The antioxidant activity of daidzein metabolites, *O*-desmethylangolensin and equol, in HepG2 cells

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Abstract. Daidzein and its glycoside form daidzin, are known to have potential health benefits and are metabolized to *O*-desmethylangolensin (O-DMA) and equol following consumption. In the current study, the antioxidant activity and cytotoxicity of O-DMA, equol, daidzein and daidzin was investigated and their effects on HepG2 human hepatocelluar carcinoma cells were compared. For cytotoxicity assays, lactose dehydrogenase (LDH) release and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based cell viability, cells were exposed to various concentrations of each compound (5-200 μM) for 24, 48 or 72 h. O-DMA and equol did not affect LDH release, but higher concentrations (<75 μM) showed inhibition of cell growth. By contrast, daidzein and daidzin (200 μM) increased LDH release and cell growth. All compounds stimulated catalase and total superoxide dismutase (SOD) (CuZn- and Mn-SOD) activity, and mRNA and protein expression. This phenomenon was most pronounced for O-DMA and equol, as their effects were similar. These data suggested that O-DMA and equol possess greater antioxidant properties compared with daidzein and may, thus, be beneficial for human health.

Introduction

Among several hypotheses that seek to explain how flavonoids benefit human health, perhaps the most persuasive is antioxidant activity, which has been reported *in vitro* and *in vivo*. Flavonoids have been extensively studied for their antioxidant capacity, a biological function that involves scavenging and blocking of reactive oxygen species (ROS) (1,2). An imbalance of oxidative stress may result in a condition in which cellular antioxidant defenses are insufficient to maintain the levels of oxidants below a risk threshold. These oxidative species, which include ROS, such as superoxide ($O_2^{\cdot-}$, OOH·), hydroxyl (OH·) and peroxyl (ROOH·) radicals and reactive nitrogen species and sulfur-centered radicals may cause chronic diseases, including cancer, diabetes and cardiovascular conditions (3,4).

Isoflavoneis a member of the flavonoids family and exists in nature in commonly consumed plants. Daidzein, which occurs as daidzin, its glycoside form, in nature, is a primary component of isoflavones and is metabolized to the reduced forms equol and *O*-desmethylangolensin (O-DMA) and oxidative forms, 3',4',7-trihydroxy-isoflavones and 4',6,7-trihydroxyisoflavone, by gastrointestinal bacteria in humans (5). Only 30-50% of the population may produce equol, while 80-90% of the population produce O-DMA (5-7).

A number of studies have hyopthesized that metabolites may be important for the health effects associated with isoflavone consumption. However, only a few studies have focused on equol, which is a more potent antioxidant compared with daidzein or genistein when measured *in vitro* (8-10).

Therefore, the current study evaluated the effects of O-DMA, equol, daidzein and its glycoside daidzin on the antioxidant defense system by assessing antioxidative parameters *in vitro*.

Materials and methods

HepG2 cell line culture. Human hepatocellular carcinoma HepG2 cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were routinely maintained in minimum essential medium [Life technologies (Molecular Probes), Carlsbad, CA, USA], supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Sigma-Aldrich Co. LLC., St. Louis, MO, USA) at 37˚C in a humidified atmosphere containing 5% CO_2.

Preparation of O-DMA, equol, daidzein and daidzin. Equol, daidzein and daidzin were purchased from LC Laboratories® (Woburn, MA, USA) and synthesized O-DMA was a gift from Dr Lee (Professor of Chemistry Department, Duksung Women's University, Republic of Korea), these were dissolved in dimethylsulfoxide (final concentration 0.1% in medium).

Cytotoxicity. Cytotoxicity was evaluated by lactate dehydrogenase (LDH) release and an 3-(4,5-dimethylthi-

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azol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated at a density of $1x10^5$ cells/well in a 96-well tissue culture plate (Corning Incorporated Life Sciences, Tewksbury, MA, USA) and incubated at 37˚C for 24 h. Plated cells were treated with indicated concentrations of the molecules, O-DMA, equol, daidzein and daidzin. Following 72 h treatment, to determine the LDH release, 100 μl/well supernatant medium was transferred into corresponding wells of an optically clear 96-well flat bottom microtiter plate and an LDH cytotoxicity detection kit (Takara Bio Inc., Shiga, Japan) was used. Following treatment and incubation, plated cells were incubated with MTT (Sigma, St. Louis, MO, USA; 0.5 mg/ml final concentration) for 4 h at 37˚C. When all medium from the plates had been discarded, 100 μl DMSO was added to each well. The plates were placed at room temperature for 5 min with agitation, so that complete dissolution of formazan was achieved. The absorbance of MTT formazan was determined at 540 nm by a ultraviolet/visible spectrophotometric plate reader (Emax; Molecular Devices, Sunnyvale, CA, USA).

Enzymes activity. Catalase activity was assayed according to Aebi (11). Catalase activity was calculated as nmol of H_2O_2 decomposed/min/mg/protein. Superoxide dismutase (SOD) activity was assayed according to the pyrogallol autoxidation method of Marklund and Marklund (12). Each unit of SOD activity was defined as the quantity of enzyme that inhibited the auto-oxidation of pyrogallol by 50% under experimental conditions. Protein concentration was determined by a Bradford protein assay kit II (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblotting assay. Cells were lysed in RIPA buffer [1% NP-40, 150 mM NaCl, 0.05% deoxycholic acid, 1% SDS and 50 mM Tris (pH 7.5)] containing protease inhibitor at 4˚C for 1 h. The supernatant was separated by centrifugation and protein concentration was determined by a Bradford protein assay kit II. Proteins (25 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% bovine serum albumin solution for 3 h and washed twice with phosphate-buffered saline containing 0.2% Tween-20 and incubated with the primary antibody at 4˚C overnight. Antibodies against catalase, CuZn-, Mn-SOD and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and used to probe the separate membranes. The following day, the immunoreaction was continued with the secondary goat anti-rabbit horseradish peroxidase-conjugated antibody Santa Cruz Biotechnology, Inc. following washing for 2 h at room temperature. The specific protein bands were detected with an Opti-4CN Substrate kit (Bio-Rad Laboratories).

Relative mRNA expression by quantitative PCR. Samples were homogenized with TRIzol (Gibco-BRL, Carlsbad, CA, USA) and mRNA was extracted according to the manufacturer's instructions. First-strand cDNA was synthesized using SuperScript First-Strand Synthesis system (Invitrogen Life Technologies, Carlsbad, CA, USA). Each target mRNA expression was quantified by quantitative PCR with the use of CFB-3120 MiniOpticon™ system (Bio-Rad Laboratories, Inc.). The CFB-3120 MiniOpticon system uses an array of 48-light-emmitting diodes, which efficiently excite fluorescent dyes with absorption spectra between 470 and 505 nm. PCR reactions were performed with 2X SYBR®-Green mix (Finnzymes, Vantaa, Finland). Each mRNA level was calculated by means of the comparative cycle threshold (Ct) method using $2^{-\Delta\Delta Ct}$, according to the manufacturer's instructions. GAPDH was used as an endogenous control (internal control). The fold change in target gene relative to the endogenous control was determined 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 quantities of catalase, CuZn-SOD and Mn-SOD transcripts in the other samples were assigned arbitrary units relative to the levels in the calibrator sample.

Statistical analyses. All values are expressed as means ± standard deviation. Data were analyzed by unpaired Student's t-test or one-way analysis of variance followed by Dunnett's multiple comparison test (SigmaStat, Jandel Corporation, San Rafael,

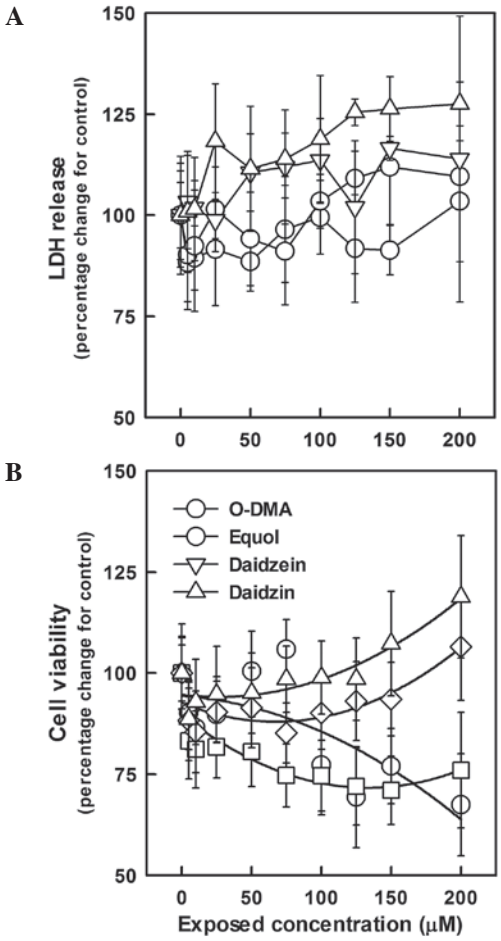

Figure 1. Cytotoxicity of O-DMA, equol, daidzein and daidzin. Cytotoxicity was evaluated by (A) LDH release and (B) MTT assay. Similar results were obtained in three independent experiments and representative data are shown as the mean ± SD. All data are reported as the percentage change in comparison with the vehicle-only group. *P<0.05, vs. vehicle-only group. O-DMA, *O*-desmethylangolensin; LDH, lactose dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

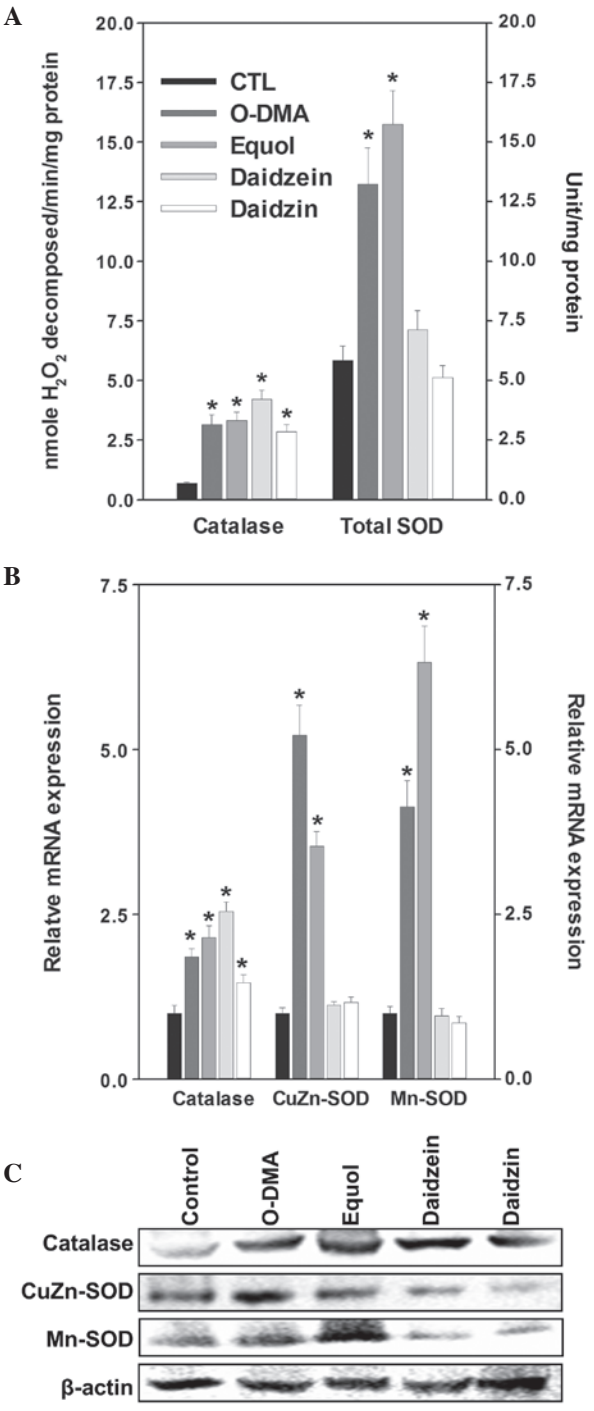

Figure 2. Regulation of (A) antioxidant enzymes activity, (B) relative mRNA and (C) protein expression by O-DMA, equol, daidzein and daidzin. Similar results were obtained in three independent experiments and representative data are shown as the mean ± SD. *P<0.05, vs. the vehicle-only group. O-DMA, *O*-desmethylangolensin; SOD, superoxide dismutase; CTL, control.

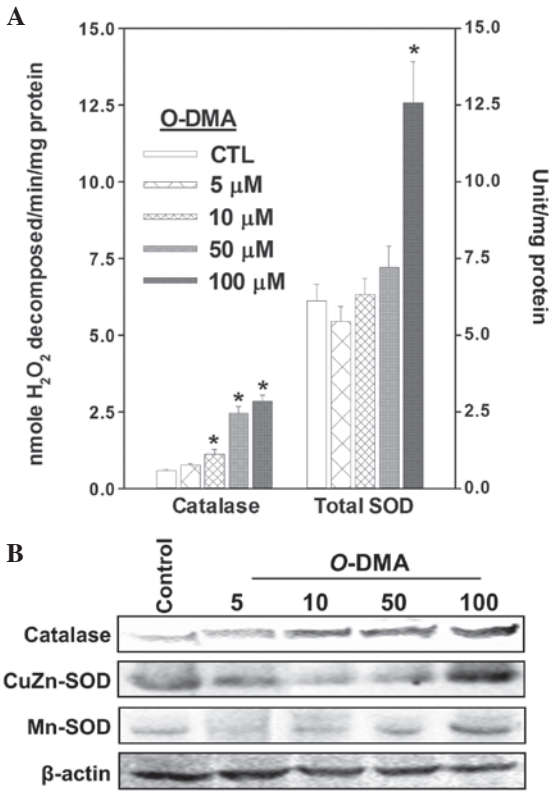

Figure 3. Regulation of (A) antioxidant enzymes activity and (B) protein expression by O-DMA. Similar results were obtained in three independent experiments and representative data are shown as the mean ± SD. *P<0.05, vs. the vehicle-only group. O-DMA, *O*-desmethylangolensin; SOD, superoxide dismutase; CTL, control.

CA, USA). For all comparisons, P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxicity. The cytotoxicity of O-DMA, equol, daidzein and daidzin was assessed by LDH release and MTT assays in HepG2 human hepatocellular carcinoma cells exposed to each compound at concentrations of 5-200 μM for 24, 48 or 72 h. As shown in Fig. 1, O-DMA and equol did not affect LDH release. By contrast, daidzein and daidzin resulted in an increase in LDH release by 10-28% following exposure for 72 h; however, the difference was only statistically significant for daidzin at a concentration of 200 μM. In terms of daidzein and daidzin, this result was in agreement with the increased growth of HepG2 induced by daidzein and daidzin (7 and 18% for daidzein and daidzin, respectively, at 200 μM for 72 h). O-DMA and equol at >75 μM significantly inhibited the viability of HepG2 cells. At concentrations <100 μM, cell growth was not altered by the addition of O-DMA or equol.

Antioxidant enzyme activity and expression. When antioxidant function was investigated in HepG2 cells exposed to 100 μM of each compound, all showed significant activation of antioxidant activity compared with control levels (Fig. 2A, P<0.05). Catalase activity was significantly increased by O-DMA and equol (each 4.7-fold compared with control). Daidzein increased catalase activity by 6.2-fold. Daidzin also increased catalase activity, although it had no effect on total SOD. This result was supported by the mRNA (Fig. 2B) and protein expression data (Fig. 2C).

Antioxidant activities were assayed in cells exposed to O-DMA or equol at 5, 10, 50 and 100 μM for 72 h (Figs. 3 and 4). O-DMA significantly increased the activity and expression of catalase at concentrations >10 μM. Total SOD activity was increased by >2.0-fold at 200 μM alone and

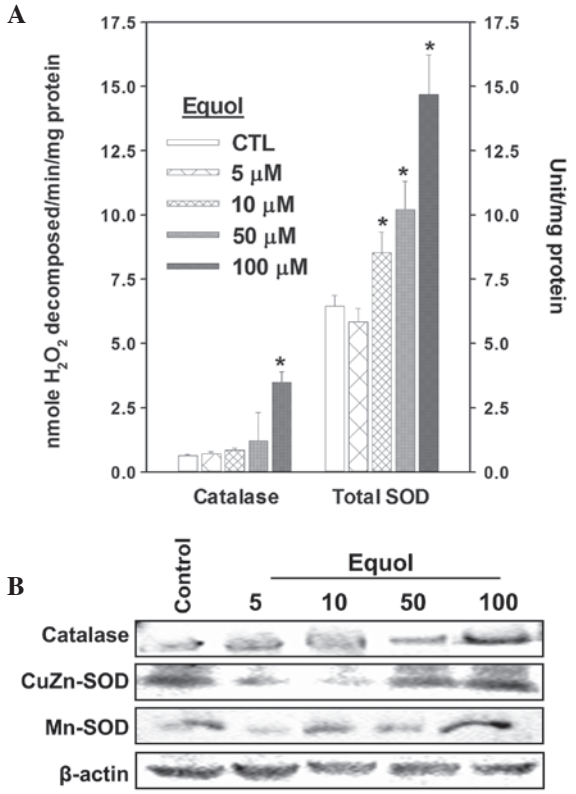

Figure 4. Regulation of (A) antioxidant enzymes activity and (B) protein expression by equol. Similar results were obtained in three independent experiments and representative data are shown as the mean ± SD. *P<0.05, vs. the vehicle-only group CTL, control; SOD, superoxide dismutase.

CuZn-SOD expression was more pronounced compared with Mn-SOD. By contrast, 200 μM equol significantly increased the catalase activity by 5.6-fold compared with control levels. In addition, total SOD activity was significantly increased in a dose-dependent manner at concentrations between 10 and 100 μM. The increased CuZn-SOD expression induced by equol showed a similar pattern to total SOD activity and Mn-SOD expression was markedly increased with the addition of 200 μM equol.

Discussion

Although daidzein, produced from daidzin, is a bioactive molecule in the body, a number of studies have reported that its biological activity is more pronounced than that of daidzin (13-15). Moreover, previous studies have suggested that the clinical effectiveness of isoflavones may be due to the activity of their metabolites (16-18). The current study was designed to investigate and compare the antioxidant characteristics of the daidzein metabolites, O-DMA and equol, in HepG2 cells. HepG2 cells, which were derived from a hepatocelluar carcinoma, are used *in vitro* to study toxicity as a number of the characteristics of normal hepatocytes are retained, including phase I and II and the expression of antioxidant enzymes (19,20).

When cells were exposed to O-DMA or equol, LDH release and cell viability were investigated. LDH release was not altered by exposure to O-DMA and equol at any concentration or exposure time. In addition, although LDH release was increased by daidzein and daidzin, no significant difference was observed and this result may have been due to altered cell growth. However, O-DMA and equol inhibited the growth of HepG2 cells at higher doses (~30% decrease at 75 and 100 μM for O-DMA and equol, respectively).

Our previous *in vivo* studies (21,22) indicated that equol may act a prooxidant, as well as an antioxidant. Long-term administration of equol at higher doses to mice increased serum equol concentrations and may lead to prooxidant effects. In addition, serum ALT activity was marginally increased, however, this difference was not statistically significant. These results are consistent with the present study and indicate that O-DMA and equol may possess prooxidant cytotoxicity, albeit extremely weak.

A number of antioxidants, including flavonoids, are hypothesized to possess opposing anti- and prooxidant actions (23-26). One mechanism of antioxidant activity may involve the termination of chain radical reactions by donating hydrogen atoms to the peroxy radical, forming a novel radical, which in turn reacts with free radicals, thus, terminating the propagating chain (27). Although the reactivity of the formed radicals is weak, they may function as prooxidants, depending on the circumstances. Nevertheless, the hypothesis that such dual functions contribute to tumor apoptosis and cancer chemotherapy has recently become widely accepted (28,29).

In the present study, O-DMA, equol, daidzein and daidzin exhibited antioxidant activities. Oxidative stress leads to an increase in free radicals and ROS and a decrease in antioxidant defense system-associated molecules and enzymes. Cellular oxidative stress has been implicated in the etiology and pathology of a number of diseases (30). Increased consumption of antioxidants, which are important in the prevention of human diseases and maintenance of good health by protecting against oxidative stress, has been suggested. Among the antioxidant defense systems, SOD is the first and most important line of enzymatic defense against oxidative stress and particularly oxygen radicals. SOD scavenges superoxide by converting it to peroxide. Peroxide, in turn, is destroyed by catalase, which is widely distributed in all animal tissue. SOD and catalase act in a mutually supportive way with antioxidant enzymes to protect against ROS.

The antioxidant activities of O-DMA, equol, daidzein and daidzin were in descending order, daidzein > equol > O-DMA > daidzin for catalase and equol > O-DMA > daidzein > daidzin for total SOD. mRNA and protein expression was similar and CuZn- and Mn-SOD were more highly induced by O-DMA and equol, respectively. Based on these data, these four compounds may act at different points in the antioxidant defense system. In all assays used in the current study, the antioxidant activity of daidzin was weaker than that of its metabolites, which is consistent with previous studies (13,31) stating that the antioxidant activity of daidzein was stronger than that of daidzin. Also, aglycone flavonoids appear to have a higher accessibility to the sites of trapped radicals (32).

Moreover, O-DMA and equol, which are derived from daidzein, generally showed a significantly greater antioxidant activity compared with daidzein itself. This is supported by the observations that metabolites, including equol, may be key for the clinical effectiveness of isoflavones (33-36). O-DMA

and equol activated catalase and SOD in a dose-dependent manner.

There are a number of studies that support the hypothesis of role of antioxidants in the prevention of chronic disease, including cancer (37-39). These data suggest that O-DMA and equol exert their antioxidant activities by stimulating catalase and SOD activity and expression. Therefore, further studies are required to determine the exact mechanisms underlying the antioxidant effects of O-DMA and equol and to understand the anticancer effects.

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