Original Research

Dietary soy isoflavones increased hepatic protein disulfide isomerase content and suppressed its enzymatic activity in rats

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Abstract

Protein disulfide isomerase (PDI) is a multifunctional protein and plays important roles in protein folding, triglyceride transfer, insulin degradation, and thyroid hormone transportation. This study examined the modulation of PDI expression by soy consumption using rat as a model. Sprague-Dawley male and female rats at 50 days (d) of age were fed diets containing either 20% casein or alcohol-washed soy protein isolate (SPI, containing 50 mg isoflavones (ISFs)/kg diet) or SPI plus ISF (250 mg/kg diet) and mated at age of 120 d. The offspring (F1) were fed the same diets as their parents. Addition of ISF to SPI diet markedly increased PDI protein content in the liver and testis of the adult rats compared with the casein or SPI diet. PDI mRNA abundance in the liver and protein content in the brain, thyroid, heart, and uterus were unchanged by the diets. Two-dimensional Western blot showed that the rats fed diets containing SPI had a diminished hepatic PDI protein with an isoelectric point (pI) of 6.12, a dephosphorylated form, compared with the rats fed diets containing either casein or SPI with supplemental ISF. Soy ISF added into SPI diet remarkably suppressed hepatic PDI activity of the rats compared with the casein diet. Moreover, soy ISF dose-dependently increased PDI and thyroid hormone receptor (TR) β protein content, whereas reduced TR DNA binding ability in human hepatocytes. Overall, this study shows that soy ISF increased hepatic PDI protein content, but addition of ISF into SPI diet inhibited its enzymatic activities and this effect may be mediated through a post-transcriptional mechanism.

Keywords: Rat, soy protein, isoflavones, protein disulfide isomerase, gene expression

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Introduction

Soy intake has been linked to a variety of health benefits such as improvement of lipid profiles,[1–3] decreasing the risk of cardiovascular diseases[4–6] and certain types of carcinogenesis, relief of menopause symptoms, lowering blood pressure, prevention of osteoporosis, and diabetes.[7,8] However, potential adverse effects have also been reported. For example, soy may increase the risk of breast cancer, elevate calcium requirement in women and overall mineral requirements, and cause goiter and/or thyroid cancer.[8,9]

The effects of soy on thyroid were first observed in rats fed raw soybeans.[10] Thereafter, goiter and hyperthyroidism were also observed in infants fed soy formulas.[11] Fort *et al.* demonstrated that the incidence of auto-immune thyroid diseases in children doubled when they were fed soy formula.[12] These effects on the thyroid have been reversed in animals fed soy by supplementing iodine to the diet.[13,14] leading to the discovery that soy consumption increases the iodine requirement for preventing thyroid disorders.[15] As a result of this, currently available soy-based infant formulas are fortified with iodine.

These anti-thyroid effects of soy are well documented, but still not completely understood. Several mechanisms have been proposed to explain the observations. Thyroid peroxidase (TPO) is an enzyme found in follicle cells of the thyroid that catalyzes the iodination of thyroglobulin to produce T4. Soy isoflavone (ISF), consisting mainly of genitein and daidzein, has been shown to inhibit TPO both *in vitro*[16,17] and *in vivo*,[18] leading to the belief that they are the goitrogenic component of soybeans. However, the *in-vivo* study showed no hypothyroidism in the rats,[18] and later studies have demonstrated that defatted soybean components rather than ISF are responsible for thyroid hyperplasia in iodine-deficient rats.[19,20] These findings suggest that both the ISF and other components in the soy may act in synergism with iodine-deficiency to cause hypothyroidism in rats.[21] Thyroid hormone receptors (TRs) are members of the nuclear receptor superfamily. They can

bind to the thyroid hormone response element of many genes and are responsible for mediating the effects of thyroid hormones in target cells. Our previous studies showed that dietary soy increased the abundance of hepatic TRβ1 protein, the major liver isoform, and however reduced the binding ability of the receptor to its target genes.[22,23] This suggests that many of the TR proteins in the livers of the soy-fed rats are not functional, which will result in the decreased sensitivity of the livers to thyroid hormones; this will cause the same effect as anti-thyroid hormone production. However, the underlying cellular events responsible for these actions of soy components remain unclear.

Protein disulfide isomerase (PDI) is a 55 kDa highly expressed endoplasmic reticulum (ER) protein,[24] and a major oxidoreductase responsible for the formation of disulfide bridges in newly synthesized proteins and the rearrangement of incorrectly-formed disulfide bonds.[25] PDI is also an important chaperone protein that binds to unfolded proteins and prevents their aggregation, and may act as an anti-chaperone by causing some misfolded proteins to aggregate.[26] Along with these ER functions, more recent study has demonstrated that PDI has a variety of other roles in the cell.[27] It is a required component of the enzymatic complexes prolyl-4-hydroxylase[28] and microsomal triglyceride transfer protein.[29] Moreover, it can bind to thyroid hormones,[30,31] estradiol,[32] and estrogenic compound genistein.[33] The purpose of this study was to investigate the effect of dietary soy protein isolate (SPI) and soy ISF on PDI gene expression and enzymatic activity in the livers of the rats as well as potential mediating effects of PDI on TR function in cultured human hepatocytes.

Materials and methods

Chemicals and reagents

Alcohol-washed SPI (Pro Fam 930, containing 90% protein and 0.23 mg ISF/g) and Novasoy (soy ISF concentrate) were purchased from Archer Daniels Midland Company (Decatur, IL). Casein protein (90% total protein) was from ICN (St. Laurent, Canada). Glycine, protein assay kit, nitrocellulose membranes, donkey anti-goat, and goat anti-rabbit immunoglobin G (IgG) (H + L)-horseradish peroxidase (HRP) conjugated antibody were from Bio-Rad (Hercules, CA). E-gel 2% agarose, 8–16% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, Novex Tris-Gly SDS running buffer, and M-MLV reverse transcriptase were from Invitrogen Corp (Grand Island, NY). Complete mini protease inhibitor cocktail tablets were obtained from Roche (Indianapolis, IN). Taq DNA polymerase was from New England Biolabs (Ipswich, MA). ECL Western blotting detection kits and CL-X Posure™ films were purchased from Thermo Scientific (Rockford, IL). Rabbit polyclonal anti-human PDI, β-tubulin, and goat anti-human TR β antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and QuantumRNA™ 18S Internal Standards Kit from Ambion (Austin, TX). Genistein, daidzein, bovine insulin, and PDI were from Sigma-Aldrich (St. Louis, MO). A human hepatocyte cell line, HepG2, was from American Type Culture Collection (Manassas, VA).

Animals and diets

Animal protocol was approved by Health Canada-Ottawa Animal Care Committee, and all animal handling and care followed the guidelines of the Canadian Council for Animal Care. Sprague-Dawley rats at 50 days (d) of age were randomly divided into three groups (8 males and 8 females/group) and fed different diets. The diets were formulated according to the AING93 diet[34] except that in diets 2–3, 20% casein was replaced by 20% alcohol-washed SPI. The ISF content in diet 2 was 50 mg/kg diet. In addition, diet 3 was supplemented with 250 mg ISF/kg diet. At 120 d of age, males and females from each dietary group were mated to produce pups (offspring; F1). The pups were weaned at 21 d of age and then fed the same diets as their parents until they were necropsied at an age of 28, 70, 120, or 240 d. All tissues were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Protein extraction and Western blot analysis

Total protein extraction from brain, thyroid, heart, liver, kidney, testicle, or uterus and Western blot analysis of PDI were carried out as previously described[22,23] with minor changes. Briefly, tissue samples were homogenized in 500 μL of cold lysis buffer containing protease inhibitors, centrifuged (15,000 × *g*, 20 min) and the supernatant was collected. Protein content of the extract was quantified using the Bio-Rad protein assay reagent. Total proteins (20 μg) were separated using 8–16% gradient SDS-PAGE and electrotransferred (25 V, 4°C, 90 min) onto nitrocellulose membranes. After blocking for 1 h with nonfat milk powder (5%) in Tris-buffered saline (10 mmol/L Tris, 150 mmol/L NaCl; TBS) and Tween-20 (0.05%; TBST-T), membranes were incubated overnight at 4°C with a 1:2000 dilution of the primary antibody in TBS-T with nonfat milk powder. Subsequently, the HRP-conjugated secondary antibody was incubated 1:5000 in TBS-T with nonfat milk powder for 45 min at room temperature. Immunoreactivity was detected by chemiluminescence autoradiography following the instructions of the product supplier and the images were scanned. The blots were re-probed with β-tubulin antibody. The intensities of the target bands were quantified using Scion Image software and normalized by either their respective β-tubulin.

Quantitation of hepatic PDI mRNA

Total RNA was isolated from liver as previously described[22] and reverse transcribed for cDNA synthesis using random primers. One-tenth of the cDNA was amplified using the following primers: rat PDI (forward: 5′-GACAACCA GCGCATACTTGA-3′ [892–911], reverse: 5′-TCAACAG CTTCCACCTCATTG-3′ [1309–1290]) and universal 18S rRNA primers and competimers (Ambion). Polymerase chain reaction (PCR) cycle conditions were: 94°C for 5 min, 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 30 cycles, and 72°C for 10 min. Samples were resolved on 2% agarose gels prestained with ethidium bromide. The PDI mRNA level was normalized against its 18S rRNA using densitometry.

Two-dimensional Western blot analysis of PDI

Two-dimensional gel electrophoresis was conducted according to the method of O'Farrell.[35] Briefly, liver total proteins (200 μg) pooled from three male rats fed the same diets and killed at 70 d of age were subjected to isoelectric focusing (IEF) in 2% glass tube gels of pH 3.5–10 (Amersham Pharmacia Biotech, Piscataway, NJ) for 9600 volt-h. One microgram of an IEF internal standard, tropomyosin, with a molecular weight of 33 kDa and isoelectric point (pI) of 5.2 was added to each sample. After equilibration for 10 min in the equilibration buffer (10% glycerol, 50 mmol/L dithiothreitol, 2.3% SDS and 0.0625 mol/L Tris, pH 6.8), the tube gels were sealed to the top of stacking gels on top of 10% acrylamide slab gels and SDS slab gel electrophoresis was carried out for about 4 h at 12.5 mA/gel. The gel was transblotted onto polyvinylidene fluoride (PVDF) membrane overnight at 200 mA and approximately 100 V/two gels and stained with Coomassie Brilliant Blue R-250. The membranes were immunostained with rabbit anti-human PDI polyclonal antibody (1:500 dilution) and detected using ECL kit. The pI values of PDI were calculated using a linear regression of pI and the location (distance) of the protein on the 2D gel, which was established with the internal standard.

PDI isomerase activity measurement

Hepatic PDI activity was measured following the standard method provided by Sigma using bovine insulin as a substrate. Briefly, liver tissue was homogenized in homogenization buffer (0.25 mol/L sucrose, 5 mmol/L $MgCl_2$) containing protease inhibitors (1 cocktail tablet/10 mL buffer, Roche) and centrifuged at 800 × *g* for 10 min at 4°C to collect the supernatant. The homogenates were sonicated and their protein concentration was determined by Bio-Rad protein assay kit. Liver protein homogenate (100 μg) was incubated with reaction buffer (100 mmol/L sodium phosphate, pH 7.5, 3 mmol/L EDTA and 1 mmol/L diothiothreitol (DTT)) containing bovine insulin (1.3 mg/mL) at 25°C. The turbidity was determined by reading at OD_{650}. The PDI enzymatic activity was expressed as percentage of those in casein control.

Effects of genistein, daidzein, and soy ISF on the activity of PDI *in vitro*

Genistein, daidzein, and soy ISF were dissolved in dimethyl sulfoxide (DMSO) and added into the reaction cocktail containing 1.3 mg/mL insulin, 100 mmol/L DTT, and 10 μg/mL PDI to final concentrations of 10 μmol/L for genistein and daidzein, or 5 μg/mL for soy ISF. The mixtures were incubated for 10 min at 25°C and then measured at OD_{650}. The PDI enzymatic activity was expressed as percentage of that in DMSO (vehicle) control.

Effects of soy ISF on PDI and TRβ content and TR DNA binding ability in human hepatocytes

HepG2 cells were plated at a concentration of 1×10^6 cells/100 mm dish in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin for overnight. Culture media were changed and cells were treated with 0, 5, and 50 μg/mL soy ISF or 500 μmol/L bacitracin, a cyclic peptide antibiotics which can inhibit the enzymatic activity of PDI through specific binding to its protein/peptide binding site, for 24 h. The media were removed, and the cells were rinsed with cold phosphate-buffered saline. The cells were either lysed with radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors for total protein isolation or extracted for nuclear proteins using the nuclear extraction kit (Signosis, Inc, Santa Clara, CA). Total proteins (40 μg) were separated by SDS-PAGE as described above and immune-stained with PDI, TRβ1, and β-tubulin antibodies.

The binding ability of nuclear TR to the specific sequence of its target genes was measured using the TR filter plate assay (Signosis, Inc.). Briefly, nuclear proteins (10 μg) were incubated with binding buffer mix and TR probe at 16°C for 30 min in a PCR machine. The TR-DNA complex was separated from free probes using the filter plate. The bound probes were eluted and detected in the luminometer. The TR DNA binding ability was expressed as percentage of DMSO control.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA). Differences between individual group means were determined by Fisher's least significant difference test. A probability of $P < 0.05$ was considered to be significant. Results are presented as the means ± standard error of the mean (SEM). Means with different letters significantly differ. Statistical analyses were performed using Statistica 8.0 software (StatSoft, Tulsa, OK).

Results

Hepatic PDI protein content in F1 rats

The offspring (F1) were analyzed to determine the effect of early exposure (prenatal and neonatal) of rats to soy on PDI expression. Supplementation of SPI with ISF (250 mg/kg diet) markedly increased PDI protein levels compared with casein and SPI alone diets in male rats ($P < 0.05$, Figure 1a), but had no significant effect in females measured at d 28 ($P > 0.05$, Figure 1b). Dietary addition of ISF to the SPI-based diet markedly elevated PDI content by 1.3–2.6 folds compared with casein and SPI alone diets at d 70, 120, and 240 in both male and female rats ($P < 0.05$, Figure 1a and b). PDI mRNA steady-state abundances in the livers of both male and female rats were not different among dietary groups ($P > 0.05$, data not shown).

PDI protein in other tissues

PDI protein levels in the kidneys were significantly lowered by dietary SPI compared with casein (0.5 and 0.4 folds for male and female, $P < 0.05$), and restored by the addition of supplemental ISF compared with SPI alone in both male (1.5 folds) and female (3.4 folds) rats at 70 d of age ($P < 0.05$, Figure 2a and b). Testicle PDI protein content was markedly increased by the supplementation with

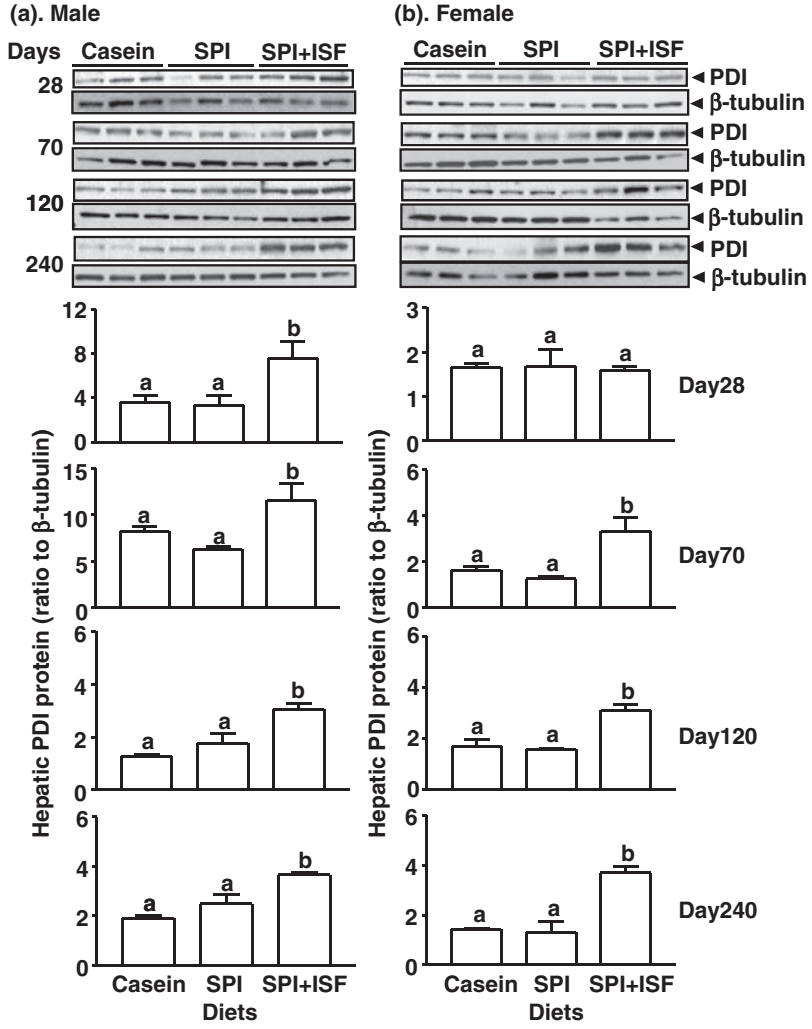

Figure 1 Hepatic PDI protein levels in F1 male (a) and female (b) rats fed either 20% casein or 20% alcohol-washed SPI with or without supplemental ISF (250 mg/kg diet) and then killed at d 28, 70, 120, or 240. The images shown are representative of six replicates. Values are mean ± SEM, $n = 6$. Means with different letters significantly differ, $P < 0.05$. ISF: isoflavone; PDI: protein disulfide isomerase; SEM: standard error mean; SPI: soy protein isolate

soy-derived ISF to the SPI-based diet compared with casein (1.6 folds) or SPI (2.5 folds) alone ($P < 0.01$, Figure 2c). However, PDI levels in the brain, thyroid, heart, and uterus did not differ among dietary groups ($P > 0.05$, data not shown).

2D Western blot analysis of hepatic PDI

The male rats fed diets containing either 20% casein or 20% SPI with added ISF and measured at 70 d of age had two PDI proteins with the same molecular weight but distinct pI (5.48 and 6.12, respectively) in the liver. The PDI protein with pI = 6.12 was diminished in the rats fed 20% SPI alone (Figure 3).

PDI enzymatic activity

Both male and female rats fed diets containing 20% alcohol-washed SPI and ISF (250 mg/kg diet) had significantly lower hepatic PDI activities than those fed a casein diet ($P < 0.05$, Figure 4) measured at ages of 70 and 240 d. Moreover, treatment of PDI with either 10 μmol/L of genistein, or daidzein or 5 μg/mL soy-derived ISF significantly suppressed its enzymatic activities ($P < 0.001$, Figure 5).

PDI, TRβ protein content, and TR DNA binding ability in HepG2 cells

Treatment with soy-derived ISF dose-dependently increased PDI and TRβ protein content in cultured human hepatocytes ($P < 0.05$, Figure 6). Interestingly, the DNA binding abilities of the nuclear TR of the HepG2 cells were significantly suppressed by ISF. A similar effect was observed with 500 μmol/L bacitracin, a known PDI inhibitor.

Discussion

Approximately 25% of American infants consume soy protein-based formula as a replacement for milk protein-based formulas.[36] Various soy components are known to be biologically active and affect expression of many genes

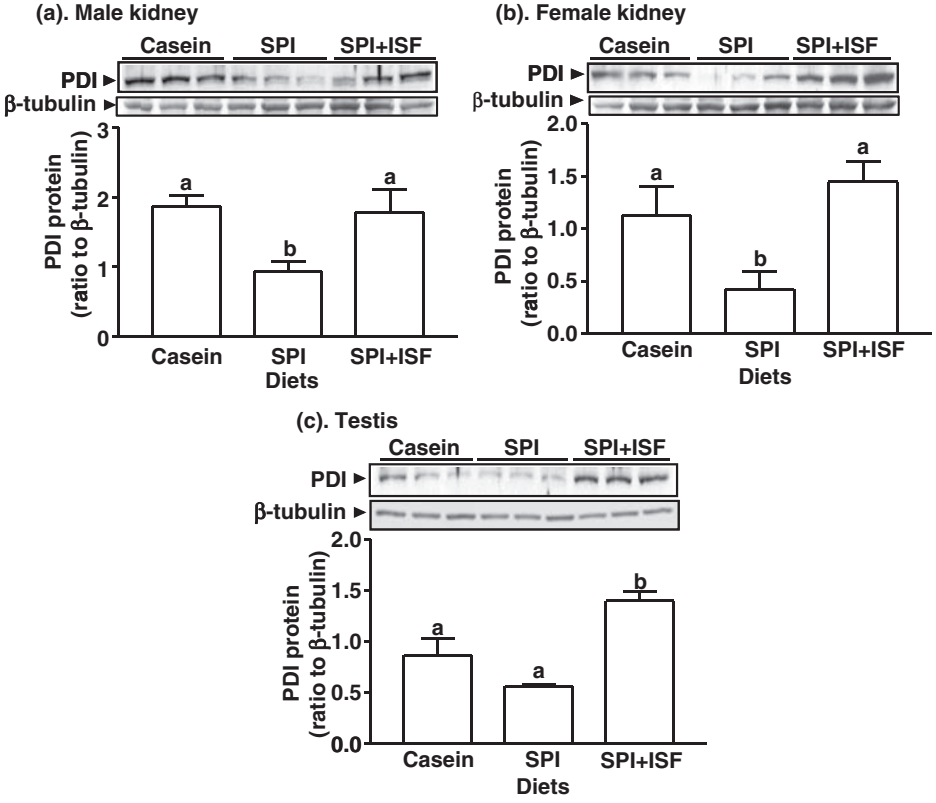

Figure 2 PDI protein content in the kidneys (a, b) or testes (c) of the rats fed diets containing either 20% casein or 20% alcohol-washed SPI with or without supplemental ISF (250 mg/kg diet) and killed at 70 d of age. The images shown are representative of six replicates. Values are mean ± SEM, $n = 6$. Means with different letters significantly differ, $P < 0.05$. ISF: isoflavone; PDI: protein disulfide isomerase; SEM: standard error mean; SPI: soy protein isolate

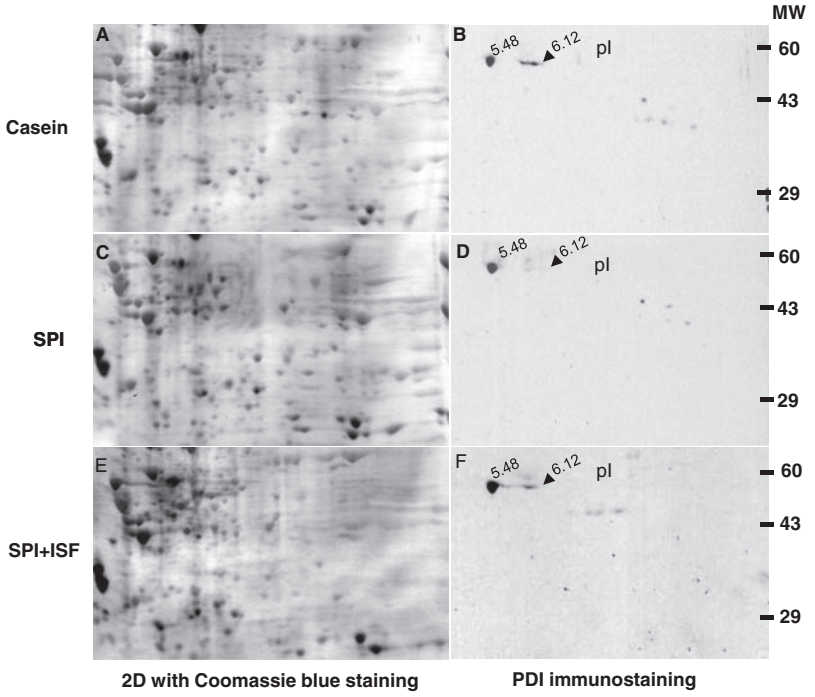

Figure 3 Two-dimensional Western blot analysis of hepatic PDI protein in the male rats fed diets containing either 20% casein (A and B) or 20% alcohol-washed SPI without (C and D) or with supplemental ISF (250 mg/kg diet) (E and F) and killed at age of 70 d. The isoelectric points (pI) were calculated using a linear regression. ISF: isoflavone; PDI: protein disulfide isomerase; SPI: soy protein isolate

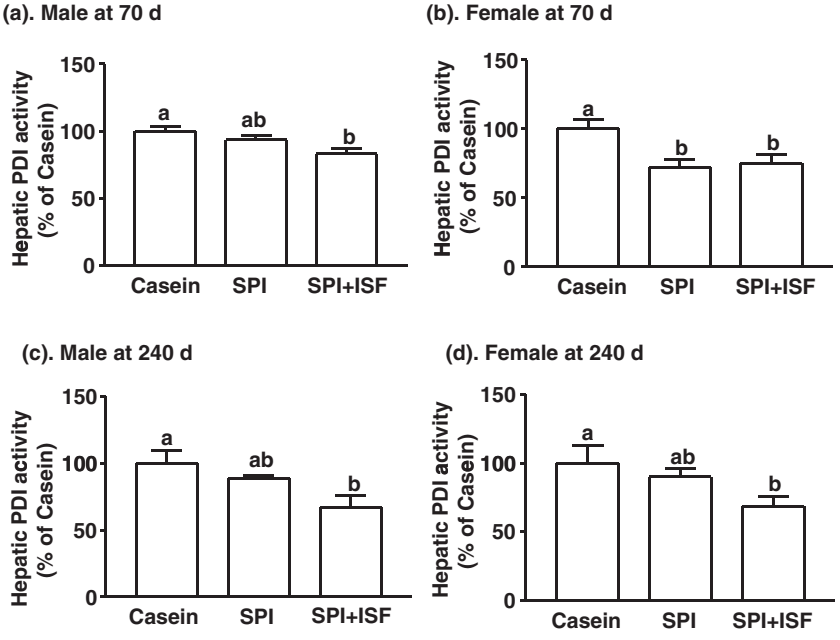

Figure 4 Hepatic PDI activity in the rats fed diets containing either 20% casein or 20% alcohol-washed SPI in the absence or presence of supplemental ISF (250 mg/kg diet) and killed at ages of 70 d (a, b) or 240 d (c, d). Values are mean ± SEM, $n = 6$. Means with different letters differ, $P < 0.05$. ISF: isoflavone; PDI: protein disulfide isomerase; SEM: standard error mean; SPI: soy protein isolate

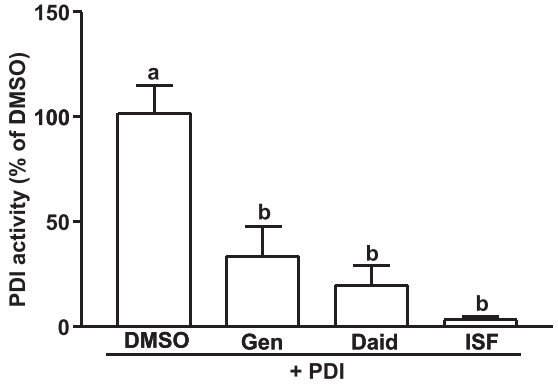

Figure 5 Inhibition of PDI activity by genistein, daidzein, and soy ISF. PDI protein was co-incubated with either dimethyl sulfoxide (DMSO, vehicle) or 10 μmol/L genistein (Gen), or 10 μmol/L daidzein (Daid) or 5 μg/mL of soy ISF for 10 min. Values are mean ± SEM, $n = 6$. Means with different letters differ, $P < 0.05$. ISF: isoflavone; PDI: protein disulfide isomerase; SEM: standard error mean

involved in different metabolic pathways and modulate the function of physiologically important proteins. Our previous studies showed that rats fed 20% dietary SPI had significantly elevated levels of TRβ1, the dominant isoform of TRs, in the liver and inhibited binding of nuclear TRs to the target DNA sequence.[22,23] In this study, PDI, a multifunctional protein involved in the protein folding and a known cellular thyroid hormone binding protein, was investigated to determine if dietary SPI or ISF had any effect on its abundance and enzymatic activity in the livers of the rats.

Maternal and neonatal exposure to dietary ISF markedly increased hepatic PDI protein content in the males, but had no significant effect in the females measured at 28 d of age. Although the mechanisms involved in this sex difference remain unclear, it has been shown that the abundances of a second class of estrogen-binding proteins with higher-capacity, lower-affinity binding sites in addition to estrogen receptors in the livers of males and females are very different.[37] This difference may contribute to the gender-dependent responsiveness of livers to the estrogenic ISF. However, this needs to be further investigated. The hepatic PDI protein content measured at ages of 70, 120, and 240 d were significantly increased by dietary ISF in both sexes. Dietary ISF failed to up-regulate PDI mRNA expression. 2D Western blot results showed that dietary casein and SPI differentially modified PDI protein. Feeding the alcohol-washed SPI diet diminished the dephosphorylated hepatic PDI, pI = 6.12, compared with the casein diet, whereas addition of ISF into the SPI diet at least partially restored the status of the dephosphorylated PDI. This suggests that soy ISF may play a role in the regulation of PDI phosphorylation and dephosphorylation. Very few studies have reported diet-induced changes in PDI expression. One study found that hepatic PDI protein levels decreased by 40% in response to fasting for three days, but they returned to normal after 3 d of refeeding.[38]

PDI has distinct binding sites for hormones and peptide/protein.[39] Estradiol inhibited isomerization activity of PDI, which might be due to the similarity of PDI parts with estrogen receptor segments.[32] Genistein suppressed the ability of PDI to prevent aggregation of folding proinsulin through inhibition of peptide binding activity.[33] There are several possible mechanisms by which soy ISF influence PDI protein levels in liver cells. ISF could directly affect PDI through its estradiol binding site. Although the binding affinity of ISF to PDI is low (2 μmol/L), a high concentration

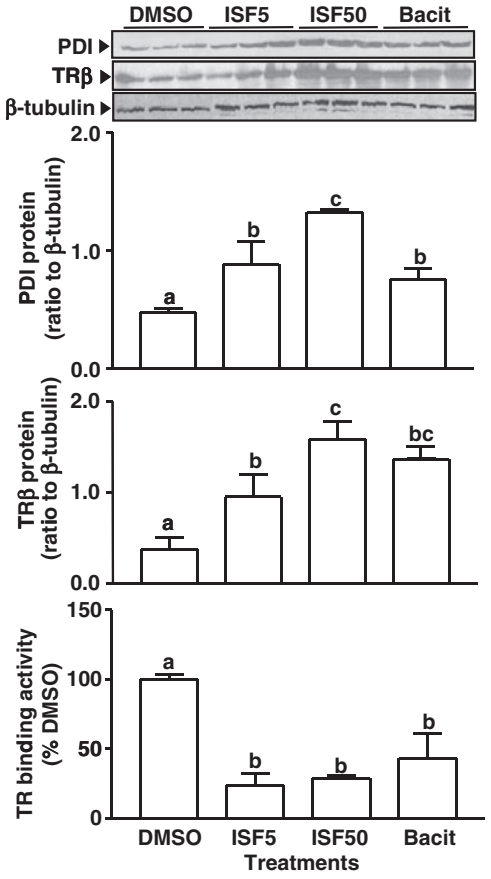

Figure 6 PDI, TRβ protein content, and TR DNA binding ability in HepG2 cells treated with 0 (DMSO as vehicle), 5, and 50 μg/mL soy ISF or 500 μmol/L bacitracin (Bacit), a known PDI inhibitor, for 24 h. Values are mean ± SEM, $n = 6$. Means with different letters differ, $P < 0.05$. DMSO: dimethyl sulfoxide; ISF: isoflavone; PDI: protein disulfide isomerase; SEM: standard error mean; TR: thyroid hormone receptor

of ISF could make the binding physiologically significant. They could either block the interaction of PDI with E2 or T3, or induce a change in conformation that alters PDI activity. Moreover, genistein is known to bind to the peptide-binding site of pancreatic PDI and prevent proper folding activity.[40] It has been shown that genistein can inhibit proper refolding of proinsulin by PDI.[33] This makes it possible that dietary genistein causes a decrease of PDI activity, leading to a feedback mechanism in which PDI is stabilized to increase protein levels. This notion has been supported by both the *in-vivo* and *in-vitro* results of the present study, showing that genistein, daidzein, and soy ISF inhibited PDI enzymatic activity *in vitro* and addition of soy ISF into SPI diet suppressed hepatic PDI activities in both male and female rats compared with a casein diet. Additionally, soy ISF dose-dependently increased PDI and TRβ protein abundances and suppressed TR DNA binding ability in the human hepatocytes. The decreased PDI activity induced by soy intake may prevent the TR from achieving its correct conformation and thereby reducing its DNA binding ability. This would correlate with our previous studies in which TRβ1 had reduced affinity for its consensus target sequence upon feeding SPI.[23]

In conclusion, this study demonstrated for the first time that dietary soy ISF increased hepatic PDI protein levels, but addition of ISF into SPI diet suppressed its enzymatic activity in rats. Soy ISF were also shown to increase the PDI protein abundance in cultured human hepatocytes. The potential physiological significance of these cellular responses and the molecular mechanism(s) involved need to be further investigated.

Author contributions: KD, OL, and CW conducted experiments, sample and data analyses. GC and IC were involved in animal study. CWX designed the experiments, interpreted the data and wrote the manuscript.

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