Inhibitory Effect of Isoflavones on Peroxynitrite-mediated Low-density Lipoprotein Oxidation

Hsi-Huai LAI and Gow-Chin YEN†

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

Received May 31, 2001; Accepted July 30, 2001

Peroxynitrite, a potent oxidant formed in vivo from the reaction of nitric oxide with superoxide, can mediate low-density lipoprotein (LDL) oxidation which is thought to increase the risk of atherosclerosis. This study investigates the inhibitory effect of the isoflavones, genistein and daidzein, together with their glycosidic forms, genistin and daidzin, on the peroxynitrite-mediated LDL oxidation and nitration of tyrosine. Genistein and daidzein were observed to dose-dependently inhibit peroxynitrite-mediated LDL oxidation, while their glucoside conjugates showed less activity. Moreover, all the isoflavones used in this study were found to be potent peroxynitrite scavengers, preventing the nitration of tyrosine. The ability of the isoflavones at 50 μM to decrease the tyrosine nitration induced by peroxynitrite (1 mM) was in the ratios of genistein (49%), daidzein (40%), daidzin (41%) and genistin (42%) when compared to the control (tyrosine incubated only with peroxynitrite). These results suggest that an intake of isoflavones could contribute to protecting against cardiovascular diseases and chronic inflammatory diseases.

Key words: isoflavone; peroxynitrite; LDL; tyrosine nitration

A high blood cholesterol level is a well-known risk factor for heart disease. However, it has been shown that approximately 50% of the myocardial infarction cases had cholesterol level not considered abnormal, and 20% had a cholesterol level below 200 mg dl⁻¹.¹ Oxidatively modified low-density lipoprotein (LDL), which is taken up by scavenger receptors, has been suggested as being involved in the development of atherosclerosis.² LDL oxidation can be catalyzed by such heavy metals as Cu²⁺ and Fe³⁺ ³ and be promoted by activated cells.⁴ It is thought that only oxidized LDL-cholesterol can be taken up by macrophages within the endothelial cells lining the arterial wall to form lipid-laden foam cells that are found in atherosclerotic plaque.⁵ Peroxynitrite is formed from the reaction of nitric oxide and superoxide with a rate constant of 6.7 × 10⁹ M⁻¹ s⁻¹.⁶ The formation of both nitric oxide and superoxide occurs simultaneously in stimulated macrophages, neutrophils and endothelial cells,⁷ and has been shown to generate peroxynitrite in vivo.⁸ Peroxynitrite is a potent oxidant and can therefore oxidize protein and non-protein sulfhydryls.⁹ Since the peroxynitrite reaction with proteins yields nitrotyrosine, it is a useful marker for peroxynitrite detection.¹⁰ Peroxynitrite has also been shown to oxidize LDL, suggesting that peroxynitrite may play a significant role in atherosclerosis.¹¹ Therefore, interest is increasing in the biological importance of natural antioxidants in their ability to act as peroxynitrite scavengers.

There are a number of biologically active compounds to be found in soy products which have been associated with lowering blood lipids.¹² Several studies have indicated that soybean isoflavones may be responsible for cholesterol reduction.¹³,¹⁴ In patients with cardiovascular disease, daily soy consumption reduced the amount of copper-initiated LDL-cholesterol oxidation in vitro by about one-half.¹⁵ The major isoflavones in soybeans are genistein (4',5,7-trihydroxyisoflavone) and daidzein (4',7-dihydroxyisoflavone), together with their respective β-glycosides, genistin and daidzin, all of which have been found to have antioxidant activity in vitro.¹⁶,¹⁷ In addition, a recent study has suggested that genistein can inhibit the oxidative modification of isolated LDL by the copper and peroxyl radicals.¹⁸ Although the antioxidant properties of isoflavones have been well characterized, they have not been studied to assess whether these compounds can protect LDL against the oxidative damage caused by peroxynitrite. Since preventing the oxidation of LDL may beneficially influence atherogenesis,¹⁹ the present experiments examined the effects of soybean isoflavones (genistin, daidzin, genistein and daidzein) on peroxynitrite-mediated low-density lipoprotein and the inhibition by those isoflavones of peroxynitrite-mediated tyrosine nitration.

† To whom correspondence should be addressed. Fax: +886-4-2285-4378; E-mail: gcyen@dragon.nchu.edu.tw
Materials and Methods

Chemicals. Genistein, daidzein, genistin, tyrosine, 3-nitrotyrosine, sodium nitrite, hydrogen peroxide, butylated hydroxytoluene (BHT), and bovine serum albumin (BSA) were all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Daidzin was obtained from Extrasynthese Company (Geany Cedex, France), and Protein assay kits was obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium dihydrogen phosphate, potassium hydrogen phosphate, and dimethyl sulfoxide were all obtained from E. Merk Co. (Darmstadt, Germany). Kit Midigel Lipo was obtained from Biomidi (Toulouse, France).

Peroxynitrite synthesis. The peroxynitrite synthesis was carried out by modifying the method described by Beckman et al. Acidified hydrogen peroxide (1 M in 0.5 M HCl, 20 ml) and sodium nitrite (200 mM, 20 ml) solutions were drawn into two separate syringes, analogous to a stop-flow set up. The contents of both syringes were simultaneously injected into an ice-cold beaker, before 1.5 M potassium hydroxide (40 ml) was immediately added to stabilize the resulting peroxynitrite anion. Excess hydrogen peroxide was removed by mixing with manganese dioxide. The stock solution was filtered and then frozen overnight (−20°C), after which the upper layer of the solution was collected for the experiment. The concentration of peroxynitrite was determined by measuring the absorbance at 302 nm (ε = 1670 M⁻¹ cm⁻¹). Neutralized peroxynitrite prepared from the dilution of peroxynitrite in a phosphate buffer at pH 7.4 resulted in rapid decomposition, and this was used throughout the experiment at the same concentration as peroxynitrite used as the control.

LDL isolation from plasma. Blood from a single healthy volunteer was collected in tubes containing EDTA (1 mg ml⁻¹). Plasma was isolated by centrifugation at 3000 × g for 10 min. LDL was isolated from the plasma by density gradient ultracentrifugation (ρ = 1.006–1.063) at 10°C with a Hitachi (Himac CS 120 GX) centrifuge working at 100,000 × g for 35 min in a 0.5% agarose gel with a barbital buffer, after which the gel was fixed for 10 min and completely dried. The dried gel was stained with Sudan Black for 10 min and then destained. The Relative electrophoretic mobility (REM) and conjugated diene formation of LDL were examined.

Oxidation of LDL. LDL (100 µg of protein ml⁻¹) was incubated with 1 mM peroxynitrite in the presence or absence of various concentrations of isoflavones for 2 h at 37°C. After BHT had been added to the medium to stop the reaction, thiobarbituric acid reactive substances (TBARS), the relative electrophoretic mobility (REM) and conjugated diene formation of LDL were examined.

Thiobarbituric acid reactive substances (TBARS). The formation of TBARS in the LDL sample was measured by the method of Yagi. LDL (100 µg of protein ml⁻¹) was mixed with 50 µl of BHT (4% w/v ethanol), 500 µl of sodium dodecyl sulfate (0.3%), 2 ml of HCl (0.1 N), phosphotungstic acid (10%) and 1 ml of thiobarbituric acid (0.7%). After heating at 100°C for 45 min, the fluorescent reaction products were assayed by a spectrofluorometer (Hitachi, 650-40) with excitation at 515 nm and emission at 533 nm. The concentration of TBARS is expressed as the equivalent of 1,1,3,3-tetramethoxypropane which was used as the standard.

Relative electrophoretic mobility. The increased anodic electrophoretic mobility of LDL on agarose gel was used to evaluate the modification of the oxidatives. About 4 µl of a sample was applied to the gel surface according to the manufacturer’s instructions (Biomidi). Electrophoresis was performed at 80 V for 35 min in a 0.5% agarose gel with a barbital buffer, after which the gel was fixed for 10 min and completely dried. The dried gel was stained with Sudan Black for 10 min and then destained. The Relative electrophoretic mobility (REM) is defined as the ratio of the migrating distance of oxidized LDL to that of the control (neutralized peroxynitride-treated LDL).

Conjugated diene formation. The formation of conjugated diene in the LDL samples was measured by the method of Wallin et al. A 0.1-ml aliquot of the oxidized solution was added to 0.9 ml of isopropanol in 1.5-ml polypropylene tube. After vigorous shaking, the sample was centrifuged at 8000 × g for 5 min, and the resulting supernatant read by a Hitachi U-3000 spectrophotometer at 234 nm.

Tyrosine nitration assay. The formation of 3-nitrotyrosine was measured by the method of White and Halliwell. A stock concentration (10 mM) of L-tyrosine was prepared in 10 ml by adding 8 ml of water to 250 ml of 10% (w/v) KOH and then 250 ml of a 5% phosphoric acid solution with 1.5 ml of water. The tyrosine solution (0.1 ml), together with 10 µl of the solution of the compound to be tested, was loaded into a plastic test tube containing 0.88 ml of a buffer (500 mM K₂HPO₄/KH₂PO₄ at pH 7.4) and incubated in a water bath at 37°C for 15 min.

LDL oxidation by peroxynitrite. Isolated LDL (100 µg of protein ml⁻¹) in PBS-EDTA was incubated at 37°C in the presence of 1 mM peroxynitrite or neutralized peroxynitrite (the final volume was 1 ml), and the effects of various isoflavones on LDL oxidation were examined.
Peroxynitrite (typically 10 μl) was then added to achieve a final concentration of 1 mM, after which the tube was vortexed for 15 s and incubated for a further 15 min. The pH value was measured after the addition of peroxynitrite and found to be 7.1–7.4. Each sample was then analyzed by HPLC with a Merk RP-18 (125 mm × 4.6 mm) column and a LiChrospher 100 RP-18 (5 μm) cartridge guard column. The HPLC apparatus was from Hitachi (Tokyo, Japan) consisting of an model L-6200 intelligent pump, a Rheodyne 7125 syringe-loading sample injector, a D-2000 integrator and an L-4200 UV-vis detector. The mobile phase was 500 mM KH₂PO₄ /H₃PO₄ at pH 3.1 with 20% methanol (v/v) at a flow rate of 1 ml min⁻¹, and the UV detector was set at 274 nm. The detection of 3-nitrotyrosine was confirmed by spiking with standards. The peak height of 3-nitrotyrosine was measured and its concentration calculated from a standard curve.

Statistical analysis. Data were analyzed by using the Statistical Analysis System software package. Significant differences between means were determined by Duncan’s multiple-range test.

Results

Inhibition of LDL oxidation

To investigate the effect of isoflavones on peroxynitrite-mediated LDL oxidation, LDL was incubated for 2 h at 37°C with 1 mM peroxynitrite in the absence or presence of an isoflavone, and lipoprotein oxidation was monitored by the formation of TBARS (MDA) and conjugated dienes and by the relative electrophoretic mobility.

As shown in Fig. 1, peroxynitrite significantly increased the amount of MDA (86.5 ± 8.0 nmole mg⁻¹ of protein) when compared with the untreated LDL control and neutralized peroxynitrite control (3.7 ± 0.4 and 4.0 ± 0.5 nmole mg⁻¹ of protein, respectively; P < 0.05), and all four isoflavones (100 μM) used in our test decreased the concentration of MDA (ca. 74–86% of the peroxynitrite treatment only). A dose-response relationship for the inhibition of MDA formation by genistein and daidzein was observed (Fig. 2). The formation of conjugated dienes in polyunsaturated fatty acids within the LDL particle is taken as the biological signature of LDL oxidation. Figure 3 depicts the effect of exposing isolated human LDL to peroxynitrite as a marked increase in the absorbance at 234 nm, indicating the formation of conjugated dienes. However, this effect could be inhibited by the presence of 100 μM genistein and daidzein, but not by their conjugated glycosides, genistin or daidzin. The results from a further study show that genistein and daidzein, at 25, 50 and 100 μM, dose-dependently inhibited peroxynitrite-mediated LDL oxidation (Fig. 4).

In addition, agarose gel electrophoresis revealed that the peroxynitrite-induced increase in REM, which is a further indicator of LDL oxidative modification, was concentration-dependently inhibited in
Isoflavone Inhibition of Peroxynitrite-mediated LDL Oxidation

**Fig. 3.** Effect of Isoflavones on the Formation of Conjugated Dienes from Peroxynitrite-mediated LDL Oxidation.

LDL (100 μg of protein ml⁻¹) was incubated with 1 mM peroxynitrite (ONOO⁻) in the presence of 100 μM of each indicated isoflavone for 2 h at 37°C. Untreated LDL was exposed to the vehicle only, while neutralized ONOO⁻ contained decomposed peroxynitrite. Each data value is expressed as the mean ± SD (n = 3).

**Fig. 4.** Dose-dependent Inhibition of the Formation of Conjugated Dienes from Peroxynitrite-mediated LDL Oxidation by the Treatment with Isoflavones. LDL (100 μg of protein ml⁻¹) was incubated with 1 mM peroxynitrite (ONOO⁻) in the presence of the indicated concentration of genistein or daidzein for 2 h at 37°C. Neutralized ONOO⁻ contained decomposed peroxynitrite. Each data value is expressed as the mean ± SD (n = 3).

the presence of genistein and daidzein. The glycosidic isoflavone, genistin, also showed a protective effect against LDL oxidation, but daidzin did not (Table 1).

**Table 1.** Effect of Peroxynitrite (1 mM) on the Relative Electrophoretic Mobility (REM) of LDL (100 μg of Protein ml⁻¹) in the Presence of the Indicated Concentration of Each Isoflavone

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (μM)</th>
<th>Reduction in REM* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>25</td>
<td>23 ± 2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>36 ± 1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>44 ± 2</td>
</tr>
<tr>
<td>Daidzein</td>
<td>25</td>
<td>4 ± 1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>16 ± 1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>Genistin</td>
<td>100</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Daidzin</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

* Each value is the mean ± SD (n = 3).

**Fig. 5.** Effect of Isoflavones on Peroxynitrite-mediated LDL Electrophoretic Mobility.

LDL (100 μg of protein ml⁻¹) was incubated with 1 mM peroxynitrite (ONOO⁻) in the presence of the indicated concentration (μM) of each isoflavone for 2 h at 37°C. Neutralized ONOO⁻ contained decomposed peroxynitrite.

The agarose gel electrophoretic pattern of LDL after various treatments is shown in Fig. 5.

**Inhibition of tyrosine nitration**

The inhibition of peroxynitrite-mediated tyrosine nitration by isoflavones was also determined. The isoflavones were co-incubated with tyrosine prior to the addition of 1 mM peroxynitrite, and the 3-nitrotyrosine formation was then quantified. Figure 6 shows that all the isoflavones tested were potent scavengers of peroxynitrite due to their ability to prevent the nitration of tyrosine. Genistein showed sig-
sificant inhibition of 3-nitrotyrosine formation at a concentration of 50 μM; however, there was no clear distinction between daidzein, genistin and daidzin. The ability of the isoflavones at 50 μM to minimize the tyrosine nitration induced by peroxynitrite (1 mM) was 49.0 ± 1.2% for genistein, 39.8 ± 0.6% for daidzein, 40.9 ± 0.6% for daidzin and 41.9 ± 2.8% for genistin, when compared to the control (tyrosine incubated only with peroxynitrite).

Discussion

Attention has been focused on preventing LDL oxidation by natural antioxidants, since oxidized LDL may play an important role in the progression of atherosclerosis. One of the mechanisms for the antithrombogenic effect of soybean isoflavones has been considered to be its inhibition of the oxidative modification of LDL and subsequent inhibition of foam cell formation. Indeed, recent data shows that genistein inhibits the LDL oxidation induced by copper and the peroxyl radical (azo-initiator) in a concentration-dependent manner.18

The results presented in this study show that isoflavones increased LDL resistance to oxidation in vitro. It was found that when LDL was subjected to peroxynitrite-mediated oxidation, the addition of isoflavonoids decreased the MDA concentration (Figs. 1 and 2), conjugated diene formation (Figs. 3 and 4) and LDL electrophoretic mobility on agarose gel (Table 1). Most of these effects were concentration dependent, especially for genistein, a naturally occurring isoflavone known to have antioxidative properties. In addition, the effectiveness of preventing LDL oxidation by the isoflavones examined in this study was according to the structure of these compounds. Genistein, with hydroxyl groups at the C-5,7 and 4' positions, was the most effective antioxidant in the peroxynitrite-mediated LDL oxidation system used in our study. Daidzein, lacking the C-5 hydroxyl group of genistein, was less effective as a peroxynitrite scavenger. This suggests that the hydroxyl group at the C-5 position contributed to the peroxynitrite-scavenging activity of these compounds. As in genistin or daidzin, blocking the C-7 hydroxyl of genistein or daidzin by glucose had less effect on the peroxynitrite-scavenging activity. These results are in agreement with other studies that have suggested the major contribution to the total antioxidative activity from the single hydroxyl group at position 4' of the B ring of the isoflavone in the presence of either one or two hydroxyl groups in the A ring of the isoflavone molecule.28,29

Nitrotyrosine is a useful marker for peroxynitrite detection, since tyrosine residues are readily nitrated by peroxynitrite, and it has been observed in many pathological cases including human atherosclerosis.30 Findings from the present studies indicate that genistein, daidzein, and their glucosidic compounds, genistin and daidzin, have the potential ability to dose-dependently prevent tyrosine nitration by peroxynitrite (Fig. 6). Although the peroxynitrite-scavenging ability of isoflavones can be seen in Fig. 6 without distinction by chemical structure, genistein shows the best potential, which means that the isoflavone molecule exhibits a peroxynitrite-scavenging effect. Recent data shows that the soy isoflavones, genistein and daidzein, can be nitrated by the oxidant, peroxynitrite, and this may put peroxynitrite into competition with tyrosine due to the structural similarity between tyrosine and the isoflavones.31

There are conflicting results suggesting that the evidence for the antioxidative activity of isoflavones in vitro cannot be extrapolated to the in vivo situation.32 However, in addition to the antioxidative effects, isoflavones have also demonstrated several physiological actions including inhibited platelet aggregation,33 reduced plasma cholesterol,34 reduced LDL and VLDL, and increased HDL.35 Those factors are thought to help reduce the risk of heart diseases. Meanwhile, we cannot exclude the possibility that isoflavones ingested over a long period of time could accumulate in tissues, where they eventually may contribute significantly to antioxidative protection.

In conclusion, based on the results shown in the present study, isoflavones increased the LDL resistance to peroxynitrite-mediated oxidation, and decreased the formation of 3-nitrotyrosine while treating free tyrosine with peroxynitrite. This mechanism could help to provide a protective effect against cardiovascular diseases and chronic inflammatory diseases by a high intake of soybean and soy-
bean-based products.

Acknowledgments

This research work was supported in part by National Science Council of Republic of China under grant NSC 89-2313-B005-064.

References


