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Inhibition of Reactive Nitrogen Species Effects in Vitro and in Vivo by Isoflavones and Soy-Based Food Extracts

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Recent studies have shown that soy isoflavone inhibits inducible nitric oxide (NO) synthase activities and is reported to have peroxynitrite scavenging ability. Consequently, we investigated whether isoflavones (daidzein and genistein) and extracts from soy-based products (miso, soymilk, tofu, soy sprout, black soybean, soybean, and yuba) would inhibit the reactive nitrogen species (RNS) effect in vitro and in vivo. In the in vitro experiments [including the protection of cellular DNA from peroxynitrite or sodium nitroprusside damage, an inhibitory effect on nitric oxide production from lipopolysaccharide (LPS)-induced RAW 264.7 cells, and nitric oxide scavenging ability], extracts from soy-based foods showed a potent antioxidant activity and an inhibiting effect on RNS activity. These effects were correlated with total isoflavone content. In the in vivo experiments, rats were given isoflavones (4.0 mg/kg bw) or soy-based product extracts (1.0 g/kg bw) orally for 1 week and were injected with vehicle H_2O (1 mL/kg bw) or LPS (10 mg/kg bw) on the day 7. Twelve hours after treatment, the rats were killed, and blood serum was collected for analysis. The intraperitoneal administration of LPS resulted in an increase in serum nitrite, nitrate, and nitrotyrosine concentrations. These are stable metabolite end products of nitric oxide, to 4-, 16-, and 5-fold levels, (4, 10 μM and 58 $\pm$ 14 pmol/mL), of the placebo control, respectively. Results showed that oral administration of isoflavones and extracts from soy-based products significantly decreased serum nitrite, nitrate, and nitrotyrosine levels in LPS-induced rats. This study demonstrates that soy isoflavone supplementation may inhibit RNS-induced oxidation both in vitro and in vivo.

KEYWORDS: Isoflavones; soybean; yuba; reactive nitrogen species; nitrotyrosine; antioxidant activity

INTRODUCTION

Reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite, have several roles in mammals, but unregulated RNS production can cause adverse effects (e.g., cell damage or cell death) through reaction with biological target molecules such as DNA, lipids, and proteins. Some of these interactions are capable of causing conditions of pathological damage such as cancer, septic shock, inflammation, atherosclerosis, and ischemia-reperfusion (*1*−*3*). Furthermore, it has been reported that nitric oxide and peroxynitrite may activate cyclooxygenase, the rate-limiting enzyme for prostaglandin biosynthesis (*4*, *5*). Prostaglandins and nitric oxide are important mediators in the inflammatory process. In recent years, attention has been focused on the association between chronic inflammation and cancer risk (*6*). In fact, many phytochemicals have been screened for the chemoprevention of several chronic diseases such as cancer, inflammation, and the aging process on the basis of their attenuation of the RNS adverse effect through the inhibition of the inducible nitric oxide synthase (iNOS) activity and expression (*7*−*10*).

Epidemiological evidence and experimental data from animal studies strongly support the beneficial effects of isoflavones on human health (*11*). In mammalian systems, soy isoflavones exhibit a number of biological activities, including inhibition of cell proliferation (*12*, *13*), antioxidative effects (*14*), and enzyme−inhibitory effects (*15*). In addition, dietary soy isoflavonoids have been considered for the treatment and prevention of hormone-dependent diseases (*16*). They also show antiestrogenic activity by competing with endogenous estrogens for receptor binding (*17*, *18*). Several studies suggest that the lower incidence of breast cancer and heart disease caused by the lower estrogen levels in Asian women, compared to their western counterparts, can be attributed to their high intake of soy foods (*19*, *20*). Although the underlying mechanisms are not fully understood, the potential of isoflavones in soybean-based foods to act as antioxidants and scavengers of reactive oxygen species (ROS) and RNS is thought to be an important finding (*21*, *22*).

Since the beneficial health effects of isoflavones are of increasing interest to nutritionists and food manufacturers, it is interesting to demonstrate the correlation between the isoflavone

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content in soy-based foods and their antioxidative abilities, including their scavenging effect on ROS and RNS. In this study, we attempted to determine whether the isoflavone content in soy-based foods plays a protective role in contributing to their antioxidative abilities in vitro and to investigate the inhibitory effects of isoflavones and extracts from soy-based products on NO production and nitrotyrosine levels in rat serum induced by LPS treatment.

MATERIALS AND METHODS

Materials and Chemicals. Soybean, black soybean, soy sprout, tofu, miso, yuba, and soymilk were purchased from a local market in Taichung, Taiwan. Genistein, daidzein, tyrosine, lipopolysaccharid (LPS; *E. coli*, serotype O55:B5), *N*-(1-naphthyl)ethylenediamine dihydrochloride (NEDD), sulfanilamide (SULF), vanadium(III) chloride (VCl_3), sodium nitrite, sodium nitrate, butylated hydroxytoluene (BHT), 3-nitro-L-tyrosine (NO_2-Tyr), α-methyl-L-*p*-tyrosine (Me-Tyr), and bovine serum albumin (BSA) were all obtained from the Sigma Chemical Co. (St. Louis, MO). A protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). Sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium dihydrogen phosphate, potassium hydrogen phosphate, and dimethyl sulfoxide (DMSO) were obtained from the E. Merk Co. (Darmstadt, Germany).

Preparation of 80% Methanol Extracts from Soybean Products. All the soybean and related products were milled with a grinder (RT-08, Rong Tsong, Taichung, Taiwan) and freeze-dried. Each freeze-dried sample (20 g) was mixed with 200 mL of 80% methanol, stirred at 60 °C for 1 h, and then centrifuged at 3800*g* for 10 min. The upper solution was dried under vacuum. A 100-fold stock solution was prepared by dissolving the extracts in DMSO, such that DMSO never exceeded 1% of the final volume when added to the samples. Negative control cultures received the equivalent concentration of DMSO only.

Determination of Isoflavone Contents. Isoflavone was extracted and analyzed using the method of Coward et al. (*12*) with a modification. The 80% methanol extract from soy-based products was redissolved in 5 mL of 50% methanol and extracted with 20 mL of hexane four times to remove lipids. The methanol layer was evaporated to dryness and then redissolved in 10 mL of 80% methanol. The solution was filtered through a 0.2 μm filter paper before HPLC analysis. The HPLC system consisted of a Hitachi model L-4200 UV−vis detector and a Hitachi model D-2500 Chromato-integrator. The column was a LiChrosorb 100 RP-18 (5 μm, 250 × 4.6 mm i.d., E. Merck). The solvent system used was a gradient of 15−45% acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1.3 mL/min. The detecting wavelength was set at 262 nm. The concentration of isoflavone was calculated from standard curves of the area responses for authentic isoflavone standards.

Scavenging Effect on Nitric Oxide. The scavenging effect of extracts from soybean products on nitric oxide was measured according to the method of Marcocci et al. (*23*). Four milliliters of extract solutions at different concentrations were then added in the test tubes to 1 mL of sodium nitroprusside (SNP) solution (25 mM) and the tubes incubated at 37 °C for 2 h. An aliquot (0.5 mL) of the incubation solution was removed and diluted with 0.3 mL of Griess reagent (1% SULF in 5% H_3PO_4 and 0.1% NEDD). The absorbance of the chromophore that formed during the diazotination of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 570 nm and referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess reagent.

Nitrite Assay. Cells were seeded in 96-well plates (8×10^4/200 μL), cultured for 2 days, and then incubated both with and without LPS, in both the absence and presence of isoflavone, individually in various concentrations for 20 h. The nitrite concentration in the supernatant was assessed by the Griess reaction (*24*) and determined by comparison with a sodium nitrite standard curve.

Cell Viability. Cell respiration as an indicator of cell viability was determined by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. After removing the supernatants from the plate for nitrite determination, the cells were incubated at 37 °C with 0.5 mg/mL MTT for 45 min. The medium was aspirated and the cells were solubilized in DMSO (250 μL) for at least 2 h in the dark. The extent of the reduction of MTT was quantified by measuring the absorbance at 550 nm.

Single-Cell Gel Electrophoresis (the Comet Assay). Raw 264.7 cells, a murine macrophage cell line, were treated with extracts from soy-based products(200 μg/mL) in combination with 1 mM SNP for 2 h or peroxynitrite (0.75 mM) for 10 min at 37 °C in an incubator together with untreated control samples. Peroxynitrite synthesis was carried out and prepared by the method as described in our previous study (*25*). Cells were treated with SNP or peroxynitrite only at the same incubation conditions as the positive control. Cells were harvested by trypsinization and centrifuged at 800 rpm. The supernatant fractions were removed and the peleted cells were used to determine either cell viability or single cell gel electrophoresis as described below.

Single cell gel electrophoresis was performed according to the procedure of Singh et al. (*26*) with some modifications. Briefly, fully frosted slides were covered with 0.5% normal melting point agarose (NMA) as the first layer, with a mixture of cell suspension and 0.5% of low melting point agarose (LMA) as the second layer, and finally with 0.5% of LMA (without cell) as the third layer. After solidification at 4 °C, all the slides were immersed in lysing buffer (2.5 M of NaCl, 100 mM of EDTA, pH 10, with freshly added 1% Triton X-100 and 10% DMSO) at 4 °C for 1 h, and the slides were then placed in a horizontal electrophoresis tank. The tank was filled with freshly prepared electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 10, 4 °C), and the slides were left in the solution for 20 min to allow DNA unwinding and expression of alkali-labile damage before performing electrophoresis. Electrophoresis was then conducted at 4 °C for 20 min using 25 V and 300 mA. After electrophoresis, the slides were neutralized in a neutralization buffer, stained with ethidium bromide, kept in a humidified airtight container, and examined using a fluorescence microscope (Nikon EFD-3, excitation filter BP 543/10 nm, emission barrier filter 590 nm). The Comet analysis system (Komet 3.1, Kinetic Imaging Ltd., Liverpool, UK) was applied to quantify the DNA damage. Triplicate samples were prepared and examined. Images of 100 randomly selected cells from each slide were analyzed. The degree of DNA damage was expressed as the tail moment value: tail moment = (tail length × tail DNA %)/100.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The automated ORAC assay was carried out on a FLUOstar galaxy spectrophotometer (BMG Labtechnologies Ltd., Offenburg, Germany) with fluorescent filters (ex, 540 nm; em, 565 nm). The procedure was based on the previous report of Cao et al. (*27*). Briefly, in the final assay a mixture (200 μL total volume), β-phycoerythrin (β-PE) (16.7 nM) was used as a target of free radical (or oxidant) attack, with 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) (4 mM) as a peroxyl radical generator ($ORAC_{ROO}$• assay). Trolox was used as a control standard. The analyzer was programmed to record the fluorescence of β-PE every 2 min after AAPH was added. All fluorescence measurements were calculated using the difference of areas under the β-PE decay curves between the blank and a sample and are expressed as the μmol of Trolox equivalent/g of extracts.

LPS Treatment and Sample Preparation. Male Wistar rats (180−200 g) were obtained from National Laboratory Animal Breeding and Research Center (Taiwan) and allowed to acclimate to their surroundings for 1 week before the experiments. Rats were randomly allocated to 10 groups, each consisting of 6 rats, and housed in a room maintained under the following conditions: temperature of 22 ± 2 °C, relative humidity 65 ± 5%, and a 12-h light cycle. The animals were fed a soy-free diet, and their diet was comprised of the following ingredients: lactalbumin, 20%; sucrose, 62.2%; corn oil, 10%; cellulose, 3%; mineral (AIN 76), 3.5%; vitamin (AIN), 1%; and choline bitartrate, 0.3%. Test compound (GE or DE) and extracts from soy-based products (SE or YE) were suspended with 0.9% NaCl solution by ultrasound. The animals were given the test compounds (4.0 mg GE or DE/kg bw day) or extracts (1.0 g of SE or YE/kg bw day) by stomach incubation and given deionized water ad lib. Placebo group were administered

Table 1. The Groups of Treatment Animal

treatment[a]	oral administration	intraperitoneal injection
placebo	0.9% NaCl	H_2O (1 mL/kg bw)
LPS only	0.9% NaCl	LPS (10 mg/kg bw)
soybean extracts (SE)	1.0 g of SE/kg bw	H_2O (1 mL/kg bw)
SE + LPS	1.0 g of SE/kg bw	LPS (10 mg/kg bw)
yuba extracts (YE)	1.0 g of YE/kg bw	H_2O (1 mL/kg bw)
YE + LPS	1.0 g of YE/kg bw	LPS (10 mg/kg bw)
genistein (GE)	4.0 mg of GE/kg bw	H_2O (1 mL/kg bw)
GE + LPS	4.0 mg of GE/kg bw	LPS (10 mg/kg bw)
daidzein (DE)	4.0 mg of DE/kg bw	H_2O (1 mL/kg bw)
DE + LPS	4.0 mg of DE/kg bw	LPS (10 mg/kg bw)

[a] The animals were given the test compounds (4.0 mg of GE or DE/kg bw day) or extracts (1.0 g of SE or YE/kg bw day) by stomach incubation, and given deionized water ad lib. Placebo group were administered only vehicle.

only vehicle (ca. 2 mL of 0.9% NaCl solution). Rats were injected ip with either vehicle (sterilized distilled water) or LPS (10 mg/kg bw). The groups of treatment animals are described in **Table 1**. A plasma sample was obtained 12 h following injection; the animals were then sacrificed and liver tissue collected.

Nitrotyrosine Analysis. Nitrotyrosine levels in serum were measured by the method of Kamisaki et al. (*28*). The separated serum (120 μL) was mixed with ethanol (280 μL) containing Me-Tyr (300 pmol), as an internal standard. After centrifugation at 10000*g* for 10 min, the resulting supernatant was kept at −80 °C until the chromatographic analysis. The stored samples (100 μL) were derivatized with 20 μL of 0.1 M sodium borate buffer, pH 8.7) and 20 μL of 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) (50 mg/mL in ethanol) and incubated at 60 °C for 2 min. The reaction was terminated by addition of 30 μL of 0.1 M HCl, and an aliquot (100 μL) was injected onto the column. The HPLC system consisted of a Hitachi model F-1050 fluorescence spectrophotometer, and a Hitachi model D-2500 Chromato-integrator. The column was a LiChrosorb 100 RP-18 (5 μm, 250 × 4.6 mm i.d., E. Merk). The solvent system used was 0.1 M sodium phosphate buffer (pH 7.2)−methanol (45:55, v/v) at a flow rate of 1.0 mL/min. After 30 min elution, the mobile phase was switched to 80% methanol solution for the wash. The fluorescence detector was set at 470 and 530 nm for excitation and emission wavelengths, respectively. The calculation of NO_2-Tyr concentration in each sample was based on the ratio to Me-Tyr, the internal standard. When the reproducibility of the method was studied by injecting six samples containing NO_2-Tyr (50 pmol) and Me-Tyr (100 pmol), the coefficient of variation was 4.6%.

Nitrate and Nitrite Analysis. Nitrate and nitrite levels in the serum were measured by the method of Miranda (*29*). The rat serum was deproteinized to reduce turbidity by centrifugation through a 30-kDa molecular weight filter at 14 000 rpm for 3 h at 4 °C for 500-μL samples. Typically, a nitrate standard solution (100 μL) was serially diluted and duplicated in a 96-well, flat-bottomed, polystyrene microtiter plate. The diluting medium was used as the standard blank. After loading the plate with the sample (100 μL), VCl_3 (100 μL saturated solution in 1 M HCl) was added to each well, rapidly followed by the addition of the Griess reagents, SULF (50 μL, 2% w/v in 5% HCl) and NEDD (50 μL, 0.1% w/v in H_2O). Sample blank values were obtained by substituting the diluting medium for the Griess reagent. Nitrite was measured in a similar manner, except that samples and nitrite standards were only exposed to the Griess reagents. In both cases the absorbance at 570 nm was measured using a plate reader following incubation of 30 min.

Determination of Serum Concentrations of Isoflavone. The free isoflavone was enriched from serum by solid-phase extraction according to the method of Janning et al. (*30*). HPLC analysis was based on the method of Coward et al. (*12*) with a modification. In brief, upon addition of 200 μL of hydrolysis buffer I [0.1 M sodium acetate pH 5 containing 0.1% (w/v) ascorbic acid and 0.01% (w/v) EDTA], 50 μL of β-glucuronidase (1000 U/mL), and 50 μL of sulfatase (500 U/mL) to 500-μL serum aliquots, samples were allowed to hydrolyze at 37 °C for at least 12 h. Samples were then passed at room temperature over 0.5 g of Lichrolut RP18 cartridges (Merck, Darmstadt, Germany), which were subsequently washed with 5 mL of ammonium acetate buffer (pH 5; 0.01M) and 5 mL of water. The absorbed isoflavones were eluted with 1.5 mL of methanol. The methanol elutes were evaporated to dryness under a stream of nitrogen at 37 °C, dissolved in 100 mL of 80% methanol, and stored at −20 °C until HPLC analysis. For HPLC analysis, the conditions were the same as those described above.

Table 2. Amount of Isoflavones in Extracts from Soybean and Related Products

	isoflavones (mg/g of extract)[a]					
samples	daidzin	genistin	daidzein	genistein	glycitein	total
soybean	1.08	0.75	0.31	0.75	0.06	2.95
	(0.53)[b]	(0.37)	(0.15)	(0.37)	(0.03)	(1.45)
black soybean	0.98	0.73	0.50	0.24	0.21	2.66
	(0.72)	(0.53)	(0.37)	(0.18)	(0.15)	(1.95)
soy sprout	0.88	0.72	0.59	0.24	0.04	2.47
	(0.19)	(0.14)	(0.11)	(0.05)	(0.008)	(0.47)
soy milk	0.33	0.33	0.13	0.04	0.01	0.84
	(0.18)	(0.18)	(0.07)	(0.02)	(0.005)	(0.45)
miso	0.30	0.28	0.12	0.04	0.01	0.75
	(0.07)	(0.06)	(0.03)	(0.009)	(0.002)	(0.17)
tofu	0.76	1.19	0.20	0.17	0.01	2.33
	(0.36)	(0.56)	(0.10)	(0.08)	(0.005)	(1.11)
yuba	5.31	6.23	1.14	0.78	0.19	13.7
	(0.63)	(0.74)	(0.14)	(0.09)	(0.02)	(1.62)

[a] Values are mean of duplicate analyses. [b] Amount of isoflavones in original soybean and related products before freeze-drying, which was expressed as mg/g of wet weight.

Statistical Analysis. To verify the statistical significance of all parameters, the values of mean and standard deviation (mean ± SD) and 95% confidence intervals (CI) were calculated. If necessary, data were tested by two-way ANOVA. *P*-Values of <0.05 were assumed to be statistically significant. All data are expressed as the means of three measurements.

RESULTS

In Vitro Studies. 1. Isoflavone Contents in Extracts from Soybean and Its Related Products. Table 2 lists the amount of isoflavones in extracts from soybean and its related products, which were analyzed in this study. The amount of total isoflavones in miso, soy milk, tofu, soy sprout, black soybean, soybean, and yuba were 0.75, 0.84, 2.33, 2.47, 2.66, 2.95, and 13.79 mg/g of extracts, respectively. The value in parentheses denotes the original isoflavone content of the samples before freeze-drying. The results showed that the soy products that provided the best dietary source of isoflavones were soybean, black soybean, and yuba. The distribution of the individual isoflavones within each product is similar, while isoflavone content varied in the soy-related products. For instance, the levels of the glycon form (daidzin and genistin) are higher than its related aglycon form (daidzein and genistein). The content of daidzin and genistin in the yuba extracts was 5.31 and 6.23 mg/g, respectively. For extracts from soybean, black soybean, soy sprout, and tofu, the content of daidzin and genistin was between 0.76 and 1.08 mg/g and 0.72−1.19 mg/g, respectively. Both the daidzin and genistin content in the miso and soymilk extracts was about 0.3 mg/g. There were relatively higher levels of glycitein found in black soybean and yuba (about 200 μg/g of extract).

2. Inhibitory Effect of Extracts from Soy-Related Products on RNS-Mediated Cellular DNA Damage. Single cell gel electrophoresis (comet assay) is a simple and rapid method to evaluate the level of DNA damage in individual cells by the length of the dragging comet tail image after electrophoresis

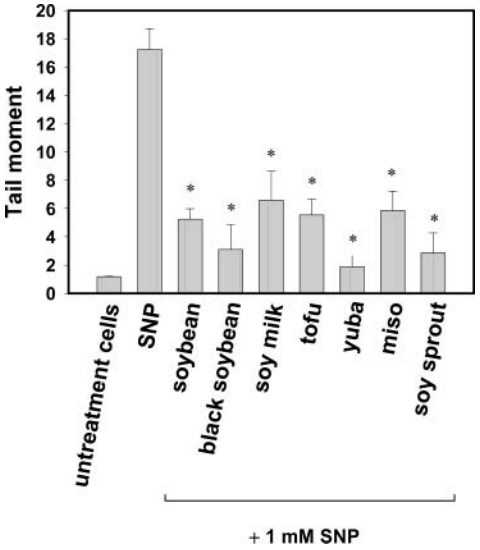


Figure 1. Inhibitory effect of 80% methanol extracts from soybean and its related products on DNA damage of RAW 264.7 cell lines induced by sodium nitroprusside (SNP). RAW 264.7 (3×10^5 cells/mL) was treated with SNP (1 mM) in the presence of 200 μg/mL of extracts for 1.5 h at 37 °C. The data are expressed as the mean ± SD ($n = 3$).

(*26*). As shown in **Figure 1**, control cells (native RAW 264.7) had a tail moment value of 1.2 ± 0.3, whereas RAW 264.7 cells incubated with SNP (1 mM) at 37 °C for 1.5 h had a tail moment value of 17.3 ± 1.5, indicating serious DNA damage. However, incubation of RAW 264.7 cells with SNP in the presence of various extracts from soy-based foods significantly inhibited the induction of SNP-mediated macrophage genotoxicities (**Figure 1**). The tail moment of SNP-treated cells in the presence of various extracts was 1.89 (yuba), 2.87 (soy sprout), 3.10 (black soybean), 5.25 (soybean), 5.55 (tofu), 5.85 (miso), and 6.58 (soymilk), respectively.

Treatment of RAW 264.7 cells with peroxynitrite (0.75 mM) also induced substantial DNA damage, having a tail moment value of 21 ± 2 at 37 °C for 10 min. Similarly, such DNA damage was significantly inhibited by incubation of RAW 264.7 cells with peroxynitrite in the presence of tested extracts (**Figure 2**). The tail moment of peroxynitrite-treated cells in the presence of various extracts was 9.3 (yuba), 12.0 (black soybean), 12.8 (soybean), 14.3 (soy sprout), 15.0 (miso), 16.1 (tofu), and 16.9 (soymilk), respectively.

3. Effect of Extracts on the Production of Nitric Oxide in LPS-Activated Macrophages. Macrophages will produce nitric oxide when activated by LPS. Nitric oxide can react with oxygen to produce the stable products nitrate and nitrite, which can be determined with Griess reagent. As shown in **Figure 3**, extracts from soy-based foods also suppress NO production in supernatants of LPS-activated macrophages as measured by nitrite accumulation. Yuba could completely suppress the LPS effect at a concentration of 100 μg/mL, and the other tested extracts from soy-based foods also showed inhibitory effects greater than 70%.

4. The Scavenging Effect of Extracts on Nitric Oxide. When dissolved in water, SNP will release nitric oxide. Therefore, an SNP solution in the presence of various extracts with Griess reagent can be used to evaluate the nitric oxide scavenging effect of extracts (*23*). In a preliminary study, extracts from soy-based foods (100 μg/mL) showed a very low scavenging effect on nitric oxide resulting from the decomposition of SNP (data not shown). However, the result in **Figure 4** shows that the scavenging effect of extracts from yuba on nitric

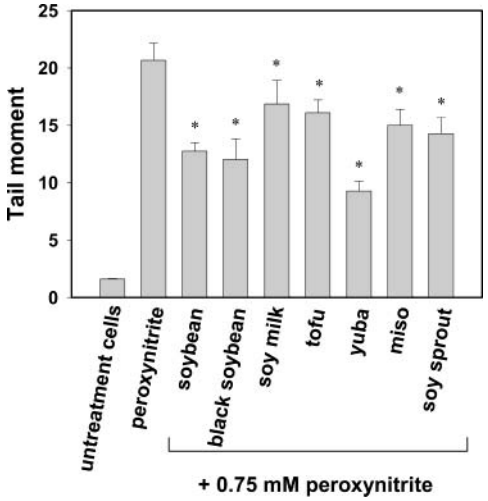


Figure 2. Inhibitory effect of 80% methanol extracts from soybean and its related products on DNA damage of RAW 264.7 cell lines induced by peroxynitrite. RAW 264.7 (3×10^5 cells/mL) was treated with peroxynitrite (0.75 mM) in the presence of 200 μg/mL of extracts for 30 min at 37 °C. The data are expressed as the mean ± SD ($n = 3$).

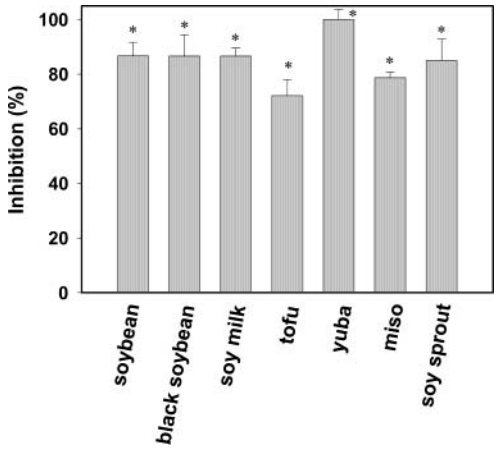


Figure 3. Effects of extracts from soybean related products on the production of nitric oxide from RAW 264.7 cells induced with LPS (1 μg/mL). Concentration of extracts used for treatment was 100 μg/mL. The incubation was carried out for 20 h at 37 °C. Results are shown as mean ± SD ($n = 3$). The nitrite levels of the control and LPS-only treated samples were 4.7 ± 0.4 and 22.8 ± 1.4 μM, respectively. An asterisk indicates significant difference from the LPS-only treated sample ($P < 0.05$).

oxide was about 26%, while the other extracts showed a 13−21% scavenging effect at a concentration of 500 μg/mL.

5. Oxygen Radical Absorbance Capacity (ORAC) Activity of Extracts. ORAC is a relatively simple but sensitive method to measure the ability to trap free radicals in a sample (*27*). Results are expressed as ORAC units, where one ORAC unit equals the net protection produced by 1 μM Trolox. As shown in **Figure 5**, the ORAC value of extracts from miso, soymilk, soybean, black soybean, soy sprout, tofu, and yuba are 3.3, 3.4, 3.6, 2.7, 3.9, 4.7, and 4.7 units, respectively, at a concentration of 25 μg/mL. The results indicate that these extracts from soy-based foods are potential antioxidants.

6. Correlation Analysis. Linear regression analysis of data in the present study showed a strongly positive correlation between the isoflavone content in soy-based foods and the inhibition of SNP or peroxynitrite-mediated DNA damage; of LPS-mediated Raw 264.7 cells producing nitric oxide,

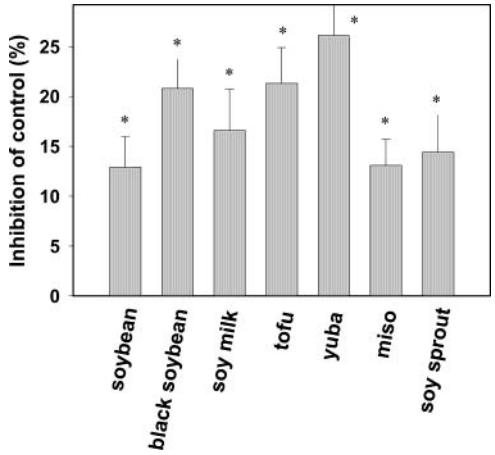


Figure 4. Effect of extracts (500 μg/mL) from soybean-related products on the accumulation of nitrite upon decomposition of sodium nitroprusside (SNP; 5 mM). Incubation time, 2 h; temperature, 25 °C. Control: 5 mM SNP in the absence of the extracts. Data are means ± SD ($n = 3$). The nitrite levels of the SNP-only group was 13.9 ± 0.2 μM. An asterisk indicates significant difference from the SNP only group ($P < 0.05$).

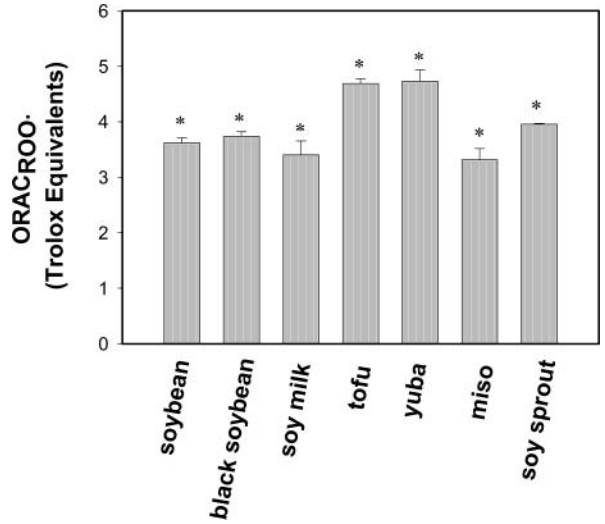


Figure 5. Effect of soybean-related product extracts on measured oxygen-radical absorbance capacity (ORAC) activity. Concentration of extracts used for treatment was 25 μg/mL. One ORAC unit equals the net protection produced by 1 μM Trolox.

Table 3. Correlation between Total Isoflavones Content and the Inhibition of Sodium Nitroprusside or Peroxynitrite-Mediated DNA Damage, Lipopolysaccharide (LPS)-Mediated Raw 264.7 Cells Producing Nitric Oxide, (ORAC$_{ROO\cdot}$Value), and the Inhibition of Sodium Nitroprusside (SNP)-Mediated Nitrite Accumulation of Soybean Related Product Extracts

	correlation coefficient (probability)
inhibn of SNP-mediated DNA damage	0.71 (<0.01)
inhibn of peroxynitrite-mediated DNA damage	0.81 (<0.01)
inhibn of LPS-mediated NO production	0.76 (<0.02)
ORAC$_{ROO\cdot}$ value	0.68 (<0.05)
scarvenging effect of NO	0.74 (<0.05)

ORAC$_{ROO\cdot}$ value; and of SNP-mediated nitrite accumulation of soybean-related product extracts. The correlation coefficients for these items were 0.71 ($P < 0.01$), 0.81 ($P < 0.01$), 0.76 ($P < 0.02$), 0.68 ($P < 0.05$), and 0.74 ($P < 0.05$), respectively (**Table 3**).

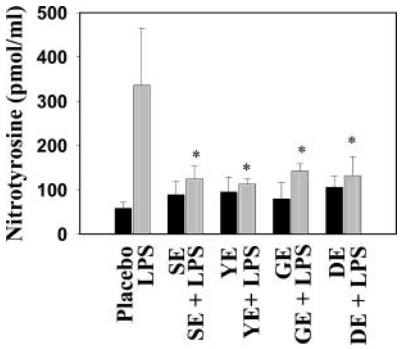


Figure 6. Effect of 1-week oral administration of authentic isoflavones, genistein (GE) or daidzein (DE) (4.0 mg/kg bw day), or 80% methanol extracts from soybean (SE) or yuba (YE) (1.0 g/kg bw day) on serum nitrotyrosine levels in lipopolysaccharide (LPS)-treatment rats. Rats were killed 12 h after LPS injection (ip 10 mg/kg bw) on day 7. An asterisk indicates significant difference from the LPS-only treated rats ($P < 0.05$).

In Vivo Effects of Authentic Isoflavones and Extracts from Soy-Based Products in LPS-Treated Rats. Intraperitoneal administration of LPS (10 mg/kg bw), 12 h before sacrifice, resulted in an increase in serum nitrotyrosine concentrations in rats (**Figure 6**). The serum nitrotyrosine concentrations in LPS-treated rats (340 ± 130 pmol/mL) increased to 5-fold the level of the placebo control (58 ± 14 pmol/mL). However, after oral administration of isoflavones (4.0 mg/kg bw day) or extracts from soy-based products (1.0 g extracts/kg bw day) for 1 week before intraperitoneal administration of LPS, we observed a significant decrease in LPS-induced serum nitrotyrosine concentrations. The LPS-induced serum nitrotyrosine concentrations in rats after oral administration of isoflavones (genistein or daidzein) and extracts from soy-based products (soybean or yuba) were 140 ± 16, 130 ± 16, 125 ± 29, and 113 ± 11 pmol/mL, respectively, or about 33−41% of the level in the rats treated with LPS alone. As shown in **Figure 7**, intraperitoneal administration of LPS also resulted in an increase in serum nitrate and nitrite concentrations in rats (160 ± 16 and 16 ± 1 μM, respectively), which were about 16 and 4 times the level of the placebo control (10 ± 2 and 4 ± 2 μM, respectively). Again, with oral administration of isoflavones or extracts from soy based products for 1 week before intraperitoneal administration of LPS, we observed a significant decrease in LPS-induced serum nitrite and nitrate concentrations.

Table 4 shows the mean serum isoflavone levels of oral administration of authentic isoflavones or extracts from soybean or yuba in LPS-treated rats. As expected, neither genistein nor daidzein was detectable in placebo or LPS-only treated rats, which had been fed a soy-free diet. On the other hand, the mean serum isoflavone concentrations in LPS-treated rats, after oral administration of extracts from soybean or yuba, were 76 and 188 ng/mL of serum, respectively, and values of 163 and 218 ng/mL of serum resulted from the oral administration of genistein and daidzein, respectively.

DISCUSSION

Much attention has recently been focused on the potential anticarcinogenic or antiatherogenic effects of isoflavone, the major phytoestrogen in soybean (*31*, *32*). There is an increasing body of evidence to suggest that the ROS and RNS are related to several diseases, including inflammation, degeneration, and cancer (*33*). Indeed, a number of studies have linked the antioxidant activity of isoflavone with its associated anticarcinogenic (*34*) or antiatherogenic effects (*35*, *36*). Our recent

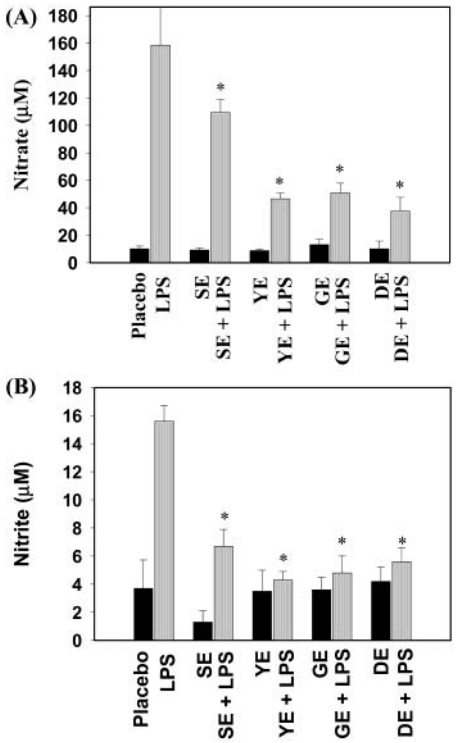


Figure 7. Effect of 1-week oral administration of authentic isoflavones, genistein (GE) or daidzein (DE) (4.0 mg/kg bw day), or 80% methanol extracts from soybean (SE) or yuba (YE) (1.0 g/kg bw day) on serum nitrate (A) and nitrite (B) levels in lipopolysaccharide (LPS)-treatment rats. Rats were killed 12 h after LPS injection (ip 10 mg/kg bw) on day 7. Data were presented as means ± SD from six rats ($n = 6$). An asterisk indicates significant difference from the LPS-only treated rats ($P < 0.05$).

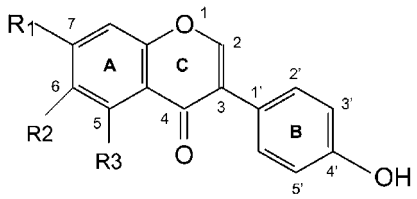
Table 4. Serum Isoflavone Concentrations[a] in Rats Treated with Oral Administration of Authentic Isoflavones or 80% Methanol Extracts from Soybean or Yuba

	isoflavone concn (μM)	
groups	genistein	daidzein
placebo (0.85%)	ND[b]	ND
LPS	ND	ND
soybean + LPS	0.18 ± 0.14	0.11 ± 0.11
yuba + LPS	0.43 ± 0.23	0.29 ± 0.26
genistein + LPS	0.60 ± 0.59	ND
daidzein + LPS	ND	0.86 ± 0.72

[a] Mean ± SD for serum isoflavone concentrations determined in six rats treated with 1 week oral adminstration of authentic isoflavones, genistein or daidzein (4.0 mg/kg bw day), or 80% methanol extracts from soybean or yuba (1.0 g/kg bw day). Rats were killed 12 h after lipopolysaccharide (LPS) injection (ip 10 mg/kg bw) on day 7. [b] ND, not detected.

studies have also demonstrated that the antioxidant activity of isoflavones can provide protection against nitric oxide or peroxynitrite-mediated DNA damage in intact cells and in plasmid DNA (*25*) and can also protect against LDL oxidation induced by peroxynitrite (*37*).

The plant family most abundant in isoflavones is the Leguminosae. However, of all the beans of the Leguminosae family, the soybean and its related products have attracted most attention. We were interested in determining whether the antioxidant activities of the extracts from various soybean and related products correlated with their isoflavone content, since the isoflavone content varied from product to product, depending on the processing conditions (*38*). As shown in **Table 2**, although the amounts and composition profile of total isoflavones differed among these extracts, the glycosidically bound isoflavones, i.e., genistin and daidzin, were the predominant form. This result is consistent with the findings of Wang and Murphy (*39*). In the present study, high levels of glycitein (ca. 200 μg/mg of extracts) were found in yuba and black soybean extracts, which was 5−20 times higher than those found in other extracts. Glycitein is one of the isoflavones in soy food products that also showed weak estrogenic activity (*40*) and antioxidant activity (*41*). Furthermore, the content of total isoflavone in yuba extracts was 5−15 times higher than in other sample extracts, suggesting that yuba is a good source of isoflavones.



Compounds	R1	R2	R3
genistein	OH	H	OH
genistin	glucose	H	OH
daidzein	OH	H	H
daidzin	glucose	H	H
glycitein	OH	OCH_3	H

Figure 8. Structures of the main isoflavons in soy products.

While determining the efficacy of antioxidant activity in an isoflavone mixture, we should also consider the isoflavone composition. Indeed, the antioxidant activity of isoflavones is associated with their structure, depending on the number and the position of hydroxyl groups (*42*). The structures of main isoflavones are shown in **Figure 8**. The authors found that the positioning of the B ring in the 3-position adjacent to the 4-keto group in the isoflavonoids, compared to the 2-position in the flavonoids, allows the 4-hydroxyl on the B-ring to contribute more significantly to antioxidant activity in terms of hydrogen donation and subsequent electron delocalization. Furthermore, glycosylation in the A-ring structure of isoflavone may attenuate the antioxidant activity. Heijnen et al. (*8*) studied the peroxynitrite scavenging of substituted phenols and several flavonoids. They found that the 3-OH group in the AC-ring was the reactive center and that the reactivity of this group was positively influenced by electron-donating groups at the 5 and/or 7 positions. However, as shown in **Table 3**, a good correlation was found between the total isoflavone content in extracts from soy-based foods and the inhibition of RNS and ROS activity, suggesting that total isoflavone content could be an indicator for evaluating the efficacy of antioxidant activities in soy-based foods.

Administration of LPS activates iNOS, which results in the formation of high levels of nitric oxide in many cells, including activated macrophages, neutrophils, and vascular smooth muscle (*43*). In an aqueous plasma solution, the reaction of nitric oxide and oxygen yields nitrite, which in turn rapidly oxidizes to nitrate (*44*, *45*). Therefore, serum nitrate and nitrite concentrations are used as markers for NO synthesis. Moreover, the subsequent interaction of NO with superoxide anion forms peroxynitrite, a powerful one-electron and two-electron oxidizing agent capable of causing pathological damage in conditions such as sepsis, inflammation, and atherosclerosis (*46*, *47*). Peroxynitrite can attack proteins and then cause nitration on the ortho position of tyrosine to form a stable product, nitrotyrosine. Therefore, the serum concentration of nitrotyrosine may reflect the degree of peroxynitrite-dependent damage (*48*).

In the present study (**Figures 1** and **2**), the extract from yuba represented a significant effect on inhibiting RNS-induced DNA damage. But, the inhibitory level on DNA damage induced by SNP is different from the DNA damage induced by peroxynitrite. The differences existing between SNP and peroxynitrite treatment might be because peroxynitrite reacts rapidly with other biological moleculars and is prone to decompose with neutral pH value. On the contrary, the SNP decomposes relatively slowly to release nitric oxide. Therefore, a longer incubation time was required for SNP to induced the DNA damage. Meanwhile, there will be more than enough time for extracts to scavenge nitric oxide produced by SNP. This is maybe the reason the inhibitory level on DNA damage induced by SNP (**Figure 1**) is different from the DNA damage induced by peroxynitrite (**Figure 2**).

To elucidate whether isoflavones and extracts from soy-based products will inhibit the RNS effect in vivo, rats were given the isoflavone or soy-based product extracts by oral administration for 1 week and were injected with vehicle (H_2O) or LPS on the day 7. As shown in **Figures 6** and **7**, when the animals were treated in vivo with LPS, 12 h before sacrifice, increases of serum nitrite, nitrate and nitrotyrosine concentrations were observed; such increases were attenuated by oral administration of isoflavones and extracts from soy-based products for 1 week prior to treatment, suggesting that dietary supplementation with isoflavones may regulate RNS metabolism in LPS-treated rats. It has been reported that genistein exhibits mild antiinflammatory properties that may, in part, involve the attenuation of nitric oxide release via iNOS and the formation of peroxynitrite, which involves a tyrosine kinase-dependent mechanism (*21*, *49*).

Xu et al. (*50*) and King et al. (*51*) have reported in tests involving humans and rats that 4−6 h after isoflavone dosing, daidzein and genistein reached maximal concentrations in plasma and thereafter decreased. Twenty-four hours after dosing, there were only trace concentrations of daidzein and genistein in the plasma. In our in vivo study, serum isoflavones were significantly increased in rats given authentic isoflavones and extracts from soy-based products for 1 week (**Table 4**), as compared to the relationship between the mean serum isoflavone concentrations and the levels of nitrite, nitrate, and nitrotyrosine in LPS-treated rats, where a highly adverse correlation was found. The correlation coefficients were −0.95 ($P < 0.01$), −0.99 ($P < 0.01$), and −0.82 ($P < 0.05$), respectively.

As described above, the antioxidant behavior of isoflavones is related to the structure of the compound, i.e., the number and position of hydroxyl groups and hydrogen atoms or electron-donation ability. Other evidence of antioxidant activity for isoflavones also has been proposed. It has been demonstrated that the isoflavones genistein, daizein, and biochanin-A can be chlorinated and nitrated by oxidants such as hypochlorous acid and peroxynitrite, which indicated that isoflavones could react with oxidant species under conditions where these reactive species are generated (*52*). The role of isoflavones acting as prooxidants scavenger was precluded due to the low plasma concentrations of isoflavones in a soy-enriched diet (less than 1 μM). However, soy isoflavones are capable of suppressing formation of plasma lipid oxidation products in vivo, although they react with hypochlorous acid and peroxynitrite, involving an isoflavone-derived phenoxyl radical (*53*). Furthermore, daidzein and genistein were readily metabolized to hydroxylated products by the action of hepatic cytochrome P450 enzymes, which would affect the biological activity of isoflavone (*54*). For example, the intestinal metabolites daidzein and equol are more potent antioxidants (*55*). This may be able to explain why the degree of the RNS inhibitory effects of SE and YE in vivo are similar, despite the large difference in isoflavone content.

In conclusion, our results demonstrated that there is a major contribution to the inhibition of RNS and ROS activity from total isoflavone content in soy-based foods in vitro, suggesting that total isoflavone content could be used as an indicator to evaluate the efficacy of antioxidant activity in soy-based foods. In our in vivo study, an elevation of serum nitrite, nitrate, and nitrotyrosine concentrations in LPS-treated rats can be reduced by pre-oral administration of isoflavones and extracts from soy-based products, suggesting that dietary supplementation with isoflavones might regulate RNS metabolism in LPS-treated rats. Since ROS and RNS are related to several diseases, these results may indicate the possible beneficial effects of high dietary intake of soy-based foods with respect to their ROS and/or RNS scavenging abilities.

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