (lanes 3 and 9) or 20% (lanes 4 and 10) SPI were lower than in those fed the casein diet (lanes 2 and 8; P < 0.05).

**Tissue distribution of RARβ proteins.** Distinct RARβ isoforms were expressed in different tissues of both female (Fig. 6) and male (data not shown) rats. The 51-kDa RARβ2 protein was the predominant isoform in the liver and remarkably increased by dietary SPI compared with casein. In kidney and heart, the predominant form was RARβ4 (48 kDa) and was unchanged by the diets (Fig. 6A). RARβ2 protein content was very low in thyroid tissue and was not different between dietary groups (Fig. 6B).
protein with changed pI. The location (distance) of the protein on the 2D gel, which was established with the absence (SPI; C, D) or presence (SPI + ISF; E and F) of supplemental ISF (250 mg/kg diet) for 70 d. The pl were calculated using a linear regression of pl and the location (distance) of the protein on the 2D gel, which was established with an internal standard of a known pl. The arrows in (D) and (F) represent the RARβ protein with changed pl.

**Figure 4** 2D western-blot analysis of hepatic RARβ protein in the male rats fed diets containing either 20% casein (A, B) or 20% alcohol-washed SPI in the absence (SPI; C, D) or presence (SPI + ISF; E and F) of supplemental ISF (250 mg/kg diet) for 70 d. The pl were calculated using a linear regression of pl and the location (distance) of the protein on the 2D gel, which was established with an internal standard of a known pl. The arrows in (D) and (F) represent the RARβ protein with changed pl.

**Figure 5** DNA binding activity of hepatic nuclear RAR in rats fed diets containing either 20% casein (lanes 2 and 8) or 5% (lanes 3 and 9) or 20% (lanes 4 and 10) alcohol-washed SPI for 90 d. The binding specificity was determined by cold probe competition (100×) (lanes 6 and 12) and supershifting with rabbit anti-human RARβ polyclonal antibody (lanes 5 and 11). The images shown are representatives of 3 replicates of each.

**Figure 6** Total RARβ protein content in liver, thyroid, kidney, and heart (A) of the female rats fed diets containing either 20% casein or 20% alcohol-washed SPI for 70 d. The image shown in (A) is a representative of 3 replicates. RARβ2 expression in thyroid was further analyzed separately (B).

### Discussion

In this study, we showed that consumption of alcohol-washed SPI (containing minimal amount of ISF) significantly elevated hepatic RARβ2 protein content in both male and female rats. This effect was further demonstrated to be dose dependent. However, the protein content of the other RAR, including RARα and RARγ, did not differ among dietary groups, indicating that the effect of SPI is RAR isoform-specific. Supplementation with increasing amounts of soy-derived ISF to SPI-based diets had no consistent additional effect.

Although the SPI used in this study was subjected to alcohol extraction to remove the associated ISF, a minimal level of ISF (31.7 mg/kg diet) remained in the diet containing 20% SPI. Soy ISF, especially the major component genistein, are capable of modulating RARβ gene expression in various cancer cells via inhibition of DNA methyltransferase activity (38). This may imply that the effect of SPI observed could be a result of ISF contamination. To exclude this possibility, we added a similar amount of ISF (42.8 mg/kg diet) from Novasoy to the casein-based diet in Expt. 2. The results showed that added ISF had no significant effect (P > 0.05) on the nuclear RARβ2 protein in the liver, confirming that SPI rather than ISF accounts for the increase in hepatic RARβ2 protein.

To understand the potential underlying molecular event(s) by which SPI elevated hepatic RARβ2 protein content, we further measured the RARβ2 mRNA steady-state levels and found that dietary SPI failed to upregulate the RARβ2 gene expression throughout the feeding periods. In contrast, the long-term feeding (310 d) of the rats with the SPI-based diet decreased the RARβ2 mRNA abundances in the liver compared with the casein diet. This indicates that modulation of hepatic RARβ2 protein content by SPI might be post-transcriptional. This notion was supported by the evidence obtained from 2D western-blot analysis showing that the rats fed SPI-based diets have 2 unique hepatic proteins with the same molecular weight (51 kDa) as RARβ2 but different pls. These proteins cross reacted with rabbit anti-human RARβ antibody, suggesting that they might be modified forms of RARβ2 protein.

This study showed that dietary SPI markedly suppressed the binding activity of the hepatic nuclear RARβ to the consensus DNA sequence of target genes. Binding of the nuclear receptors to the ligand response elements of the promoter region in the target genes is essential for the regulation of downstream gene expression and critical for the receptor-mediated functions. Thus, inhibition of RARβ function (i.e. DNA binding activity) is believed to play a pivotal role in mediating the previously reported suppressive actions of SPI on retinoid-induced hyperglycemia that was shown to be mediated through RAR (10).

We further demonstrated that nuclear content of RARβ2 protein was also consistently elevated in both male and female rats by dietary SPI (Fig. 2). This suggests that modification of RARβ2 protein by SPI may affect only the DNA binding ability but not the translocation of the receptor into nucleus. RARβ protein content in the other tissues examined were not affected by dietary SPI in this study, indicating that the effects of soy protein are liver specific. Liver plays important roles in the uptake, storage, and mobilization of retinol and stores up to 80% of the body retinoids (39). In addition, RARβ2 is the most abundant RARβ isoform in the body (40). Suppression of hepatic RARβ function by soy components may have impacts on vitamin A function and RARβ-regulated gene expression and activities. RARβ has been identified as a tumor suppressor (41–44) and silenced or reduced RARβ gene expression is closely associated with tumorigenesis (13,18,45). Whether the inhibition
of RARβ DNA binding ability induced by soy components in the liver is related to any type of carcinogenesis warrants further investigation.

Although the exact molecular mechanism(s) by which soy components elevated the hepatic RARβ2 protein level and suppressed RARβ DNA binding activity is not understood, it is believed that post-translational modification of RARβ2 protein may play a key role in this regard. Phosphorylation is one of the most common protein modifications in animal cells (46). It has been shown that phosphorylation of serine and/or threonine in proteins such as soluble CD44, the principal hyaluronic acid receptor (47), and rat valosin-containing protein (48) resulted in acidic shift in their pIs, an effect observed in hepatic RARβ2 of the rats fed SPI in this study. Moreover, phosphorylation is known to be an important mechanism regulating the transactivation and degradation of RAR (49). Particularly, the phosphorylation of RARα has been extensively studied and several kinases including Akt and c-Jun N-terminal kinase have been shown to be involved in these processes. For example, Akt phosphorylates the Ser-96 residue of RARα DNA-binding domain and inhibits its transactivation (49), c-Jun N-terminal kinase phosphorylates RARα at residues Thr-181, Ser-445, and Ser-461, resulting in RAR dysfunction and degradation through the ubiquitin-proteosomal pathway (50). In addition, several components of soy, including soybean trypsin inhibitors, ISF (genistein), and their metabolite, equol, have been shown to affect phosphorylation status of proteins and enzymes (51–53).

It was suggested that a higher arginine-to-lysine ratio in soy may be responsible for its lipid-lowering actions, because the addition of arginine to a casein-based diet reduced the severity of retinoid-induced hypertriglyceridemia, but not as effectively as replacing casein with soy protein (54). More recent evidence indicates that β-conglycinin, one of the major soybean storage proteins, may contain the bioactive peptide responsible for the hypolipidemic effects of soy (2,55–57).

In summary, this study demonstrates for the first time, to our knowledge, that intake of alcohol-washed SPI markedly increased hepatic RARβ2 protein content and suppressed the binding activity of the nuclear RARβ to the consensus DNA sequence of target genes in rats. The RARβ2 mRNA abundance was not increased by dietary SPI. The pIs of the hepatic RARβ2 protein demonstrated that more acidic isoforms were prevalent in rats ingesting SPI. These results suggest that dietary SPI may exert its effect through post-translational modification such as phosphorylation on the RARβ2 protein, thereby changing its structure or conformation and affecting the protein degradation (or stability) and DNA binding activity of RARβ. However, this remains to be confirmed. Our ongoing studies include determination of the type of protein modifications in a variety of proteins in response to dietary SPI. Further work is required to identify the bioactive component(s) in soy and their effects on protein degradation using appropriate models.

**Literature Cited**


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