The Phytoestrogen Daidzein Affects the Antioxidant Enzyme System of Rat Hepatoma H4IIE Cells

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ABSTRACT Phytoestrogens such as the soy isoflavonoid daidzein have potential health benefits. The antioxidant properties of phytoestrogens are considered to be responsible for their protective effects. The antioxidant enzyme (AOE) system plays an important role in the defense of cells against oxidative insults. To determine whether flavonoids can exert antioxidative effects not only directly but also indirectly by modulating the AOE system, we investigated the influence of the flavonoid daidzein on the expression of different AOE. Daidzein treatment of hepatoma H4IIE cells increased catalase mRNA expression two- to threefold. Expression levels of copper zinc superoxide dismutase (CuZnSOD) were not affected by exposure to daidzein. Manganese superoxide dismutase (MnSOD) mRNA expression levels decreased slightly and glutathione peroxidase (GPx) levels increased slightly after daidzein exposure. Changes in AOE mRNA expression levels were significant at 300 μmol/L daidzein. To elucidate the mechanisms underlying the strong increase in catalase mRNA, transfection experiments were performed. Transient transfection of hepatoma cells with reporter plasmids containing different parts of the upstream region of the catalase gene showed a significant one- to threefold increase in reporter gene activity after daidzein exposure. This indicates that daidzein can directly activate the rat catalase promoter region. Despite the increase in catalase mRNA, daidzein pretreatment of cells did not protect against oxidative stress resulting from H2O2 exposure. On the contrary, daidzein itself exerted a mild oxidative stress. In conclusion, the changes in the AOE system provoked by daidzein affected the oxidant rather than the antioxidant properties of daidzein. J. Nutr. 132: 370–375, 2002.

KEY WORDS: antioxidant enzymes • daidzein • expression • hepatoma cell • lipid peroxidation

Phytoestrogens are a group of naturally occurring diphenolic compounds present in legumes, whole grains, fruits and vegetables. These dietary flavonoids have drawn much attention because there are suggestions that they might benefit human health. High consumption of phytoestrogen-rich food has been linked to a reduced incidence of cancers at different sites including breast, prostate and colon (1–4). Flavonoids in regularly consumed foods may also reduce the risk of death from coronary heart disease (5,6).

Several in vitro and animal studies were undertaken to clarify the biological and physiologic processes that account for the cancer chemoprotective effects of flavonoids. The estrogenic activities of the flavonoids may play an important role in their health-enhancing properties. Flavonoids have been reported to bind to estrogen receptors (ER)3 and prevent cell growth in hormone-dependent cancer cells (1,7,8). Another proposed mechanism for cellular protection is the induction of phase II detoxification enzymes, leading to detoxification of proximate carcinogens generated by phase I metabolism. Indeed, some phytoestrogens were found to induce the phase II enzyme NADPH quinone reductase (9). In addition to these different modes of action, the protective effects of flavonoids have been attributed mainly to their antioxidant properties. Flavonoids scavenge free radicals, chelate redox-active metal ions and increase metallothionein expression (10–12). Metallothionein can protect cells from heavy-metal toxicity, but also exerts antioxidative activity.

In the defense against oxidative stress, the antioxidant enzyme (AOE) system of cells plays an important role. The antioxidant enzymes include the superoxide dismutases (SOD), catalase and glutathione peroxidases (GPx) (13–15). Catalase and GPx convert H2O2 to H2O and the SOD catalyze the dismutation of the superoxide radical anion. There are three forms of SOD in mammals, i.e., MnSOD located in mitochondria, CuZnSOD found mainly in the cytosol and an extracellular SOD localized in the extracellular fluid.

The expression of AOE can be regulated by oxidative stress (16–19). Induction of catalase mRNA expression could be elicited by H2O2 or hyperoxia in different mammalian cells (16–18,20). MnSOD expression was also found to be selectively induced by inflammatory mediators (21,22) as well as oxidative stress provoked either by H2O2 (17,23) or the redox-
cycling compound paraquat (24). However, little is known about the influence of flavonoids on the AOE system.

There are many reports relating to the reactivity of flavonoids with active oxygen species, thus emphasizing their antioxidant potential via a direct radical scavenging mechanism (10,25). On the other hand, flavonoids were found to induce the expression of enzymes such as metallothionein or NADPH quinone reductase, which may exert protective effects (9,12). We wanted to know whether flavonoids also can affect AOE expression, thus exerting indirect antioxidant activities. Therefore, we treated hepatoma cells with the flavonoid daidzein. We investigated the expression of different AOE and concurrently determined whether exposure of cells to daidzein protected against oxidative stress.

MATERIALS AND METHODS

Culture of hepatoma cells. Rat hepatoma H4IIIE cells were grown in Dulbecco’s modified Eagle’s medium (PAA Laboratories, Colbe, Germany) and human hepatoma HepG2 cells in RPMI (PAA Laboratories) supplemented with 10% fetal calf serum, penicillin (1 × 10^5 U/L) and streptomycin (100 mg/ml) in a humified atmosphere of 5% CO2 in air at 37°C. Cells were seeded in 6-well plates. Unless stated otherwise, cells were treated for 24 h with different concentrations of daidzein, which was dissolved in dimethyl sulfoxide. The final concentration of the solvent never exceeded 0.7%. Control cells were treated with dimethyl sulfoxide. For the experiments involving H2O2 exposure, cells were pretreated for 24 h with daidzein in different concentrations. Then the medium was changed to one containing 2 mmol/L H2O2, and cells were incubated for another 6 h before malondialdehyde (MDA) measurement.

Viability assay. To determine the viability of daidzein-treated cells, mitochondrial dehydrogenase activity was measured in the MTT-assay as described elsewhere (26). Additionally, the Neutral Red assay was performed according to Borensfreund and Puerner (27).

Lipid peroxidation. Hepatoma H4IIIE cells were pretreated with 200 or 300 μmol/L daidzein for 24 h followed by a 6-h treatment with 2 mmol/L H2O2. The respective controls received no treatment at all, were pretreated with medium and then with 2 mmol/L H2O2 or were given a daidzein pretreatment only. The amount of lipid peroxidation was determined by measuring the release of the breakdown product MDA into the medium. MDA was determined after reaction of cell culture supernatants with thiobarbituric acid and subsequent HPLC as previously described (24). Measurements were compared with untreated control cells. In further experiments, H4IIIE cells were exposed to different concentrations of daidzein only for 24 h and the amount of MDA released into the medium was determined.

RNA isolation and RNA analysis. Total RNA was isolated from cells using Trizol- Reagent (GIBCO BRL, Eggenstein, Germany). For Northern blot analysis, 5 μg of total RNA was resolved by electrophoresis in a 1% agarose, 2,25 mmol/L formaldehyde gel in a running buffer containing 20 mmol/L MOPS, pH 7, 0.5 mmol/L sodium acetate and 1 mmol/L EDTA. RNA was transferred to nylon membrane (Amersham, Arlington Height, IL) according to Maniatis et al. (28). Purified cDNAs were labeled with [α-32P] dCTP (111 TBq/mmol, Hartmann Analytic, Braunschweig, Germany) by random hexamer priming (Roche Diagnostics GMBH, Mannheim, Germany). Blots were prehybridized and hybridized with cDNAs for rat catalase, CuZnSOD and 18 S rRNA as described previously (24). Autoradiographs were made by exposing blots to X-ray film (Kodak XAR) with an intensifying screen at ~80°C. Blots were stripped and reprobed with the different cDNAs. Autoradiographs were analyzed by densitometric scanning using the Quantity One system from BIO RAD, Munich, Germany.

Polymerase chain reaction (PCR) after reverse transcription was performed for semiquantitative determination of MnSOD and glutathione peroxidase mRNA. Total RNA (1 μg) was transcribed into cDNA in a 25-μL final volume of reaction buffer (50 mmol/L Tris-HCL, 75 mmol/L KCl, 3 mmol/L MgCl2, 10 mmol/L dithiothreitol, 0.5 mmol/L of each dNTP) and 5 μmol/L oligo-d(T)16-primer, 1 μ RNase inhibitor and 2.5 μL MLV reverse transcriptase by incubation for 1 h at 42°C. The reaction was stopped by incubation at 99°C for 5 min. For rat MnSOD and Gpx, PCR was performed with 100 ng of the synthesized cDNA as described elsewhere (29,30). GAPDH PCR was performed with 100 ng synthesized cDNA according to El-Bahay et al. (31) The amplified PCR products were 394 bp for MnSod mRNA, 602 bp for Gpx mRNA and 450 bp for GAPDH mRNA. The conditions of PCR were set to be in the linear phase of amplification to allow for semiquantification of mRNA content. From each PCR reaction, 5 μL was electrophoresed in a 1% agarose gel in Tris-borate-EDTA buffer. The cDNA bands were visualized by UV illumination after the gels were stained with ethidium bromide. Gels were photographed and scanned densitometrically.

Transfection and reporter gene assays. To evaluate the transcriptional control of the catalase gene, deletion plasmids were constructed from a plasmid containing 1.1 kb of the rat catalase upstream sequences, were transfected separately to additionally control for transfection efficiency. (10,25). On the other hand, antioxidant potential via a direct radical scavenging mechanism (10,25). However, little is known about the influence of flavonoids on the AOE system.

Influence of daidzein on the AOE system (18). The different promoter-reporter constructs of the catalase upstream region in front of the luciferase expression vector (18). The different promoter-reporter constructs of the catalase upstream region in front of the luciferase vector are shown in Figure 4.

HepG2 cells were transiently transfected at 50–70% confluence with a modified calcium phosphate precipitation method followed by glycerol shock as previously described (32). Transfection cells were given fresh media 2–3 h before. Each well was cotransfected with 4 μg of a plasmid containing the luciferase reporter-construct and a plasmid containing the β-galactosidase gene driven by the SV40 promoter (Promega, Mannheim, Germany). After 24 h, transfection cells were treated with 50 or 100 μmol/L daidzein for another 24 h. For harvesting, the cells were washed twice with 1 × PBS and then lysed in cell culture lysis buffer (Promega). Protein concentration of the cellular extract was determined according to Bradford (33), using bovine serum albumin as standard. Cellular protein (10 μg) was used for luciferase assays. Luciferase activity was determined with a Multi-Biolumat LB 9505 C (Berthold, Bad Wildbad, Germany) using luciferase assay reagent according to the manufacturer’s protocol (Promega). With every transfection assay pGL3-basic and pGL3-control plasmid (Promega), containing SV 40 promoter and enhancer sequences, were transfected separately to additionally control for transfection efficiency. β-Galactosidase activity was determined with 0.1% 3-nitrophenyl β-D-galactopyranoside as substrate (28). To minimize variability due to the difference in transfection efficiency among plates, the activity of luciferase and chloramphenicol acetyltransferase was corrected on the basis of the activity of β-galactosidase.

Statistical methods. Results are expressed as means ± SEM. Significance was evaluated by ANOVA followed by Fisher’s Protected Least Significant Difference test. A probability of P < 0.05 was used as the criterion for significant difference.

RESULTS

Cytotoxicity. The cytotoxicity of daidzein was determined with two different assays (data not shown). In H4IIIE cells, the MTT assay was more sensitive than the Neutral Red assay. According to the MTT test, cytotoxicity was apparent with 200 μmol/L daidzein. There was a significant reduction in viable cells of ~24%. This increased to 30% with 300 μmol/L daidzein and to 54% with 500 μmol/L daidzein. With the Neutral Red assay, cytotoxicity of daidzein became apparent (40% dead cells) and was significant when cells were exposed to 500 μmol/L daidzein.

Effect of daidzein on AOE expression in H4IIIE cells. H4IIIE cells were grown to confluency and then treated with different concentrations of daidzein for 24 h. Figure 1A shows the Northern blots for catalase and CuZnSOD mRNA expression and Figure 1B the densitometric data relative to 18 S rRNA for catalase mRNA expression. In Figure 2A, the PCR for detection of MnSOD and Gpxs is depicted and in Figure 2B, the densitometric data relative to GAPDH mRNA expression. Daidzein treatment led to an increase of catalase mRNA expression (Fig. 1B). With 300 μmol/L daidzein, a
threelfold increase in catalase mRNA levels was detected. With 300 μmol/L daidzein, we found a 100% increase in catalase activity (data not shown, mean of two experiments). On the basis of the Neutral Red assay, daidzein was not cytotoxic in this concentration range, whereas the MTT assay indicated minor cytotoxicity of daidzein with 24–30% dead cells. CuZnSOD mRNA expression was not affected by daidzein exposure; thus densitometric data are not shown. There was a slight, but significant 20% decrease in MnSOD mRNA expression after exposure of cells to 300 μmol/L daidzein (Fig. 2B). An increase of ~40% in Gpx mRNA expression generally occurred at all concentrations of daidzein tested except 200 μmol/L, and this difference was significant only at the highest concentration of daidzein, 300 μmol/L (Fig. 2B).

**Influence of daidzein on the transcriptional activity of the catalase reporter gene constructs.** The promoter-reporter constructs for the catalase gene, as shown in Figure 3, were transiently transfected into Hep G2 cells. Cells were then treated with different concentrations of daidzein for 24 h. In all of the experiments, the nontreated promoter-reporter constructs showed basal activity compared with the pGL3-basic
plasmid. Figure 4 shows the luciferase activity of the transfected constructs after exposure to 50 or 100 μmol/L daidzein. In all promoter-reporter constructs tested, daidzein increased luciferase activity from one- to threefold. Interestingly, the promoter construct containing the whole 1.1-kb upstream region showed significantly lower inducible activity at a concentration of 50 μmol/L daidzein than the deletion constructs. The greatest increase in luciferase activity was observed for pLuc(-)2, which was about threefold after treatment of cells with 50 μmol/L daidzein.

Lipid peroxidation. To determine whether the increase in catalase mRNA expression levels by daidzein exposure may exert antioxidant effects, H4IIE cells were pretreated with different concentrations of daidzein followed by a treatment with 2 mmol/L H2O2. The extent of lipid peroxidation was determined by measuring the release of the breakdown product MDA into the medium (Fig. 5A). Treatment with 2 mmol/L H2O2 increased MDA levels only ~3 times compared with controls. Daidzein pretreated cells were not protected from H2O2. On the contrary, MDA levels increased ~4 times in these cells compared with controls and compared with the cells treated with H2O2 only. Treatment with 300 μmol/L daidzein alone increased MDA levels ~50% compared with controls. Further experiments (Fig. 5B) were performed to confirm this effect of daidzein on MDA levels. H4IIE cells were exposed to different concentrations of daidzein and the amount of MDA released into the medium was determined. An increase in MDA levels of ~50% was detected in H4IIE cell supernatants treated with 200 or 500 μmol/L daidzein.

DISCUSSION

The influence of phytoestrogens on the AOE system has not yet been investigated in detail. We treated rat liver hepatoma H4IIE cells with the isoflavonoid daidzein. H4IIE cells contained high levels of AOE and induction of AOE through oxidative stress has been shown previously in H4IIE cells (17); thus, they present a good model for these investigations.

The cytotoxicity of daidzein was measured with two different assays. The two highest concentrations of daidzein used for the evaluation of AOE expression levels were slightly cytotoxic in the MTT assay but nontoxic in the Neutral Red assay. The Neutral Red assay determines membrane uptake, whereas the MTT assay shows mitochondrial dehydrogenase activity (26,27). Obviously mitochondrial functions were impaired by daidzein treatment, before a marked damage of cell membranes and thus the destruction of the whole cell became evident.

Daidzein treatment of hepatoma cells dramatically increased catalase mRNA expression. Kameoka et al. (12) investigated catalase and CuZnSOD expression after daidzein treatment of CaCo2-cells and found no change in the expression pattern of either enzyme. This may have been due to the differences in cell lines because basal as well as inducible mRNA expression levels of AOE may differ depending on the cell type investigated (16,22,34). To further elucidate the mechanisms underlying the increase in catalase mRNA by daidzein, we performed transfection experiments. Transfection of hepatoma cells with reporter genes containing different parts of the rat catalase upstream region and subsequent exposure to daidzein always resulted in an increase in reporter gene activity of treated compared with nontreated cells. These results clearly indicate that the increase of catalase mRNA expression after exposure to daidzein
after daidzein treatment is due to transcriptional activation of the rat catalase promoter. Little is known about transcriptional activation of the catalase gene. In general, post-transcriptional processes were found to be important for regulation of catalase gene expression. A redox-sensitive RNA binding protein was described for rat lung, which acts by increasing RNA stability (19). In previous experiments, we found catalase gene induction to be regulated transcriptionally in hepatoma cells, whereas in hepatocytes, a translational mechanism was assumed (17).

Isoflavonoids interact with ER. For daidzein, a stronger interaction with the β than the α-subtype of the ER was shown (7,35). Similar in vitro interactions of phytoestrogens with human and rat ERα and ERβ have been reported, indicating that ER react similarly to phytoestrogens in the two species (7,35,36). Azevedo et al. (37) even found regulation of AOE to be dependent on sex steroids. Hepatoma cells such as those used for our experiments, however, are ER-negative, especially for later passages (38). Thus, a transcriptional activation of the catalase promoter via ER interactions can be excluded.

To determine whether the increase in catalase expression protects the cells from oxidative damage, cells were pretreated with daidzein and then exposed to H2O2. Unexpectedly, in addition to catalase, we also found an increase in GPx expression after daidzein exposure. Induction of the MnSOD mRNA expression after daidzein exposure. Induction of the MnSOD gene by tumor necrosis factor-α and oxidative stress has been described in several studies (49,50). Daidzein is an efficient inhibitor of casein kinase II, which phosphorylates nuclear oncogene products and a number of proteins involved in gene expression (51). It is possible that daidzein interferes with signal transduction pathways important for transcription of MnSOD.

In addition, CuZnSOD mRNA expression was not regulated coordinately with catalase and GPx expression. After daidzein exposure, no changes in CuZnSOD mRNA expression levels were detected. In previous studies, we found catalase gene induction to be different from other oxidants. This differential regulation of AOE expression is different from or daidzein exposure as well.

In conclusion, our experiments showed that daidzein affected the AOE expression pattern. Interestingly, these changes did not add to the antioxidant, but rather to the oxidant properties of daidzein. The possible effect of phytoestrogens on human health may also be linked to the AOE system; as such, our in vitro studies suggest possible mechanisms of daidzein action. Further investigations will show whether daidzein has similar effects on the AOE system in vivo. Therefore, when evaluating the antioxidant capacity of flavonoids their ability to modulate AOE expression should also be considered.

LITERATURE CITED

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