Hepatoprotective effects of daidzein against 7,12-dimetylbenz[*a*]anthracene-induced oxidative stress in mice

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Abstract. We investigated the effects of daidzein on the antioxidant defence system in mice with 7,12-dimethylbenz[a]-anthracene (DMBA)-induced oxidative stress. Daidzein was administered orally at 5 and 25 mg/kg body weight for 5 weeks. Subsequently, mice pretreated with daidzein received DMBA intragastrically twice a week for 2 weeks. As controls, mice were given vehicle or DMBA alone. In the DMBA group, biomarkers of oxidative stress (thiobarbituric acid reactive substances value, carbonyl content) were significantly increased. However, the rise in oxidative damage was significantly reduced by daidzein at the higher dose. In addition, several antioxidant enzymes were downregulated in the DMBA-treated mice. Catalase and superoxide dismutase activity was increased by daidzein in a dose-dependent manner. Although the reduced/oxidized glutathione ratio was unaffected, glutathione peroxidase and reductase were activated by daidzein, and the effect was significant at the higher dose. Further, in the DMBA-treated mice, apoptosis was induced by a decrease in Bcl-2 and an increase in Bax. These changes were restored to their normal values in the daidzein-treated mice. Upregulation of caspase-3 was also decreased by daidzein. These results suggest that daidzein exerts a hepatoprotective effect on mice with DMBA-induced oxidative stress through its antioxidant activity and the reduction of apoptosis.

Introduction

Recently, a great deal of attention is focused on the biological properties of isoflavones, including genistein and daidzein, in the hope of developing new chemotherapeutic strategies for treatment of cancer. Several epidemiological and experimental studies suggested that natural phytochemicals are very useful as food supplements and adjuvants for degenerative diseases such as cancer (1-3).

Among the phytochemicals described, isoflavones are prominent candidates for cancer chemopreventive agents. Daidzein belongs to the isoflavone subclass of flavonoids and is found in fruits, nuts, soybeans, and soy-based products (4,5). We previously showed that daidzein may act not only as an antioxidant, but also as a prooxidant (6). The prooxidant effects of daidzein, which include the promotion of apoptosis, may contribute to cancer chemotherapy and inhibition of tumour growth. Moreover, many studies reported that daidzein significantly inhibits cancer cell growth *in vitro* (7-10). The proposed biological activities of daidzein include oestrogenic/antioestrogenic activity, induction of cell-cycle arrest and apoptosis, inhibition of oxidative stress, and activation of cell death signalling (7,11-13).

However, few *in vivo* studies have assessed whether daidzein has anti-cancer effects. Therefore, we investigated the effects of the chronic administration of daidzein on oxidative damage and activation of the antioxidant defence system in the liver of mice with 7,12-dimethylbenz[a] anthracene (DMBA)-induced oxidative stress. DMBA is among the most potent chemical carcinogens and is widely used in mammary cancer chemopreventive studies (14,15). DMBA is a procarcinogen, which requires metabolic conversion to its ultimate carcinogenic metabolites. Substantial oxidative damage raises the incidence of cancer during DMBA metabolism in the liver (16,17).

Materials and methods

Daidzein treatment and sample preparation. ICR female mice (Central Lab, Animal Inc., Seoul, Korea) were acclimated for 1 week under standard environmental conditions with AIN 93M diet (Dyets, Bethlehem, PA, USA) and allowed free access to water. After an adaptation period, mice were divided randomly into four groups. Daidzein (TCI Tokyo Kasei Kogyo Co., Tokyo, Japan) was suspended in water and administered by oral gavages to two groups, each comprising of nine animals, at 5 and 25 mg/kg body weight (BW) for 5 weeks. Subsequently, the DMBA-treated groups were intragastrically administered a dose of 34 mg/kg BW in corn oil vehicle twice a week for 2 weeks. As controls, mice were divided into vehicle only and DMBA only groups and each

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group comprised of six mice. Animal care in this study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. At 6 h after final administration of daidzein, mice were rapidly anesthetized using diethylether and their livers were isolated, blotted, weighed, frozen in liquid nitrogen and stored at -70˚C until assayed. For assay, livers were homogenized for ~45 sec in 9 volume of ice-cold 10 mM phosphate buffered saline (PBS, pH 7.4) containing 1.15% KCl. Homogenates for oxidative stress biomarker (TBARS and carbonyl content) measurement were centrifuged at 800xg to remove cell debris and nuclei; the supernatants were centrifuged at 10,000xg for 10 min and transferred the portions of the post-mitochondrial fraction for GSH related analysis. To obtain the cytosolic fractions for SOD activity measurements, the remaining supernatant was centrifuged further at 105,000xg for 45 min using a 50 Ti rotor in a Beckman model L90 ultracentrifuge. Post-mitochondrial and cytosolic fractions were stored at -80˚C in aliquots until analysis within one week.

Measurement of daidzein and its metabolite equol concentrations. The contents of daidzein and its metabolite equol were analyzed by high-performance liquid chromatography (HPLC). HPLC analysis was performed using a system consisting of a Hewlett Packard 1084B liquid chromatography with electrochemical detector (Hewlett Packard, USA). Determination of daidzein and equol content in serum was performed accordingly to a modified method of King and Bursill (18). Briefly, each 200 μl of serum was mixed with 400 μl of 0.17 M ammonium acetate (pH 4.6) containing 1,000 units of ß-glucuronidase, and hydrolyzed by incubation overnight (16 h) at 37˚C. The sample solution was extracted twice with 500 μl of diethyl ether. The aqueous and organic phases were separated by centrifugation and the upper (organic) phase was dried under nitrogen gas. The dried residue was re-dissolved in 200 μl of methanol and injected into the HPLC system. The mobile phase consisted of methanol, 0.1 M ammonium acetate (pH 4.6) and 25 mM EDTA (55:50:1, v/v/v), at a flow rate of 1.0 ml/min and a potential of 700 mV. Sample was analyzed on a 4.6x250 mm RP C-18 Nova Pak column (5 μm) (Millipore, Germany) and HPLC-grade daidzein and equol (Sigma) were used as standards.

Immunoblotting assay. Liver samples were homogenized within radio-immunoprecipitation assay (RIPA) buffer (1% nonidet P-40, 150 mM sodium chloride, 0.05% deoxycholic acid, 1% sodium dodecyl sulfate, 50 mM Tris, pH 7.5) containing protease inhibitor and lysed for 1 h at 4˚C. The supernatant was separated by centrifugation and protein concentration was determined by Bradford protein assay kit II (BioRad Laboratories, CA, USA). Proteins (50 μg/well) denatured with sample buffer were separated by 10% SDS-polyacrylamide gel. Proteins were transferred onto nitrocellulose membranes (0.45 μm). The membranes were blocked with a 1% BSA solution for 3 h, washed twice with PBS containing 0.2% Tween-20, and incubated with the primary antibody overnight at 4˚C. Antibodies against Bcl-2, Bax, cleaved caspase-3 (19 kDa), and ß-actin were purchased from Santa Cruz (Santa Cruz Biotechnology Inc., CA, USA) and used to probe the separate membranes. The next day, the immunoreaction was continued with the secondary goat anti-rabbit horseradish-peroxidase-conjugated antibody after washing for 2 h at room temperature. The specific protein bands were detected by Opti-4CN Substrate kit (BioRad).

Oxidative stress measurement. Thiobarbituric acid reactive substances (TBARS) value was determined by measuring the concentration of malondialdehyde (MDA) according to the method of Ohkawa *et al* (19) and calculated according to the molar absorption coefficient of MDA, $\varepsilon=1.56\text{x}10^5$ M^{-1} cm^{-1} at 535 nm. The formation of protein carbonyl in the liver was determined using dinitrophenyl hydrazine (DNPH) according to the method of Reznick and Packer (20). The absorbance was read at 365 nm and the results were expressed as moles of DNPH incorporated/100 mg protein using a molar extinction coefficient of 21 mM^{-1} cm^{-1}.

Antioxidant enzyme activities. Superoxide dismutase activity was assayed according to the pyrogallol auto-oxidation method of Marklund and Marklund (21). Each unit of SOD activity was defined as the quantity of enzyme that inhibited auto-oxidation of pyrogallol by 50% under experimental conditions. Catalase activity was assayed by the method of Aebi (22). Catalase activity was calculated as nmol of hydrogen poroxide (H_2O_2) decomposed/min/mg protein. Activities of glutathione peroxidase and reductase were determined with a spectrophotometer by measuring the disappearance of NADPH at 340 nm, based on the methods of Flohe and Gunzler (23) and Carlberg and Mannervik (24), respectively. The enzyme activities of glutathione peroxidase and reductase was defined as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of $6.22\text{x}10^{-3}$ M^{-1} cm^{-1}. Protein concentration was determined by Bradford protein assay kit II (BioRad Laboratories, CA).

Determination of total GSH and GSSG. The concentration of glutathione was measured using o-phthalaldehyde as a fluorescent reagent according to the method of Hissin and Hilf (25). For GSSG measurement, each sample was incubated with N-ethyl maleimide to interact with GSH present in the tissue homogenate. The GSH and GSSG content (ug/mg protein) were obtained from a standard curve and then the GSH/GSSG ratio was calculated.

Relative mRNA expression by real-time PCR. Liver samples were homogenized with Trizol (Gibco-BRL, Invitrogen, USA) and mRNA was extracted according to the manufacturer's protocol. First-strand cDNA was synthesized using SuperScript First-Strand Synthesis System (Invitrogen, USA). Each target mRNA expression was quantified by real-time PCR with the use of CFB-3120 MIniOpticon™ system (BioRad Laboratories Inc., CA, USA). CFB-3120 MIniOpticon™ system uses an array of 48-light-emmitting diodes (LEDs), which efficiently excite fluorescent dyes with absorption spectra in the 470-505 nm range. PCR reactions were carried out with 2X SYBR® Green mix (Finnzymes, Finland). mRNA levels were calculated by means of the comparative cycle threshold (C_t) method using $2^{-\Delta\Delta Ct}$ according to the manufacturer's instructions. GAPDH was used as an endogenous (internal) control. The fold change in target gene relative to the endogenous control was determined

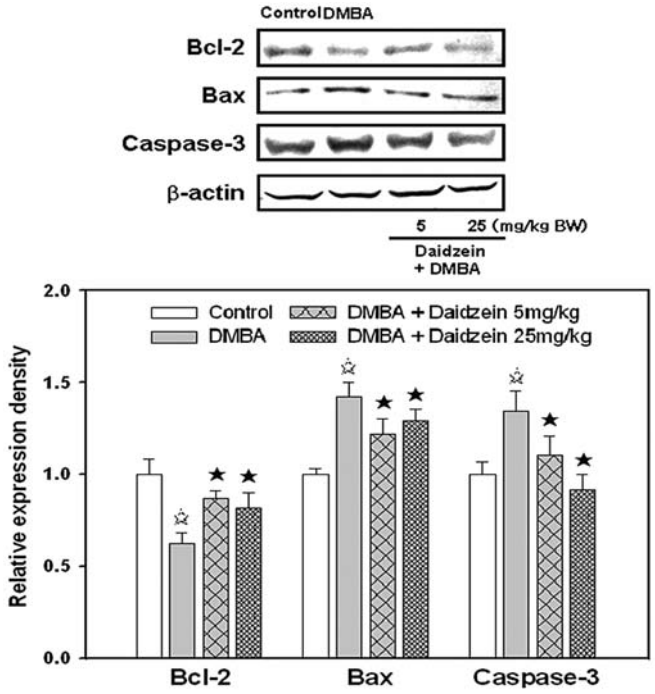

Figure 1. Effect of daidzein on expression of apoptosis related genes, Bcl-2, Bax, and caspase-3. After liver tissue was lysed with RIPA buffer, apoptosis related genes (Bcl-2, Bax, caspase-3, and ß-actin) expression were measured using immunoblotting assay. For relative expression density, the protein expressions were calculated relative to ß-actin and the value for the control (vehicle-only) was accepted as '1.0'. Values are mean ±SD (n=6). ☆P<0.05, significant difference between control (vehicle-only) and DMBA-treated group by unpaired Student's t-test; ★P<0.05, significant difference from the DMBA-treated group by one-way analysis of variance.

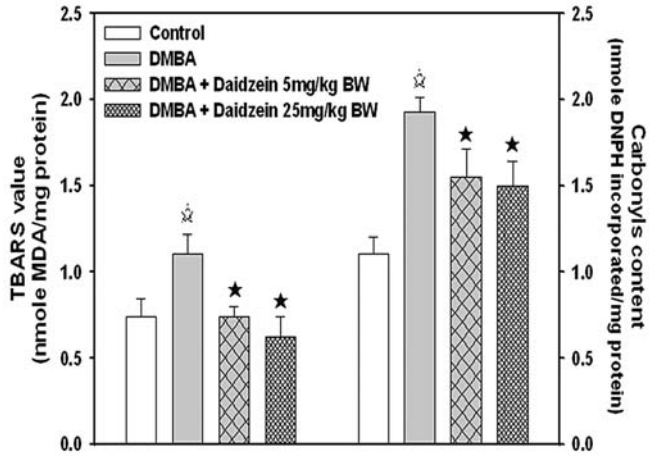

Figure 2. Effect of daidzein on lipid peroxidation (left) and protein oxidation (right). Oxidative stress biomarker (TBARS and carbonyl content) was measured in hepatic homogenates centrifuged to remove cell debris and nuclei. Values are mean ±SD (n=6). ☆P<0.05, significant difference between control (vehicle-only) and DMBA-treated group by unpaired Student's t-test; ★P<0.05, significant difference from the DMBA-treated group by one-way analysis of variance.

as Fold change = $2^{-\Delta\Delta Ct}$; where $\Delta\Delta Ct=(Ct_{target}-Ct_{endogenous})_{treated\ group}-(Ct_{target}-Ct_{endogenous})_{control\ group}$. The untreated sample (control group) was defined as the calibrator in this experiment. Therefore, the amounts of GPx, and GR transcripts in the other samples were assigned dimensionless numbers relative to the levels in the calibrator sample.

Statistical analyses. Values are presented as the mean ± standard deviation (SD) obtained from six measurements in tissue isolated from six different mice. Data were analyzed by unpaired Student's t-test or one-way analysis of variance followed by Dunnett's multiple comparison test (SigmaStat, Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at P<0.05.

Results

Anti-apoptotic effects of daidzein in mice treated with DMBA. A 37.2% decrease in Bcl-2 expression and a 43.1% increase in Bax expression were observed in the DMBA group compared to the control group (Fig. 1). In addition, cleaved caspase-3 expression was increased by 34.2% compared to the control group. These changes were reversed by pretreatment with daidzein; Bcl-2 expression was increased by ~35% at both doses compared to the DMBA group. Daidzein also significantly decreased the expression of Bax and cleaved caspase-3 in a dose-dependent manner.

Serum content of daidzein and its metabolite equol. The daidzein contents in serum from mice orally administered 5 or 25 mg/kg BW were 1.28±0.15 and 4.11±0.52 μg/ml, respectively. The equol content showed a similar pattern (0.72±0.05 and 2.74±0.18 μg/ml in mice orally administered 5 or 25 mg/kg BW, respectively).

Effects of daidzein on the oxidative damages in mice treated with DMBA. The TBARS value of the DMBA group was significantly greater than that of the control (vehicle-only) group (1.11 and 0.74 nmol/mg protein, respectively, Fig. 2). Daidzein treatment significantly decreased the value by 33.2 and 62.4% at 5 and 25 mg/kg BW, respectively, compared to the DMBA-only group. The carbonyl content in the DMBA and control groups was 1.11 and 1.93 nmol/mg protein, respectively. In the DMBA-treated groups, a significant dose-dependent decrease resulting from daidzein treatment was observed. Compared to the control value, a decrease of 28.5% was detected at the higher dose.

Effects of daidzein on the antioxidant defense enzymes in mice treated with DMBA. Catalase activity in the DMBA group was significantly decreased to less than half of that in the control group (0.46 and 1.23 nmole H_2O_2 decomposed/min/mg protein, Fig. 3). Daidzein increased the value to 1.37 and 2.06 times that seen in the DMBA group at 5 and 25 mg/kg BW, respectively. Total SOD activity in the DMBA and control groups was 0.39 and 0.58 unit/mg protein, respectively. Total SOD activity increased in a dose-dependent manner, and a significant increase was detected at the higher dose.

Effects of daidzein on the GSH related system in mice treated with DMBA. The GSH/GSSG ratio was significantly decreased by ~20% in the DMBA group compared to the control group (Fig. 4A). Daidzein had no effect on the DMBA-treated group, although a slight increase was observed. The basal activity of

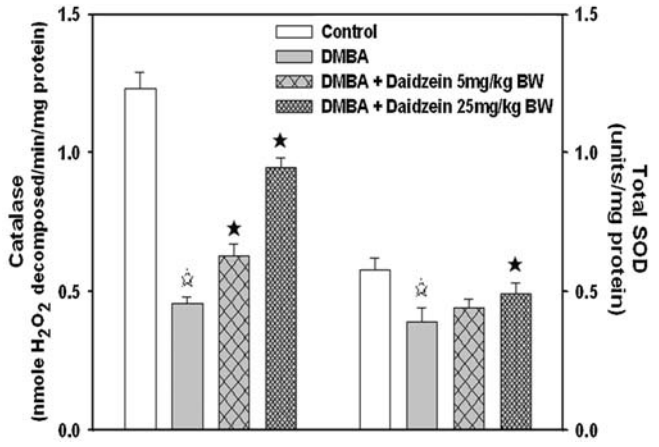

Figure 3. Effect of daidzein on catalase (left) and total SOD (right). Catalase and total SOD activity were measured in post-mitochondria and cytosolic fractions obtained from hepatic homogenates by centrifugation, respectively. Values are mean ±SD (n=6). ☆P<0.05, significant difference between control (vehicle-only) and DMBA-treated group by unpaired Student's t-test; ★P<0.05, significant difference from the DMBA-treated group by one-way analysis of variance.

GSH-px and GR in the DMBA groups was decreased by 23.6 and 22.1%, respectively, compared to the control group (Fig. 4B). The level of activity of GSH-px and GR was significantly increased by daidzein in a dose-dependent manner, although there was a significant difference only at the higher dose (19.3 and 14.5% increase for GSH-px and GR, respectively, compared with the value in the DMBA group). Similar changes were observed in the relative expression of GSH-px and GR mRNA (Fig. 4C). GSH-px mRNA expression was increased significantly in a dose-dependent manner. At higher doses, GSH-px and GR mRNA expression were increased 1.47- and 1.16-fold, respectively, compared to the DMBA group.

Discussion

Based on a previous *in vivo* study (6), the excessive use of daidzein under normal conditions was not likely to produce beneficial health effects. That is, daidzein slightly down-regulated the hepatic GSH-related system when given orally to Sprague-Dawley rats at the higher dose (20 mg/day) for 4 weeks. These studies led to the hypothesis that daidzein acts as both an antioxidant and prooxidant and the prooxidant effects of daidzein are associated with its metabolite equol. In serum samples of rats orally administered daidzein at either 2 or 20 mg/day, the concentrations of daidzein as well as its major metabolite equol were both remarkably elevated in a dose-dependent manner. The daidzein content at the higher dose was threefold higher than the content at the lower dose. This effect was more pronounced with the serum equol content, which increased 5.6-fold in rats administered the higher dose of daidzein. Equol treatment at the high concentration (over 50 μM) showed a significant increase in LDH release from cells *in vitro* (6), although equol also has antioxidant activity. LDH leakage (an increase in LDH release) is the most common indicator of membrane damage in cell cytotoxicity assays (26).

The daidzein dose in the present study was established at a level that allowed it to act as an antioxidant, but was not high enough to overwhelm its preventative action through equol produced for metabolism. The daidzein dose was approximately 32% of that used in a previous study (6), and the daidzein and equol concentrations of mice serum in the present

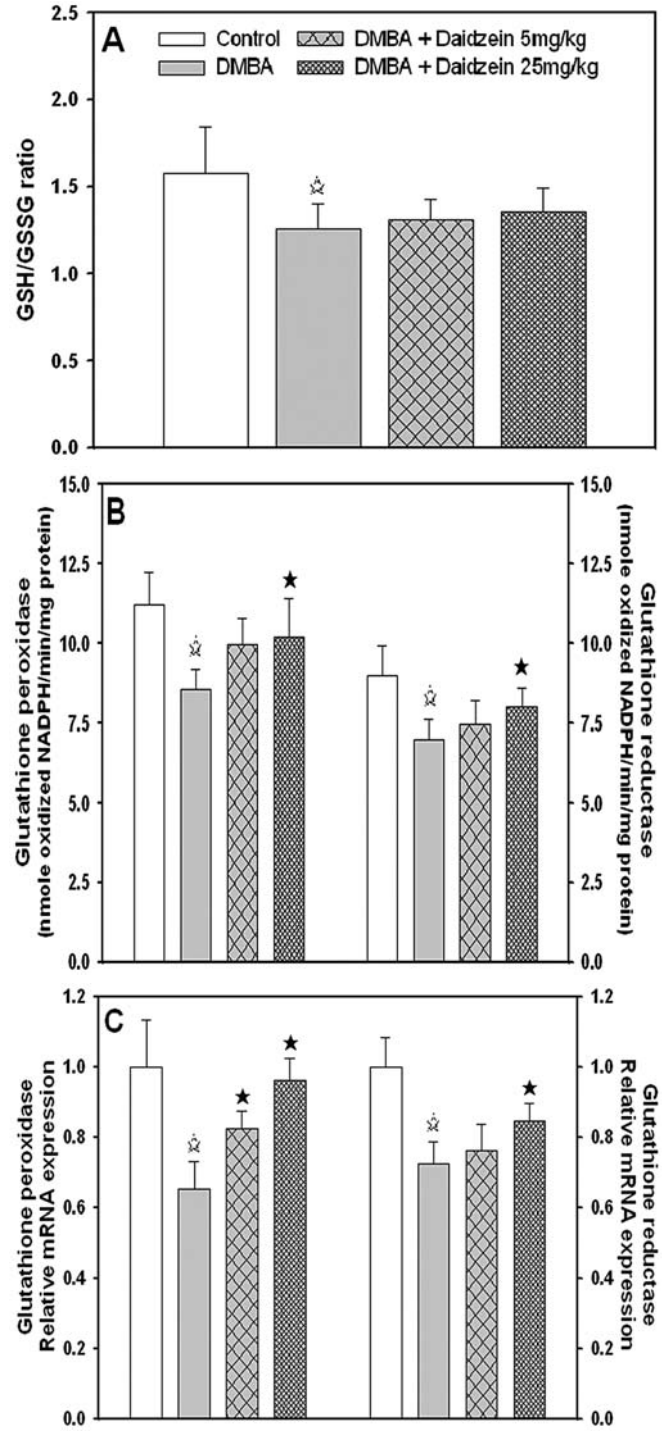

Figure 4. Effect of daidzein on GSH/GSSG ratio (A) and its related enzymes, GSH-px and GR (B, C). GSH/GSSG ratio and its related enzyme activity, GSH-px and GR were measured in post-mitochondria fractions obtained from hepatic homogenates by centrifugation. After total RNA was extracted using Trizol methods in liver tissue, GSH-px and GR mRNA expression were measured by real-time PCR. For relative mRNA expression, the value for the control (vehicle-only) was accepted to be '1.0'. Values are mean ±SD (n=6). ☆P<0.05, significant difference between control (vehicle-only) and DMBA-treated group by unpaired Student's t-test; ★P<0.05, significant difference from the DMBA-treated group by one-way analysis of variance.

study were also lower. At these physiological levels, daidzein and equol do not seem to induce a prooxidant effect.

It was reported that daidzein has antioxidant activity both *in vitro* and *in vivo* (27,28). Several epidemiological studies reported a significantly lower incidence of chronic disease in Asia, where isoflavone consumption is high, compared to that in Western countries, where isoflavone consumption is low (29,30). According to these trends, recent studies focused on the relationship between antioxidants and cancer (31-33). These studies suggest that the prooxidant effect of flavonoids, including daidzein, lead to their use as anticancer medicine causing apoptosis. Moreover, the antioxidant effect of daidzein was more pronounced under oxidation stress (6).

Therefore, we observed the effect of daidzein pretreatment under oxidative stress induced by DMBA, which induces mammary carcinogenesis in animal models. *In vitro* experiments indicate that DMBA in rat liver microsomes causes dose- and time-dependent production of H_2O_2, an oxidant closely associated with tumour promotion (34). The metabolic activation and detoxification of DMBA *in vivo* causes substantial oxidative damage in organs such as the liver and mammary glands (16,17).

The changes induced by DMBA led to the induction of apoptosis. It was reported that apoptosis was detected in the adrenal cortex of mice 24 and 36 h after DMBA treatment at 80 mg/kg BW (35,36). Apoptosis is a form of programmed cell death that is essential for tissue development and homeostasis. It involves an orchestrated series of biochemical events that lead to a variety of morphological changes, including blebbing, changes in the cell membrane such as the loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. The mechanism of apoptosis involves several factors known to induce and inhibit cell death. Consistent with these results, we found that DMBA significantly decreased Bcl-2 expression and increased Bax and cleaved caspase-3 expression. Moreover, daidzein pretreatment restored the expression of Bcl-2, Bax, and cleaved caspase-3 to their normal levels at both doses. Apoptosis is a promising target for cancer chemotherapy (37,38), and flavonoids, which act as general cell growth inhibitors and apoptosis inducers, are prime candidates in the development of new anticancer compounds (39-41). However, under pathophysiological conditions, apoptosis can induce inflammation due to the onset of secondary necrosis (42,43). In the present study, the modulation of apoptosis-related gene expression by daidzein in mice with DMBA-induced apoptosis may be the result of a protective mechanism in the liver.

The TBARS value and carbonyl content were measured as biomarkers of lipid peroxidation and protein oxidation, respectively. Lipid peroxidation and protein oxidation were significantly higher in the DMBA group than in the control group. This is consistent with previous reports that DMBA induces critical oxidative damage in the liver *in vivo* (44,45). Daidzein significantly inhibited oxidative damage, especially at the higher dose, as shown by the inhibition of lipid peroxidation (TBARS value). The rise in oxidative stress during DMBA metabolism produces deleterious effects by initiating lipid peroxidation directly or by acting as a second messenger for the primary free radicals that initiate lipid peroxidation (46). In a previous study, daidzein decreased the increase in lipid peroxidation with oxidative stress significantly to near normal levels, although daidzein also decreased lipid peroxidation under normal conditions. Thus, this result is consistent with that daidzein pretreatment prevented oxidative damage in the DMBA-treated groups, suggesting that it has protective effects.

In order to protect tissues from these forms of oxidative damage, antioxidant defense systems exist in enzymatic and non-enzymatic mechanisms. The enzymatic defense system is composed of SOD, catalase, and glutathione-related enzymes such as glutathione peroxidase, reductase, and -S-transferase. SOD and catalase act in a mutually supportive way with antioxidant enzymes to provide a protective defence against ROS. SOD scavenges superoxide by converting it to peroxide. Peroxide, in turn, is destroyed by catalase, which is widely distributed in all animal tissues. Daidzein significantly activated total SOD and catalase, each of which is inhibited by DMBA, suggesting that daidzein effectively defended the cells against oxidative damage.

GSH is a major nonenzymatic antioxidant molecule that is involved in the second line of defence against free radical damage in the body. GSH donates an electron in the reduction of peroxides catalysed by GSH-px as a component of the enzyme system containing GSH oxidase and reductase. GSH-px removes H_2O_2 produced by SOD by oxidising GSH. GR is responsible for recycling GSSG that is formed during oxidation events by reducing it back to GSH. At first glance, the GSH/GSSG ratio was unchanged by daidzein. However, at the higher dose, daidzein significantly increased GSH-px and GR activity to near-control values. Moreover, daidzein significantly increased the relative mRNA levels of GSH-px and GR in a pattern similar to the change in their enzyme activities. It is still not clear how daidzein activates the GSH-related system, although a previous study suggested two possibilities: antioxidant molecule recycling by daidzein and the involvement of equol, the major metabolite of daidzein, in the antioxidant mechanism of daidzein (6). In this study, one possible explanation for this phenomenon is the up-regulation of a GSH-related system by daidzein that serves to normalize the DMBA-induced oxidative stress. Therefore, daidzein pretreatment resulted in the activation of antioxidant enzymes in the DMBA-treated group, suggesting the preventive effects of daidzein.

Taken together, our data show that daidzein possesses antioxidant activity capable of hepatoprotective effects against oxidative stress induced by DMBA, such as lipid peroxidation and protein oxidation, and activates antioxidant defence system enzymes. In addition, daidzein showed a hepatic protective effect via the reduction of apoptosis. Note that under normal conditions, daidzein at higher doses was reported to act as a prooxidant instead of an antioxidant (6). Although the present findings suggest that daidzein is useful for human health as a food supplement or adjuvant, high-dose daidzein consumption must be carefully considered.

Acknowledgements

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