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Soy Isoflavones Protect the Intestine from Lipid Hydroperoxide Mediated Oxidative Damage

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The effects of 24 h supplementation of human colon carcinoma cells (Caco-2) with isoflavones, genistein, and daidzein and their activities against oleic acid hydroperoxide mediated oxidative stress were investigated. Genistein, at 25, 50, and 100 μM, and daidzein, at 25 and 50 μM, did not induce cell injury to Caco-2 cells. Both compounds reduced cell injury and DNA damage mediated by 5 μM oleic acid hydroperoxides in Caco-2 cells. The effects of genistein and daidzein on antioxidant enzymes were dependent upon the compound and its concentration.

KEYWORDS: Oxidative stress; antioxidants; lipid hydroperoxides; soy isoflavones; genistein; daidzein; Caco-2 cells

INTRODUCTION

The cytotoxic activity of deep fried foods has been associated with their content of lipid oxidative products (*1*). Repeated use of frying oil, which is a common practice followed by restaurants and households, leads to the accumulation of many lipid oxidation products in the frying oils and, ultimately, in the fried foods (*2*). The consumption of these oxidative compounds causes cellular damage in the liver and kidneys and enhanced cell proliferation in the gastrointestinal tract (*3*). Lipid hydroperoxides, which is one of the major products of oxidation, is associated with increased risk of many degenerative diseases in human, such as cancer (*4*), inflammation (*5*), and atherosclerosis (*6*). As reported earlier (*7*), lipid hydroperoxides at physiologically relevant concentrations of 5–25 μM are responsible for disrupting cell membranes, inducing cell lipid peroxidation, and damaging DNA in intestinal cells. Previous studies (*7*) also revealed that existing antioxidant enzyme mechanisms in the intestine are not capable of overcoming cell toxicities mediated by lipid hydroperoxides, even at a low concentration of 5 μM. As a result, the potential of external sources of antioxidants, i.e., dietary antioxidants, was investigated in terms of preventing oxidative stress-related injuries to the intestine. The current study was performed to determine the potential of two dietary isoflavones, genistein and daidzein, in reducing hydroperoxide-mediated cell injury to human intestinal cells.

Isoflavones are naturally present in whole soybean and in purified soy protein isolates (*8*). These natural compounds are powerful antioxidants because of the presence of multiple hydroxyl groups in their structures (*9*). In addition to antioxidant activity, isoflavones also elicit weak estrogen-like effects in both animals and humans through the interaction with estrogen receptors because of their structural similarity to estrogen (*10, 11*). Asian populations who consume soy-based food as the staple in their diet have a lower incidence of cardiovascular disease and hormone-related cancer than those who consume a typical Western diet (*12*). Consumption of isolated soy protein, 39 g/d for 1 year by twenty-nine human subjects who had a history of adenomatous polyps or colon cancer, has been shown to alter cell proliferations in the colonic mucosa, indicating a reduced potential for colon cancer development (*13*). These beneficial effects have been attributed to two key compounds, genistein and daidzein, present in soy products (*14*).

Plasma concentration of isoflavones after consumption of soy products varies from 1 to 10 μmol/L (*15, 16*). The intestinal luminal concentrations of isoflavones can be expected to be much higher, in the range of 10–200 μmol/L depending on the dietary composition; a value that was derived from previous studies (*16–18*).

Genistein and daidzein have been shown to reduce both in vivo and in vitro oxidation induced by reactive nitrogen species (*19*). However, genistein has not been effective in inhibiting hydrogen peroxide (H_2O_2) induced lipid peroxidation in Caco-2 human colon carcinoma cells at concentrations lower than 10 μM (*20*). These results are comparable with studies conducted in both a cell free- and an endothelial cell model- system in which genistein or daidzein did not contribute significantly to free radical scavenging activities (*21*). It could be suggested that their antioxidant capabilities could be due to their influence on cellular antioxidant enzymes. Cai and Wei (*22*) reported that dietary administration of genistein (50 and 250 ppm) for 30 days significantly increased antioxidant enzyme activities in the small intestine and skin of mice. In contrast, incubating Caco-2 cells with genistein and daidzein for 48 h did not show significant effects on cellular antioxidant enzymes, catalase, and superoxide dismutase (*23*). Whether these compounds are more effective when incubated for moderate or short time periods of 24 h or less has not been studied.

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Antioxidant activities of isoflavones are also dependent on the relationship between their chemical structures and reactive oxygen species (ROS). Genistein has been found to be more effective in inhibiting hydroxyl radical induced lipid peroxidation, whereas daidzein is more effective in superoxide anion induced lipid peroxidation (*24*). In Caco-2 colon cell cultures, low concentrations (<5 μM) of genistein increased cell proliferation, whereas high concentrations (>100 μM) lowered cell growth (*25*). Similar effects have been reported in ovarian cancer cell lines (*26*). However, the effects of genistein or daidzein on intestinal cellular antioxidant mechanisms, when incubated at moderate concentrations (25–100 μM), have not been studied. The current study provides information on the effectiveness of incubating cells for 24 h with genistein (25, 50, and 100 μM) and daidzein (25 and 50 μM) in reducing lipid hydroperoxide induced oxidative stress in the intestine using a human colon carcinoma cell line, Caco-2.

MATERIALS AND METHODS

A human colon carcinoma cell line (Caco-2) was obtained from the American type Culture Collection (Rockville, MD). Dulbecco's Modified Eagle Medium (DMEM), and fetal bovine serum (FBS) were purchased from Invitrogen Corporation (Carlsbad, CA). L-glutamine, penicillin with streptomycin, trypsin with ethylenediaminetetraacetic acid (EDTA), and phosphate buffered saline (PBS) were purchased from Fisher Scientific, (Fairlawn, NJ). Nonessential amino acid solution, xanthine, hypoxanthine, nitro blue tetrazolium, diethylenetriaminepentaacetic acid, a lactate dehydrogenase (LDH) based TOX-7 kit, and a glutathione peroxidase cellular activity assay kit CGP-1 were obtained from Sigma Chemicals (St. Louis, MO). Hydrogen peroxide, chloroform, cyclohexane, and ethanol were purchased from VWR International (Bridgeport, NJ). A Micro BCA protein assay kit was purchased from Pierce Biotechnology (Rockford, IL). Genistein and daidzein were purchased from LC Laboratories (Woburn, MA). Purities of these compounds were >98%.

Culture and Oxidation of Caco-2 Cells. Caco-2 cells were grown in DMEM supplemented with 20% FBS, 1% L-glutamine, 1% nonessential amino acids, and 100 units/mL penicillin with 100 μg/mL streptomycin. The cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and were seeded onto collagen-coated 25 or 75 cm^2 area culture flasks. Oxidation was induced by exposing Caco-2 cells to 5 μM oleic acid hydroperoxides (OAHPx) in FBS-free DMEM or PBS, supplemented with 1% L-glutamine and 1% nonessential amino acids, for 30 min on the fourth day of culturing after confluency had been reached. Cells were harvested by a brief (6 min) trypsinization and then centrifuged (Beckman GS-15R centrifuge, Beckman, Palo Alto, CA) at 200*g* for 5 min. Lipid hydroperoxides were generated by oxidation of oleic acid under fluorescent light for 21 days, and OAHPx solutions were prepared as described by Wijeratne and Cuppett (*7*). Preliminary studies showed that oxidation products other than lipid hydroperoxides were extremely low and were not in significant amounts in the oxidized oleic acid samples.

Cell Membrane Damage. Cells were grown to confluence in 25 cm^2 culture flasks and were washed with PBS prior to use. A concentration of 5 μM OAHPx in PBS supplemented with 1% L-glutamine and 1% nonessential amino acids were used to induce oxidation. After 30 min of exposure, PBS from each flask was collected. Damage to cell membrane by OAHPx was studied by measuring the release of lactic acid dehydrogenase (LDH) from injured cells. LDH leakage into PBS and total LDH activity (LDH leakage to PBS plus LDH in remaining cells) were measured with an in vitro cytotoxicity assay kit, LDH-based TOX-7 (Sigma Chemical Co.), and were corrected by the activity already present in the medium of untreated cells. The assay is based on the reduction of NAD to NADH by LDH. NADH is utilized to convert a tetrazolium dye in the assay kit to a colored compound with an absorption maximum at 490 nm. The intensity of the color is indicative of LDH activity in the assay medium, and the LDH activity was measured spectrophotometrically (Beckman Coulter DU800 spectrophotometer, Beckman Coulter Inc., Fullerton, CA) at 490 nm. LDH activity in the PBS supernatant was determined as a percentage of the total LDH activity.

Preparation of Cell Lysates. Culture medium was decanted, and cells were washed with 5–10 mL of PBS. The cells were harvested by a brief trypsinization. Cell suspensions were centrifuged at 200*g* for 5 min and were washed twice with 5 mL of PBS. Supernatants were discarded, and cell pellets were resuspended in 5 mL of PBS at 0 °C and then placed on ice. Cells were lysed using a minibead beater (Biospec Products, Bartlesvillle, OK) for 10 s at 4200 rpm. The lysates were centrifuged (Beckman GS-15R centrifuge) at 14000*g* for 10 min at 4 °C, and supernatants were immediately used for lipid peroxidation and antioxidant enzyme assays.

Lipid Peroxidation Assay. Lipid peroxidation was assayed by measuring conjugated dienes in cell lysates according to the method described by Buege and Aust (*27*). One milliliter of cell lysate in PBS was thoroughly mixed with 5 mL of a chloroform/methanol (2:1) solution, followed by centrifugation (Beckman GS-15R centrifuge) at 1000*g* for 5 min until phase separation was achieved. Most of the upper layer was removed by suction, and 3 mL of the lower chloroform layer was transferred to a test tube. The chloroform layer was removed under nitrogen infusion, and the lipid residue was dissolved in 1.5 mL of cyclohexane. The absorbance of the solution at 233 nm was measured (Beckman Coulter DU800 spectrophotometer) against a cyclohexane blank at 233 nm. Conjugated dienes were reported as absorbance at 233 nm.

Catalase Assay. Catalase was assayed spectrophotometrically at 25 °C following the extinction of H_2O_2 at 240 nm (*28*). The catalase activity per milliliter of the cell lysate was calculated as the reduction of H_2O_2 (mmol min^{-1} mL^{-1}). Nonenzymatic H_2O_2 decomposition (baseline) was subtracted from each determination.

Superoxide Dismutase (SOD) Assay. A modified version of the Nishikimi et al. (*29*) method was used to detect SOD activity in cell lysates. In this method, superoxide radicals were generated using a xanthine oxidase/hypoxanthine system, and the potential of the cell lysates to scavenge superoxide radicals was measured spectrophotometrically. The reaction mixture contained 1 mL of 3 mM hypoxanthine, 1 mL of 100 mIU xanthine oxidase, 1 mL of 178 μM nitro blue tetrazolium, 1 mL of 12 mM diethylenetriaminepentaacetic acid, and 1 mL of the cell lysate. All solutions were prepared in PBS. The absorbance of the mixtures at 560 nm was recorded initially at 0 min and thereafter at 5 min intervals up to 30 min. Superoxide radical-scavenging capacities of the cell lysates at the end of 30 min were calculated with the equation $Y = [1 - (A/B)] \times 100$ where Y is the percentage of superoxide radicals scavenged, A is the absorbance of the medium containing cell lysate at 30 min, and B is the absorbance of the medium without cell lysate at 30 min (blank).

Glutathione Peroxidase (GPx) Assay. GPx activity was measured using the GPx cellular activity assay kit CGP-1 (Sigma Chemical Co.). This kit uses an indirect method, based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH at 340 nm during oxidation of NADPH to NADP is indicative of GPx activity. The activity of GPx per milliliter of the cell lysate was calculated as the decrease in NADPH (μmol min^{-1} mL^{-1}).

DNA Damage by Comet Assay. The comet assay was performed using Trevigen's comet assay reagent kit for single-cell electrophoresis assay (Trevigen Inc., Gaithersberg, MD). Cells (1×10^5) were suspended in 1 mL of ice-cold PBS. Fifty microliters of the cell suspension was combined with 500 μL of prewarmed low melting point (LMP) agarose, and 75 μL of this mixture was immediately pipetted onto a CometSlide. Slides were placed flat at 4 °C in the dark for 30 min for gelling. After gelling was complete, slides were transferred into a prechilled lysis solution (2.5 M NaCl, 100 mM EDTA, pH 10, 10 mM Tris base, 1% sodium lauryl sarcosinate, and 1% Triton X-100) and were placed at 4 °C for 50 min. Slides were then incubated in a fresh electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) for 40 min at room temperature to allow unwinding of DNA. Electrophoresis was carried out at room temperature in fresh electrophoresis buffer for 40 min at 1 V/cm and 300 mA. After electrophoresis, slides

were gently rinsed by dipping several times in distilled water and then immersed in 70% ethanol for 5 min and air-dried. Slides were stored with desiccant at room-temperature prior to analysis. Slides were stained with SYBR green and were viewed by an Olympus AX70TRF microscope digital camera system (Olympus Optical Co. Ltd.). Digital images of DNA were analyzed, and DNA damage was quantified by measuring the tail moment using NIH Image software available at http://rsb.info.nih.gov/ij/. About 100–150 cells were scored per sample. Damage is represented by an increase of DNA fragments that have migrated out of the cell nucleus in the form of a characteristic streak similar to the tail of a comet (*30*). DNA damage can be assessed using different parameters, such as tail length, relative tail fluorescence intensity, and tail moment (*30*, *31*).

Statistical Design. A general linear fixed effects model blocked by cell passage number, which corresponds to the age of cells, was used in all test systems. All experiments were repeated four times. Statistical analysis was conducted using Statistical Analysis System (SAS) software (version 8.02, SAS Institute Inc., Cary, NC) with analysis of variance (ANOVA) followed by Tukey's HSD test for significant differences. A p value of ≤ 0.05 was considered to be statistically significant unless stated otherwise.

RESULTS

The nonstressed and oxidatively stressed Caco-2 cell cultures showed varied responses depending on type and concentration of isoflavone used to supplement the confluent cultures. In the text, 'control' always refers to cell cultures that were not supplemented with isoflavones and/or not subjected to oxidative stress treatment with OAHPx.

Cellular Responses after 24 h Incubation with Genistein and Daidzein. *Cellular Damage.* Genistein (25, 50, and 100 μM) and daidzein (50 μM) markedly reduced LDH leakage into the culture medium, indicating increased protection against base-level cell membrane damage that could occur during cell culturing and handling procedures (**Figure 1**). Supplementation with 25 μM daidzein did not alter the proportion of total LDH activity present in the culture medium as compared to that in the control. There were no significant changes in cell lipid peroxidation of cell cultures supplemented with either of the two compounds compared to that of the control (**Figure 1**). In contrast, DNA damage was significantly decreased in daidzein (25 and 50 μM) supplemented cultures as compared to the respective control and genistein (25, 50, and 100 μM) supplemented cultures (**Figure 1**). Collectively, none of the tested levels of genistein or daidzein mediated cell injury in Caco-2 cells when incubated for 24 h.

Antioxidant Enzyme Activities. Isoflavone supplementations of Caco-2 cultures for 24 h led to slight changes in the antioxidant enzyme activity profiles relative to control cultures. Daidzein increased catalase activity as compared to that of the control, but no significant changes were observed among cultures treated with genistein (**Figure 2**). Glutathione peroxidase (GPx) activity was not significantly different among any of the isoflavone supplemented and/or control cultures (**Figure 2**). In contrast, an 8–16% decrease in superoxide dismutase (SOD) activity was observed in cultures treated with 25 and 50 μM genistein and daidzein as compared to those of control cultures (**Figure 2**). However, cell cultures supplemented with 100 μM genistein showed SOD activities similar to that of the control.

Cellular Responses after Oxidative Treatment Following 24 h Supplementation with Isoflavones. *Cellular Damage.* Exposing cultures to 5 μM OAHPx caused significantly higher amounts of LDH leakage (16%) as compared to that of the control (7%) (**Figure 3**). Genistein and daidzein supplemented cultures had significantly lower LDH leakage (9–11%) as compared to nonsupplemented cultures. Genistein, at 25 μM, was less effective but not significantly different in reducing cell membrane damage as compared to the 50 and 100 μM supplemented cultures (**Figure 3**). Nevertheless, at the tested concentrations, neither genistein nor daidzein were able to lower LDH leakage to the levels of the control cultures, indicating the inability of these compounds to achieve complete detoxification of 5 μM OAHPx.

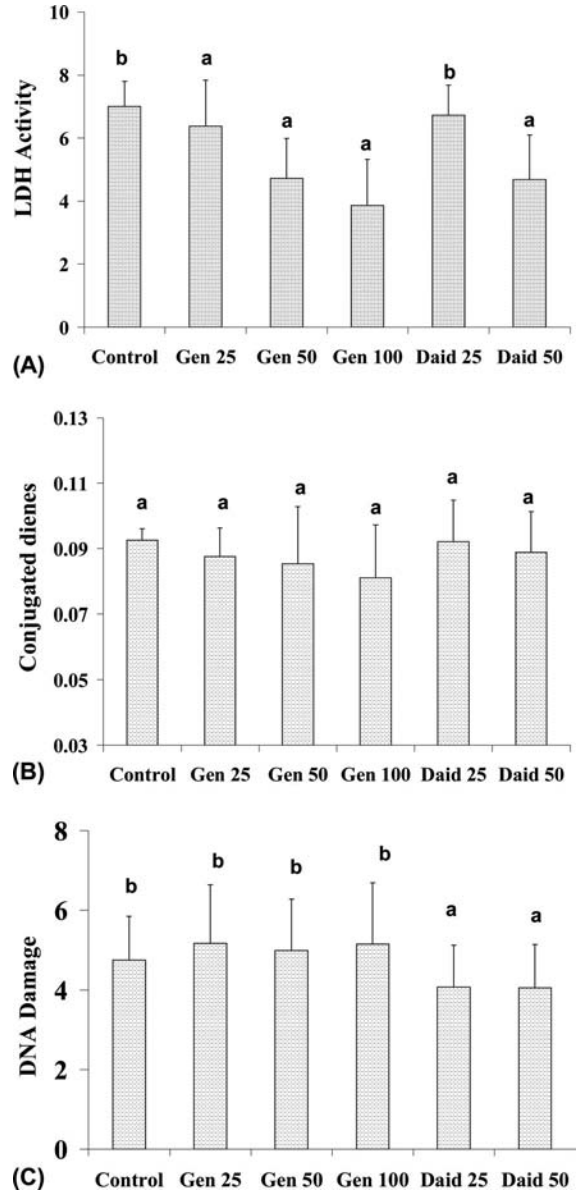

Figure 1. LDH activity in the culture medium (% of total cell LDH activity) (**A**), conjugated dienes (absorbance at 233 nm) (**B**), and DNA damage (tail moment in arbitrary units) (**C**) in cell cultures supplemented with genistein (Gen) and daidzein (Daid) for 24 h and compared to a control that had no supplements.

Conjugated diene measurements indicated that all levels of genistein and daidzein lowered lipid peroxidation as compared to the nonsupplemented cell cultures (**Figure 3**). Daidzein, at 25 μM, was the least effective and was not significantly different from the nonsupplemented oxidized cell cultures, whereas genistein at 50 and 100 μM was the most effective supplement in inhibiting cell lipid peroxidation. As observed with membrane damage, genistein and daidzein supplements were not able to completely protect Caco-2 cells against lipid peroxidation by

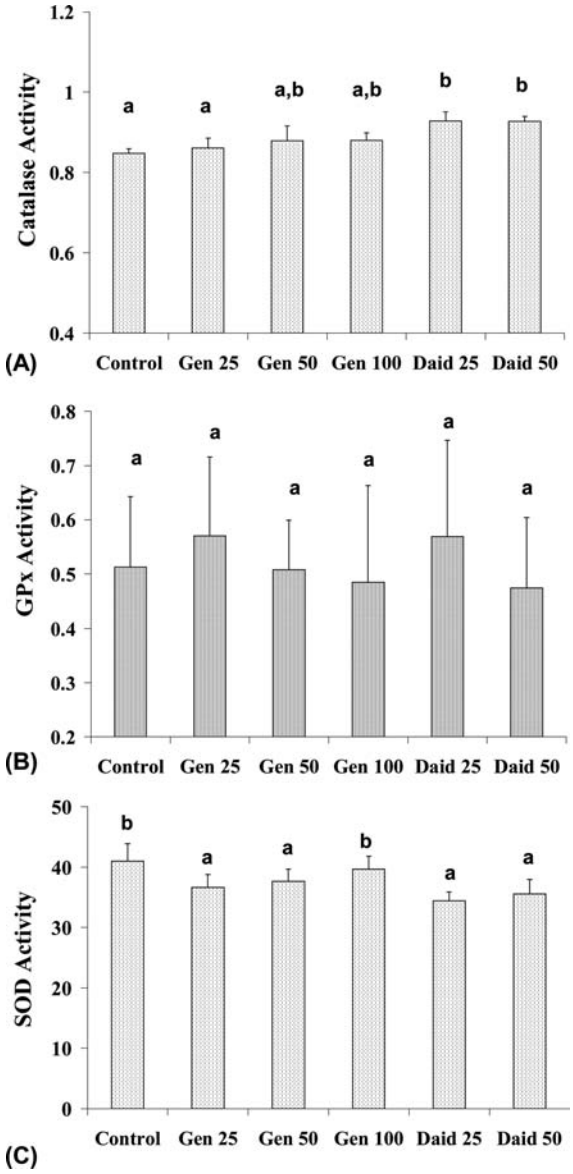

Figure 2. Activities of catalase (decrease in H_2O_2 mmol L^{-1} min^{-1} mL^{-1}) (**A**), glutathione peroxidase (decrease in NADPH μmol L^{-1} min^{-1} mL^{-1}) (**B**), and superoxide dismutase (% scavenged superoxide radicals) (**C**) in cell cultures supplemented with genistein (Gen) and daidzein (Daid) for 24 h and compared to a control that had no supplements.

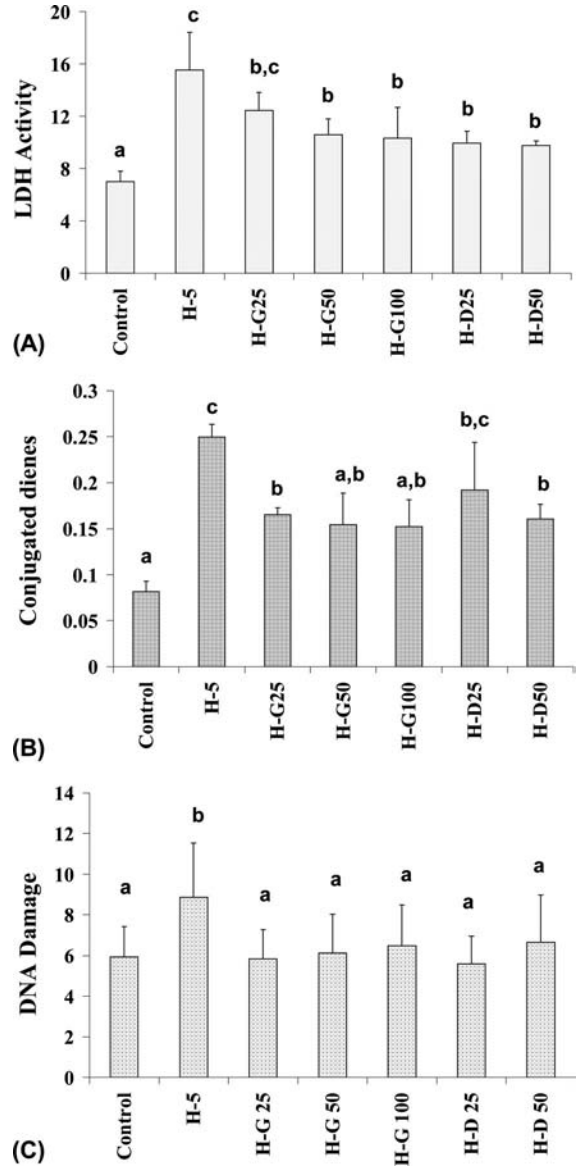

Figure 3. Changes in LDH activity in culture medium (% of total cell LDH activity) (**A**), conjugated dienes (absorbance at 233 nm) (**B**), and DNA damage (tail moment in arbitrary units) (**C**), in cell cultures supplemented genistein and daidzein for 24 h and treated with 5 μM oleic acid hydroperoxide for 30 min. Symbols are: H, hydroperoxide treated; G, genistein: D, daidzein; control refers to cultures that were not supplemented or subjected to oxidation.

OAHPx. Both compounds, at all tested concentrations, significantly lowered DNA damage to levels comparable to those of the control cultures (**Figure 3**).

Antioxidant Enzyme Activities. No significant changes were observed in catalase or GPx activity in any of the supplemented or nonsupplemented cell cultures subjected to oxidation by OAHPx (**Figure 4**). However, a trend toward decreased GPx activity with increasing concentrations of genistein and daidzein was apparent. Oleic acid hydroperoxide caused SOD activity to decrease to 18% of that of the control (**Figure 4**). Genistein, at 100 μM, and daidzein, at 50 μM, increased SOD activity levels closer to that of the control cultures, whereas other supplement concentrations had no effect.

DISCUSSION

This study assessed the effects of genistein and daidzein supplementation on nonstressed and oxidatively stressed human colon cells. Relative to control cultures, isoflavone supplementation of Caco-2 cell cultures for 24 h led to different antioxidant enzyme responses depending on isoflavone concentration. However, the expression of catalase and SOD protein genes was not affected when Caco-2 cells were incubated with 100 μM genistein and daidzein for 48 h (*23*). These results suggest that the biological activity of these isoflavones may be lost at longer incubation periods, such as 48 h as compared to 24 h incubations. Our study demonstrated that catalase activity was increased by daidzein (25 and 50 μM) and SOD activity was decreased by both isoflavones (except genistein at 100 μM) as compared to the control, whereas GPx activity was not significantly affected. Studies by Cai and Wei (*22*) showed increased activities of catalase, SOD, and GPx activities in the small intestine of mice by dietary administration of genistein at 50 and 250 ppm levels for 30 days. In contrast, Breinholt et al. (*32*) demonstrated that genistein administered to rats at a dose of 0.1g/kg body weight for 2 weeks inhibited catalase,

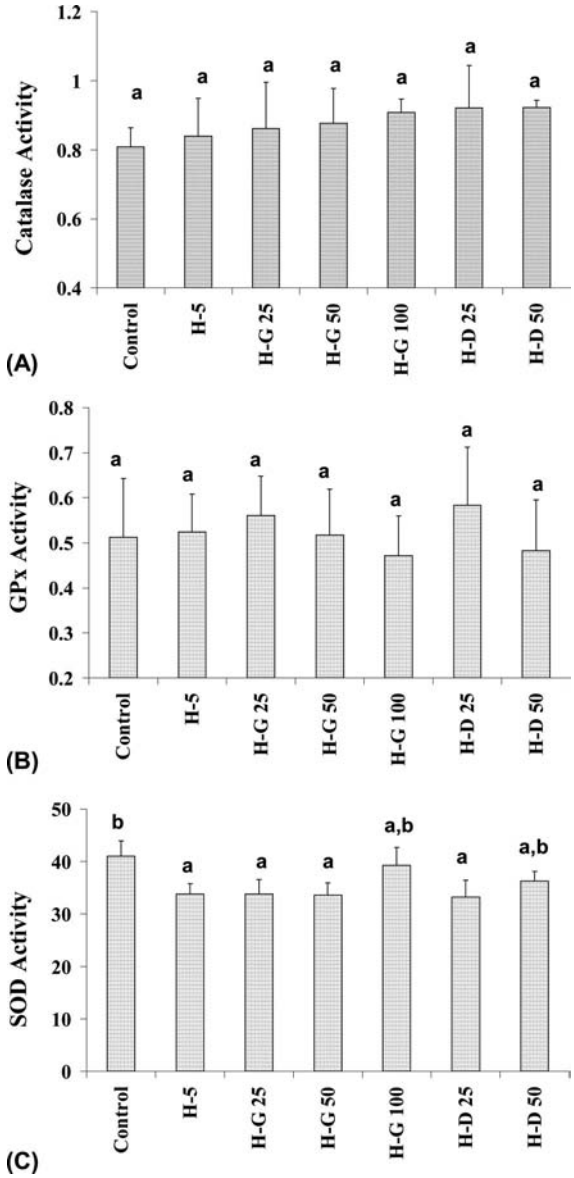

Figure 4. Activities of catalase (decrease in H_2O_2 mmol L^{-1} min^{-1} mL^{-1}) (**A**), glutathione peroxidase (decrease in NADPH μmol L^{-1} min^{-1} mL^{-1}) (**B**), and superoxide dismutase (% scavenged superoxide radicals) (**C**) in cell cultures supplemented with genistein and daidzein for 24 h and treated with 5 μM oleic acid hydroperoxide for 30 min. Symbols are: H, hydroperoxide treated; G, genistein; D, daidzein; control refers to cultures that were not supplemented or subjected to oxidation.

SOD, and GPx antioxidant enzyme activities in their red blood cells but showed no effect on liver antioxidant enzymes. These studies suggest that genistein and daidzein affect antioxidant enzyme activities in biological systems and that their activity is not only dose-dependent but also could be tissue-specific. Furthermore, Guo et al. (*21*) reported that genistein and daidzein, at levels of 100 μM concentration, do not possess substantial hydroxyl-, superoxide-, nitric oxide-, and lipid derived free radical-scavenging activities as tested in cell-free and endothelial cell model systems. Therefore, the ability of genistein and daidzein to affect antioxidant enzymes is of prime importance relative to their free radical scavenging activity in biological systems.

The antioxidant effects of these isoflavones are due to the presence of multiple hydroxyl groups in their chemical structures, which increases their ability to donate hydrogen atoms to free radicals (*33*). Furthermore, genistein is a stronger antioxidant as compared to daidzein because the 5,7-dihydroxyl structure of the "A" ring in genistein is believed to make it a better antioxidant (*9*). It has also been demonstrated that genistein is relatively more effective in inhibiting hydroxyl radical induced oxidation as compared to daidzein, which is the stronger agent against superoxide radical induced oxidation (*24*). These studies support the fact that the antioxidant activities of these isoflavones are dependent upon their structures as well as the type of reactive oxygen species.

The current study showed that the protection against OAHPx induced cell injury at higher concentrations of both genistein and daidzein were not significantly different than the protection exhibited at their lower concentrations and that daidzein was as effective as genistein. These antioxidative effects could be due to the presence of hydroxyl groups in structures of genistein and daidzein that leads to membrane stabilization and protection against lipid peroxidation due to decreased membrane fluidity (*34, 35*). However, a 24 h incubation period of Caco-2 cell cultures with genistein and daidzein failed to completely detoxify 5 μM of OAHPx. Because there is evidence for pro-oxidant activities of genistein and daidzein (*36, 37*), further studies should be conducted to investigate the effects of isoflavones at higher concentrations.

In oxidatively stressed cultures, no significant changes were observed in catalase activity in any of the supplemented or nonsupplemented cell cultures. When exposed to OAHPx, SOD decreased, but GPx did not change significantly. However, when supplemented with isoflavones, SOD activity in cultures with 100 μM genistein and 50 μM daidzein were comparable to those of the control. Lipid hydroperoxides can undergo decomposition and form peroxyl, alkoxyl, and hydroxyl radicals in the presence of metal ions and other free radicals. Furthermore, superoxide radicals are consumed during the production of alkoxyl radicals from lipid hydroperoxides (*38*). Thus, the observed antioxidant enzyme activity is the net effect of all positive, negative, and interaction effects of isoflavones and OAHPx and its decomposition products.

In conclusion, this study showed that genistein and daidzein supplementation of Caco-2 cells for 24 h reduces oleic acid hydroperoxide mediated cell injury. Genistein and daidzein also affected antioxidant enzyme activity, depending on the type and concentration of isoflavone used in supplementation.

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