Suppression Effect of Soy Isoflavones on Nitric Oxide Production in RAW 264.7 Macrophages

Fuu Sheu, Hsi-Huai Lai, and Gow-Chin Yen*

Department of Food Science, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan, Republic of China

Genistein, daidzein, and glycitein, as primary isoflavones in soybeans, are reported to have beneficial effects on atherosclerosis, chronic inflammatory diseases, and cancers that are conducted by nitric oxide (NO) injury. The objectives of this study were to investigate the effects and mechanisms of these soy isoflavones on the inducible nitric oxide synthase (iNOS) system in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. Genistein, daidzein, and glycitein dose-dependently suppress NO production ($IC_{50} = 50\ \mu M$) in supernatants of LPS-activated macrophages as measured on the basis of nitrite accumulation. In addition, direct inhibition of iNOS activity, determined by means of the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, and markedly reduced iNOS protein and mRNA levels, evaluated by means of Western blot and RT-PCR, respectively, were found in homogenates of LPS-activated cells treated with each isoflavone. Moreover, genistein was found to have a greater inhibitory effect on NO production but no significant effect on iNOS activity or protein and gene expression to daidzein and glycitein. These observations reveal that the suppression of NO production by genistein, daidzein, and glycitein might be due to the inhibition of both the activity and expression of iNOS in LPS-activated macrophages. The result suggests that soy isoflavones might attenuate excessive NO generation at inflammatory sites.

Keywords: *Nitric oxide; soy isoflavone; genistein; daidzein; glycitein*

INTRODUCTION

Much attention has been focused on the endogenous formation of nitric oxide (NO) because it has an important role in many physiological and pathophysiological processes. As a messenger molecular, NO is involved in vasorelaxation regulated by the endothelium and has a function in neurotransmission. In the immune system, NO is a defense molecule with cytotoxic, cytostatic, microbicidal, and microbiostatic activities (*1*). It can be formed by the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS, EC 1.14.13.39). Although these functions of NO are beneficial in maintaining proper physiological homeostasis, NO has been found to contribute to many diseases, such as cancer, septic shock (*2*), cerebral injury (*3*), atherosclerosis (*4*), rheumatoid arthritis (*5*), cell apoptosis, and necrosis (*6*). Under pathological conditions, macrophages can greatly increase their production of both NO and superoxide anion simultaneously. Interaction of NO with superoxide leads to the formation of peroxynitrite ($ONOO^-$), which is able to exert stronger oxidant effects and might, through further reactions, lead to chronic pathological conditions (*7*). Peroxynitrite has been found to be a highly reactive oxidant and to have the ability to cause DNA damage (*2, 8*). Moreover, it has been reported that peroxynitrite may contribute to atherosclerosis through oxidation of LDL within the arterial walls (*9*). Peroxynitrite and other reactive nitrogen oxide species are considered to be potentially cytotoxic and to be capable of injuring the surrounding cells and tissues indiscriminately. Thus, inhibition of iNOS activity and/or expression is an important goal in all cases of uncontrolled production of NO (*10*).

A number of different types of polyphenolic phytochemicals, such as flavonols (*11*), flavones (*10*), and catechins (*12*), have been found to inhibit NO production of inducible nitric oxide synthase (iNOS). With respect to those in soybeans, the isoflavones genistein (4′,5,7-trihydroxyisoflavone), daidzein (4′,7-dihydroxyisoflavone), glycitein (7,4′-dihydroxy-6-methoxyisoflavone), and their β-glucosides are regarded as major antioxidative components (*13*). These soy isoflavones have been reported to exibit marked bioactivity of scavenging radicals, thus preventing lipoprotein oxidation in serum (*14*) and inhibiting copper and peroxyl radical mediated low-density lipoprotein (LDL) oxidation (*15*). They have also been found to play a predominant role in cancer chemoprevention (*16*−*19*). We have recently demonstrated that genistein and daidzein are peroxynitrite scavengers that have the ability to prevent the peroxynitrite-mediated oxidation of LDL (*20*). On the other hand, genistein has been reported to be a potent inhibitor of tyrosine kinase, which is involved in the signal transduction pathways of iNOS expression. Genistein causes dose-dependent inhibition of TNF-induced intracellular adhesion molecule I and vascular cell adhesion molecular I up-regulation (*21*) and causes inhibition of TNF and iNOS, which are regulated by NF-κB (*22*). It would furthermore be interesting to determine the effects and mechanisms of genistein and the other two primary and structurally related soy isoflavones, daidzein and glycitein, on lipopolysaccharide (LPS)-induced NO production in macrophages.

In this study, we attempted to determine whether soy isoflavones play a protective role in the overproduction

* Author to whom correspondence should be addressed (fax 886-4-2285-4378; e-mail gcyen@mail.nchu.edu.tw).

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of NO and whether this effect is related to inhibition of iNOS activity or expression. In the present study, the effects of genistein, daidzein, and glycitein on NO synthesis in LPS-activated macrophages and the underlying mechanism of action were investigated.

MATERIALS AND METHODS

Reagents and Cell Culture. Genistein, daidzein, glycitein, 2-ethyl-2-thiopseudourea hydrobromide (SEITU), and LPS were purchased from Sigma (St. Louis, MO). The murine monocyte/macrophage cell line RAW 264.7 was obtained from the Culture Collection and Research Center (CCRC, HsinChu, Taiwan). RAW 264.7 cells were cultured in 25 or 75 cm^2 plastic flasks with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and antibiotics (Gibco/BRL Life Technologies, Eggenstein, Germany). These cells were activated with 1 μg/mL LPS (*Escherichia coli*, serotype 0.55:B5) and cultured for 20 h at 37 °C in an atmosphere of 5% CO_2.

Nitrite Assay. Cells were seeded in 96 well plates (8×10^4/200 μL), cultured for 2 days, and then incubated with or without LPS in the absence or presence of isoflavone individually at various concentrations for 20 h. The nitrite concentration in the supernatant was assessed by means of the Griess reaction (*23*) and determined through comparison with a sodium nitrite standard curve.

Cell Viability. Cell respiration as an indicator of cell viability was determined on the basis of mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. After removal of 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 insoluble formazan product was dissolved in dimethyl sulfoxide (DMSO) (250 μL) for at least 2 h in the dark. The extent of MTT reduction was quantified by measuring the absorbance at 550 nm.

iNOS Enzyme Activity. The enzyme preparation was obtained from RAW 264.7 cells cultured in flasks (75 cm^2) after the LPS activation (1 μg/mL) for 20 h. The cells were collected and washed three times with cold PBS, frozen immediately, and stored at −70 °C until the iNOS activity was measured. The effects of isoflavones and inhibitor SEITU on iNOS activity were determined by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline according to the method of Kobuchi (*10*) with a minor modification. Briefly, the cells were homogenized in 50 mM Tris (pH 7.6) containing EDTA (0.1 mM), EGTA (0.1 mM), and the protease inhibitor phenylmethanesulfonyl fluoride (PMSF, 1 mM). The reaction mixture (150 μL) consisted of L-arginine (10 μM), NADPH (1 mM), L-valine (50 mM), L-[^{3}H]-arginine (0.2 μCi, 0.033 μM), and 20 μL of homogenates. After incubation for 1 h at 37 °C, the reaction was stopped by adding 200 μL of cold stop buffer (50 mM Tris-HCl, pH 5.5, 4 mM EDTA). The reaction mixture was separated by means of ion exchange on Dowex 50W (Na^+ form) column, and the radioactivity of the eluted L-[^{3}H]citrulline was quantified by means of scintillation counting (Packard liquid scintillation analyzer 1500). Bio-Rad protein assay solution was used for protein determination, with BSA employed as a standard.

Western Blotting of iNOS Enzyme. Raw 264.7 cells were incubated in a 25 cm^2 flask with or without LPS in the absence or presence of genistein, daidzein, or glycitein at different concentrations for 12 h. Cells were washed, harvested, and homogenized. The lysate was centrifuged at 15000*g* for 20 min and standardized to contain 1.0 mg/mL total protein. Western blotting was performed according to the method of Rothe et al. (*24*). Using the acrylamide gel (7−12.5%) with Tris−glycine buffer, electrophoresis was carried out using a Mini-Protean II apparatus (Bio-Rad, Hercules, CA). Bands were transferred into a Hybond-C nitrocellulose membrane (Amersham, Braunschweig, Germany) using a Bio-Ray Trans-Blot semidry transfer cell. iNOS antibody (PA3-030A, ABR, CO) was incubated, and iNOS bands were visualized through treatment with secondary antibody (31340, Pierce, IL) and an NBT/BCIP detection system (34042, Pierce, IL). Signal intensities were evaluated through densitometric analysis.

Gene Expression. RT-PCR was performed to determine the level of iNOS gene expression (*12*). Briefly, after 4 h of LPS stimulation, total RNA was extracted from cultured cells using TRIzol reagent (Life Technologies, Rockville, MD). From each sample, 200 ng of RNA was reverse-transcribed using 200 units of Superscript II reverse transcriptase, 20 units of RNase inhibitor, 0.6 mM of dNTP, and 0.5 μg/μL of oligo(dT_{12-18}). Then, PCR analyses were performed on the aliquots of the cDNA preparations to detect iNOS and G3PDH (as an internal standard) gene expression using the FailSafe PCR system (Epicenter Technologies). Murine iNOS and G3PDH 5′ and 3′ oligonucleotide primers was purchased from Clontech (Palo Alto, CA). The reactions were carried out in a volume of 50 μL containing (final concentration) 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MnCl_2$, 0.2 mM dNTP, 2 units of Taq DNA polymerase, and 50 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 95 °C, 30 cycles of amplification (at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min) were performed, followed by a 7-min extension at 72 °C.

Analysis of PCR Products. A 10 μL aliquot from each PCR reaction was electrophoresed in a 1.5% agarose gel containing 0.2 μg/mL ethidium bromide. The gel was then photographed under ultraviolet transillumination. For quantification, the PCR bands on the photograph of the gel were scanned using a densitometer linked to a computer analysis system. We normalized the iNOS signal relative to the corresponding G3PDH signal from the same sample and expressed the data as the iNOS/G3PDH ratio.

Statistical Analysis. Each experiment was performed in triplicate and repeated three times. The results were expressed as means ± SE. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test. Differences were considered significant when the P values were <0.05.

RESULTS

Soy Isoflavones Suppress NO Production by LPS-Activated Macrophages. The effects of genistein, daidzein, and glycitein on NO synthesis in RAW 264.7 macrophages were investigated. The nitrite levels in the culture medium as an index of NO production were measured using Griess reaction (*23*). As shown in Figure 1, genistein, daidzein, and glycitein showed dose-dependent inhibition of nitrite accumulation in LPS-activated (1 μg/mL) RAW 264.7 cells. After 20 h of stimulation, LPS-treated cells produced a high level of nitrite (68.2 ± 2.32 μM) that was 30-fold higher than that produced by the unstimulated cells (2.43 ± 0.74 μM). Moreover, this LPS-activated nitrite production could be significantly ($P < 0.05$) reduced by incubation with isoflavones at various concentrations. A marked reduction in nitrite production (67.7%) was observed in the genistein treatment (100 μM). Among the isoflavones examined, genistein had a stronger suppression effect ($IC_{50} \sim 50$ μM) than did daidzein or glycitein ($IC_{50} \sim 100$ μM). When the cells were incubated with each isoflavone, the amounts of nitrite in the medium stayed at a level similar to the control (data not shown). Cell viability, examined using the MTT assay, was analyzed to exclude the possibility that the inhibitory effect of isoflavones was due to their potential cytoxicity. The viability of all the LPS-activated cells treated with or without isoflavones at different concentrations was >90%. This result indicates that the inhibition of nitrite production by genistein, daidzein, and glycitein is not due to cell death.

Soy Isoflavones Inhibit iNOS Enzyme Activity. Decreased NO release in isoflavone-treated cells implied

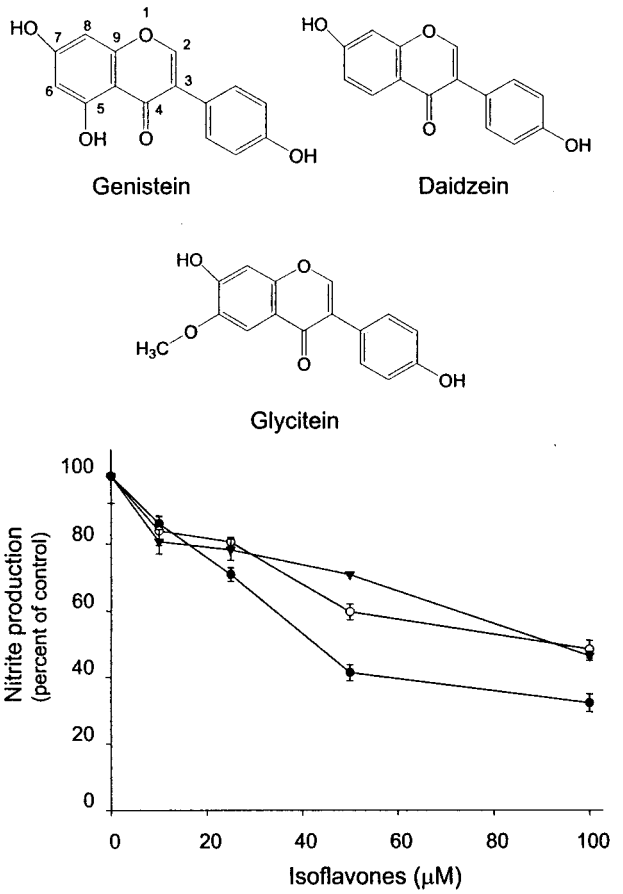

Figure 1. Dose-dependent inhibition of nitrite production in the cell culture supernatants of LPS-activated RAW 264.7 macrophages by structurally related genistein (●), daidzein (▼), and glycitein (○). RAW 264.7 macrophages were activated with LPS (1 μg/mL) and isoflavone (0−100 μM) for 20 h. The nitrite concentration was determined on the basis of the Griess reaction. The percentage of inhibition was deduced by dividing the amount of nitrite produced in the isoflavone-treated sample by that in the LPS-only-treated sample. The nitrite levels of the control and LPS-only-treated sample were 2.43 ± 0.74 and 68.20 ± 2.32 μM, respectively. Results are expressed as means ± SE; $n = 3$. All points are significantly different from those of the LPS-only-treated sample.

that the synthesis of NO could be altered in these cells. Therefore, the effect of isoflavones on iNOS activity was determined by measuring the conversed L-[H^3]citrulline. Figure 2 shows that the activity of iNOS from cell homogenates was dose-dependently inhibited by genistein, daidzein, and glycitein. However, this direct inhibition was significant ($P < 0.05$) only when iNOS was treated with high-dose isoflavones (100 μM). Genistein showed inhibition (36.5%) superior to that of daidzein and glycitein (26.7 and 19.9%, respectively) at a concentration of 100 μM ($P < 0.05$). There was no significant difference ($P > 0.05$) in the inhibitory effect of these isoflavones at concentrations of 25 and 50 μM. This result indicates that the direct inhibition of iNOS activity results in reduced NO production in RAW 264.7 macrophages.

Soy Isoflavones Reduce iNOS Protein Production. Although isoflavones are able to inhibit iNOS activity directly, suppression of gene expression of iNOS is still a possible reason for the reduction in NO production in the cells. To evaluate this possibility at the protein/translation level, iNOS protein expression was examined by means of Western blot analysis. RAW

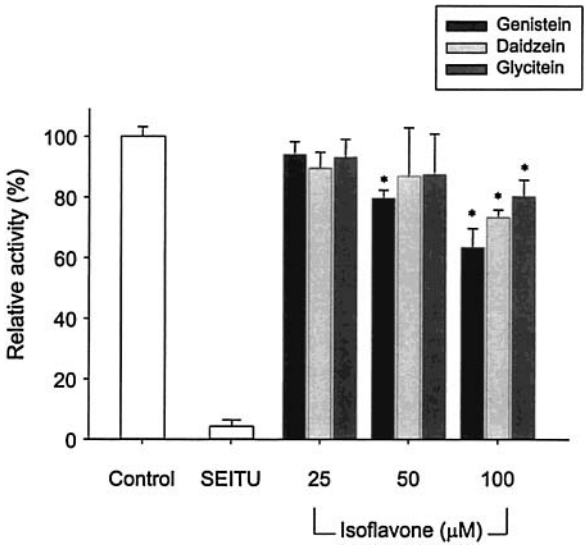

Figure 2. Inhibitory effect of isoflavones on iNOS activity. RAW 264.7 macrophages were activated with LPS (1 μg/mL) for 20 h, and the homogenates were prepared as the source of iNOS. The conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline was measured. The percentage of inhibition was deduced by dividing the activity of iNOS in the isoflavone-treated sample that had been treated with isoflavanone or inhibitor SEITU by that in the control. The activity of iNOS in the LPS-only-treated sample was 56.91 pmol/min/mg of protein. Data are represented as means ± SE; $n = 3$. An asterisk indicates significant difference from the control.

264.7 cells were activated with LPS (1 μg/mL) in the absence or presence of each isoflavone at different concentrations, and the cell extracts were examined for iNOS proteins with iNOS antibodies. Figure 3 shows reduced iNOS protein levels in genistein-, daidzein-, and glycitein-treated cells compared to the unactivated control and LPS control. Dose-dependent effects of three isoflavones (25−100 μM) on the expression inhibition of iNOS protein are clearly demonstrated (Figure 3A). Evaluation of the signal intensity (Figure 3B) by means of densitometric analysis showed similar decreases ($P > 0.05$) in iNOS protein expression: 89.0, 82.3, and 83.7% for genistein, daidzein, and glycitein at a concentration of 100 μM.

Soy Isoflavones Diminish iNOS mRNA Production. The diminution of iNOS protein expression suggests that the transcription of iNOS mRNA might have been inhibited by isoflavones. Therefore, iNOS mRNA levels were further examined by means of RT-PCR analysis. As shown in Figure 4A, RAW 264.7 cells did not express detectable levels of iNOS mRNA with medium alone, whereas LPS induced an obvious increase in iNOS mRNA expression. In addition, an inhibitory effect on iNOS mRNA expression was found in isoflavone-treated cells (Figure 4A). Genistein, daidzein, and glycitein at a concentration of 100 μM significantly ($P < 0.05$) inhibited 66.4, 57.8, and 57.2% of mRNA expression, respectively (Figure 4B). Moreover, the difference in the suppression effect among the three examined isoflavones was not significant ($P > 0.05$). These results indicate that inhibition of iNOS mRNA expression by isoflavones contributed to the decrease in NO production in RAW 264.7 macrophages.

DISCUSSION

Soybean-related foods have been found to possess antioxidative ability, to prevent atherosclerosis and

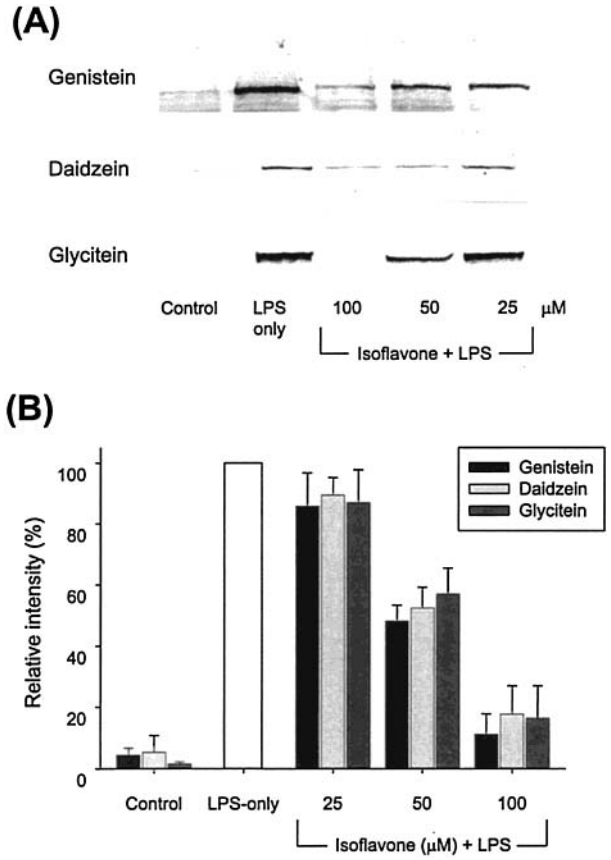

Figure 3. Western immunoblotting analysis of iNOS protein in LPS-activated RAW 264.7 macrophages. Cells were activated with LPS (1 μg/mL) and incubated in the absence or presence of isoflavones (0−100 μM) for 12 h. Equal amounts of cellular proteins (1 mg/mL) from control or isoflavone-treated cells were separated on an SDS−10% polyacrylamide gel, followed by electrophoretic transfer of proteins from the gel to a nitrocellulose membrane. (A) The bands of iNOS were identified by means of Western immunoblotting with anti-iNOS antibodies. (B) The immunoblots were analyzed by means of densitometry, and the data were generated as integrated density units. The relative intensity was deduced by dividing the intensity in the control and the isoflavone-treated sample by that in the LPS-only-treated sample. Data are represented as means ± SE; $n = 3$. The isoflavone-treated samples at concentrations of 50 and 100 μM are significantly different ($P < 0.05$) from the LPS-only-treated sample.

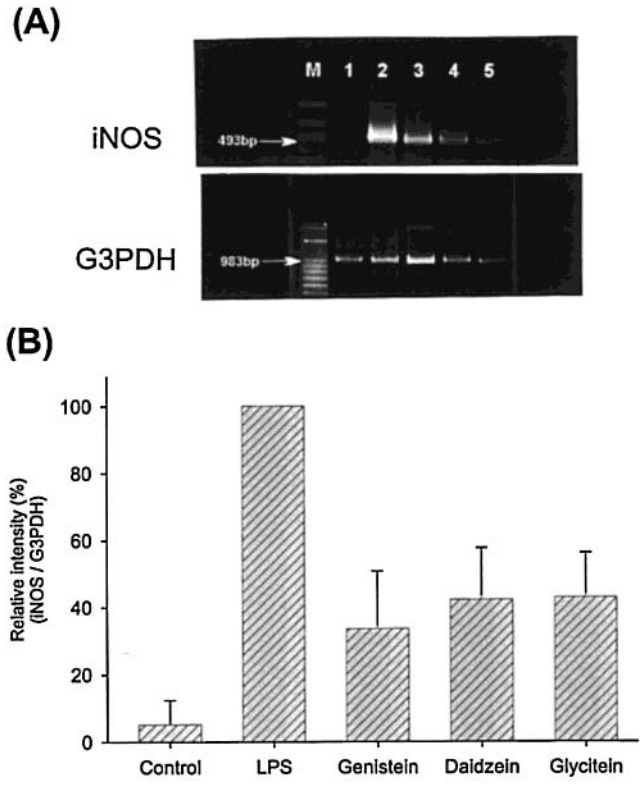

Figure 4. Effect of isoflavones on the expression of iNOS mRNA in LPS-activated RAW 264.7 macrophages. Cells were activated with LPS (1 μg/mL) and incubated in the absence or presence of isoflavones (100 μM) for 4 h. The expression of mRNA for iNOS was determined by means of RT-PCR analysis. (A) 1.6% agarose gel electrophoresis of PCR-amplified cDNA derived from iNOS and G3PDH stained with ethidium bromide: (lane 1) control; (lane 2) LPS-activated iNOS; (lane 3) genistein treatment; (lane 4) daidzein treatment; (lane 5) glycitein treatment. (B) Quantitation of mRNA levels by means of densitometry. The levels of iNOS mRNA and G3PDH mRNA were quantified by means of densitometry. The iNOS expression was normalized by expressing the data as the ratio of iNOS to G3PDH. Results are expressed as means ± SE; $n = 3$. All isoflavone-treated samples are significantly different ($P < 0.05$) from the LPS-only-treated sample.

stroke, and to reduce cancer risk (*14*−*19*). To our knowledge, isoflavones primarily consisting of genistein, daidzein, and glycitein are antioxidative components providing these biological protections (*13*). Because NO may contribute to carcinogenicity and atherosclerosis through peroxynitrite formation (*2*, *4*, *8*), the effect of soy isoflavones on NO production and its mechanism was investigated in the present study. Results reveal that genistein, daidzein, and glycitein are able to dose-dependently suppress NO synthesis in LPS-activated murine macrophages (Figure 1B). To investigate this suppression mechanism, the enzyme activity and protein/mRNA expression of iNOS were further analyzed. Analyses of iNOS activity demonstrated that the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in LPS-activated RAW 264.7 cells could be directly reduced by these isoflavones. It has been reported that the high affinity of polyphenols for proteins and a possible subsequent conformational change of enzyme might be associated with the observed inhibitory effect by flavonoids (*10*). Therefore, the affinity between isoflavones and proteins might contribute to the suppression of iNOS enzyme activity by isoflavones.

Moreover, Western blot and RT-PCR analysis showed markedly decreased iNOS protein and mRNA levels after treatment with each isoflavone was applied to LPS-activated control cells. It is known that the expression of iNOS mRNA is regulated at the transcriptional level (*25*), so it would be interesting to investigate whether these isoflavones regulate LPS-induced iNOS expression through iNOS transcription factors, such as NF-κB, IFN-r response element (r-IRE), IFN regulatory factor-binding element (IRF-E), or nuclear factor-IL-6 (NF-IL-6) (*26*−*30*). This should be addressed in a future study. However, we cannot exclude the possibility that isoflavones may decrease the stability of iNOS mRNA or affect translational regulation. Some flavonoids have been reported to inhibit protein kinase C (*31*), which might be involved in post-transcriptional modification of iNOS mRNA (*32*). This is another possible mechanism whereby isoflavones suppress iNOS mRNA in macrophages.

Among these three structurally related isoflavones, genistein with an additional C-5 hydroxyl substitution, had a stronger inhibitory effect on NO production (Figure 1) and enzyme activity (Figure 2) than did

daidzein and glycitein at a concentration of 100 μM. Our previous study demonstrated that daidzein, lacking the C-5 hydroxyl group of genistein, was less effective as a peroxynitrite scavenger (*20*). These results are also in agreement with those of another study, which found that the hydroxyl residues in flavonoids correspond to the inhibition of iNOS activity (*10*). However, no significant difference ($P > 0.05$) was observed among these isoflavones in terms of their inhibition of iNOS expression (Figures 3 and 4), which indicates that the inhibitory effects of different soy isoflavones on iNOS transcription/translation might be quite similar. Moreover, we did not evaluate the glucosides of soy isoflavones in this study because the glucosides were previously found to not be able to efficiently scavenge peroxynitrite (*20*) or inhibit NO production in RAW 264.7 macrophages (*32*, *33*).

As antioxidants, the soy isoflavones exhibit anticancer effects by inhibiting the growth of cancer cells (*34*), by inhibiting cellular signal transduction systems (*35*), by reducing oxidative DNA damage, and by inhibiting angiogenesis (*36*). This study had found an additional physiological action, that is, inhibition of NO production in macrophages, which might have potential therapeutic value when related to inflammation/immunoregulation and peroxynitrite responsible for cancer risk. On the other hand, in atherosclerosis, these isoflavones have been shown to inhibit copper-, peroxyl-, and peroxynitrite-mediated LDL oxidation and to reduce LDL as well as VLDL but to increase HDL (*15*). The suppression of NO production reveals another advantage, that is, prevention of NO-mediated LDL oxidation. However, because endothelial dysfunction accompanied by impaired bioactivity of NO has been found to coincide with induction of iNOS and subsequent formation of peroxynitrite (*37*, *38*), it is desirable to specifically inhibit iNOS without impairing eNOS. Further study is needed to investigate the effect of isoflavones on enzyme activity and gene expression of eNOS.

In summary, the soy isoflavones genistein, daidzein, and glycitein have been shown to suppress NO production in RAW 264.7 macrophages. The mechanisms associated with the reduction in NO production were found to be (1) directly scavenging NO radicals, (2) directly inhibiting iNOS enzyme activity, and (3) inhibiting iNOS gene expression. These results may indicate possible protective effects against cardiovascular diseases and chronic inflammatory diseases through the high intake of soybean-related foods.

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