

Expression of antioxidant proteins in human intestinal Caco-2 cells treated with dietary flavonoids

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Abstract

Dietary flavonoids are known to scavenge free radicals but little information is available on their roles in antioxidant protein gene expression. The goal of this paper is to investigate the effect of flavonoid treatment on the antioxidant protein expression in human intestinal Caco-2 cells. The antioxidant proteins of interest were metallothionein (MT), catalase (CAT), and superoxide dismutase (SOD). Treatment of Caco-2 cells with 100 μ M genistein, biochanin A, daidzein or kaempferol significantly increased MT mRNA up to 15 fold. On the contrary, CAT mRNA level was not affected by various flavonoids. We also developed gel activity assays to determine the specific activities of CAT and Cu/Zn SOD in flavonoid-treated Caco-2 cells. Compared to the conventional spectrophotometric assays, the gel assays allow a separation of antioxidant activities of the enzymes from that of the flavonoids. CAT and Cu/Zn SOD were found not to be affected by 48-h treatment of 100 μ M dietary flavonoids (genistein, biochanin A, daidzein, flavone, quercetin, or kaempferol). In conclusion, the effects of flavonoids on antioxidant protein expression are structure- and gene-specific. When evaluating antioxidant capacity of flavonoids, their ability to modulate antioxidant protein expression should also be taken into consideration. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Antioxidant protein; Flavonoid; Caco-2 cells; Metallothionein; Catalase; Superoxide dismutase

1. Introduction

Animal and epidemiological studies have identified flavonoids as potential cancer chemopreventive agents [1–4]. The protective effects of flavonoids have been attributed, in large part, to their antioxidant properties [5]. Besides scavenging free radicals [6–9], flavonoids can also chelate redox-active metal ions [10] and/or modulate antioxidant proteins [11–13].

The latter property of the flavonoids is the focus of this paper.

Metallothionein (MT) expression can protect cells from heavy-metal toxicity. It was also shown to be an antioxidant protein [14,15]. Previous studies in our laboratory found that the MT level was increased in human intestinal Caco-2 cells treated with dietary isoflavone genistein or biochanin A [12,13]. On the contrary, flavonol quercetin decreased MT level in Caco-2 cells [12,13]. In this study, we measured the MT mRNA level in Caco-2 cells treated with various

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flavonoids including isoflavones, flavonols, and flavanols.

Antioxidant protection against oxidative damage in cells is achieved by the coordination among several different antioxidant proteins [16]. Therefore, we also investigated whether the levels of other antioxidant proteins, catalase (CAT) and superoxide dismutase (SOD), were affected by dietary flavonoids. It was reported that dietary administration of genistein in mice increased CAT activity in small intestine [11]. Besides Northern blot for mRNA determination, we performed gel activity assays to determine the levels of CAT and SOD in Caco-2 cells treated with various dietary flavonoids. Compared to the conventional spectrophotometric assays, the gel activity assays allow a separation of antioxidant activities of the enzymes from that of the flavonoids. This gel assay is thus more specific and also allows a better separation of Cu/Zn SOD and MnSOD activities.

Of the flavonoids studied in this paper, flavonols quercetin and kaempferol are widely present in common vegetables and fruits [17]. Isoflavones genistein, daidzein and biochanin A are present in high concentrations in soybean products [18]. Flavanols, catechin, epigallocatechin, and epigallocatechin gallate, are present mostly in the green tea [1]. Flavone is a synthetic flavonoid.

2. Materials and methods

2.1. Materials

All chemicals used were of analytical grade. Genistein, daidzein (Calbiochem), biochanin A, (\pm)-catechin, quercetin, flavone (Fluka), ($-$)-epigallocatechin, ($-$)-epigallocatechin gallate, and kaempferol (Sigma-Aldrich) all had greater than 95% purity (based on the information provided by the companies). All cell culture reagents with the exception of fetal bovine serum were from Gibco-BRL. Fetal bovine serum was from Hyclone laboratories. MT IIA was shown to be the most prevalent MT isoform in several cell lines [19]. The human MT IIA 63-mer oligonucleotide probe used in this study [19] was custom-synthesized by Gibco-BRL. The mouse cDNAs for CAT and Cu/Zn SOD were kind gifts of Dr. S. Koury (Department of Clinical Laboratory

Science, SUNY at Buffalo). The length of CAT and Cu/Zn SOD cDNA was 548 and 323 bp, respectively. The sequence of CAT and Cu/Zn SOD mouse cDNA had 86 and 84% homology, respectively, to the corresponding human sequence (BLAST Sequence Similarity Search, National Center for Biotechnology Information). For the purpose of normalization, the levels of 28S rRNA were also determined by a 40-mer oligonucleotide probe (Oncogene Science). In the activity gel assays, human erythrocyte CAT (1 and 3 units) (S-3556, Sigma-Aldrich) and human erythrocyte SOD (0.5, 1 and 2 units, mainly Cu/Zn SOD) (S-9636, Sigma-Aldrich) were used as the standard.

2.2. Flavonoid treatment

Human colon adenocarcinoma-derived Caco-2 cells were purchased from the American Type Culture Collection and passages 25–45 were used. These cells were cultured as previously described [20] and used at post-confluence stage as a model of human enterocytes [21]. All flavonoids, except daidzein, were stored as 100% ethanol stock solutions at concentrations of 10 or 25 mM depending on their solubility. Daidzein was first dissolved in dimethyl sulfoxide (DMSO) at 100 mM. The DMSO solution was then diluted with ethanol to a final daidzein concentration of 25 mM and used for treatment. Cells were seeded in six-well plates at 4×10^4 cells/cm². Seven days after seeding, flavonoids were added at a final concentration of 100 μ M after a medium change. The total ethanol concentration in the medium was always less than 1%. With the exception of the time-dependent study, cells were incubated for 24 h for Northern blot analysis and 48 h for gel activity assays. The control group was incubated with ethanol to exclude the potential vehicle effect.

2.3. Northern blot analysis

Total RNA was isolated by a modified Chomczynski and Sacchi method [13,22] for the experiment comparing various flavonoids. Further modification of the RNA isolation procedure was applied in the time-dependent genistein treatment experiment. Briefly, the second chloroform/isoamyl alcohol extraction was replaced by a guanidium thiocyanate extraction in the presence of sodium citrate buffer and β -

mercaptoethanol. The modification increased the apparent yield (as determined by OD_{260}) and purity of RNA (as determined by $OD_{260/280}$ ratio). Total RNA (10 μ g) was loaded in a 1% agarose gel in the presence of 1.1 M formaldehyde and 20 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS) (pH 7.0). ChromaTrack (Sigma-Aldrich) and 0.24–9.5 kb RNA ladder (Gibco-BRL) were used as molecular size markers. After electrophoresis, RNA was transferred to a nylon membrane (Nytran Membrane, Schleicher and Schuell) and UV cross-linked. The prehybridization and hybridization were performed following the protocol from Calbiochem-Oncogene Science. MT IIA and 28S rRNA probes were end-labeled with [γ - 32 P]ATP (NEN/DuPont) using RTS T4 kinase labeling system (Gibco-BRL). The specific activity was 0.476 and 0.877 μ Ci/pmol, respectively. CAT and Cu/Zn SOD probes were generated by using RTS rad prime DNA labeling system (Gibco-BRL) with [α - 32 P]dCTP (NEN/DuPont). The specific activity was 0.674 and 0.724 μ Ci/pmol, respectively. Hybridization solutions contained 0.3 μ Ci/ml MT IIA, 0.2 μ Ci/ml 28S rRNA, 0.8 μ Ci/ml CAT, or 0.9 μ Ci/ml Cu/Zn SOD probes. Visualization and quantification of the radiolabeled probes on the blots were accomplished by phosphor imager and molecular analysis software (Bio-Rad Laboratories). Representative lanes were shown in the figures but the quantification of the bands was performed with the results of triplicate samples.

2.4. Gel activity assay

Cells were washed three times with Dulbecco's phosphate buffer saline solution and scraped into 0.2–0.5 ml of 10 mM Tris (pH 7.0) buffer with 0.25 mM phenylmethylsulfonyl fluoride (PMSF). They were lysed by three cycles of freezing/thawing and then sonicated on ice with a microprobe (Vibra Cell, Sonics and Materials) for 18 s at 50% duty cycle with a pulse. No centrifugation was performed after the cell lysis. Cell lysates were kept on ice and CAT and SOD activity gel assays were performed within 48 h after the lysate preparation. The protein concentration of the lysate was determined using Bio-Rad protein assay with BSA as the standard. Native polyacrylamide gel electrophoresis was performed with 10% lower gel and 5% upper gel according to a modified

method of Davis [23]. Sucrose was included in the upper gel (7% for CAT and 21% for SOD gel) to prevent the diffusion of the bands. The electrophoresis was performed at 25 mA constant current for 2.5 h at 4°C. The gels were processed immediately after the run to determine the enzyme activities.

Different sets of gels were used for CAT and SOD activity determination. The CAT activity in the gel was determined following a modified method of Woodbury et al. [24]. In our studies, the gel was incubated in 1% ferric chloride and 1% potassium ferricyanide solution for 5 min. A single achromatic band on the green background demonstrated the presence of CAT activity. The SOD activity in the gel was determined following the method of Beauchamp and Fridovich [25]. Achromatic bands on the purple background demonstrated the presence of SOD activity. Cu/Zn SOD and Mn SOD bands could be differentiated on the activity gel [26]. The activities of CAT and SOD in the cell lysate were calculated by comparing sample band intensity with those of the standard enzymes. All experiments were performed in triplicate and all treatments were repeated at least once.

3. Results

3.1. Time-dependent effect of genistein on MT and CAT mRNA levels in Caco-2 cells.

Genistein was found to increase MT level in Caco-2 cells [12,13]. In this study, Northern blot analysis was performed to determine the time-dependent changes of MT mRNA levels in Caco-2 cells treated with 100 μ M genistein. Representative lanes of the triplicate are shown in Fig. 1A,B demonstrates the percentage increase of MT IIA mRNA over the control cells at each time point. Steady-state MT mRNA level began to increase within 4 h after the initiation of genistein treatment and reached a maximal 9-fold increase at 24 h. Although only 100 μ M genistein was used here as the treatment, an induction by genistein can be observed at 10 and 30 μ M as well [13].

Genistein increased CAT but not total SOD activities in the small intestine of mice fed with genistein-supplemented diet [11]. In our study, the CAT mRNA level was not affected by genistein at any time point (Fig 1A,B). We also performed blotting using the Cu/

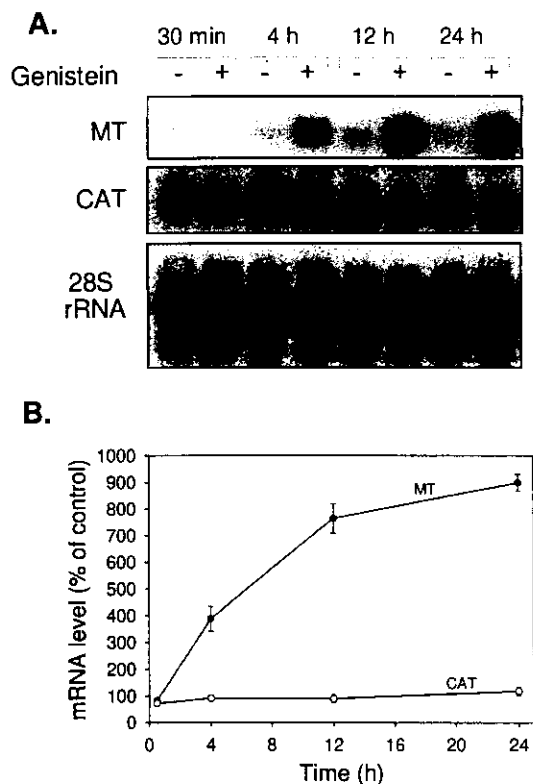


Fig. 1. Time-dependent effect of genistein on the levels of MT and CAT mRNA in Caco-2 cells. (A) Caco-2 cells were treated with 100 μ M genistein. RNA was isolated at different time points. Northern blot was performed with radiolabeled MT IIA, CAT and 28S rRNA probes. Representative lanes of the triplicate are shown. -, control cells. (B) The positive signals on the blots were quantitated by densitometry and normalized for differences by the amount of the 28S rRNA. Data are expressed as the percentage of control. Each point represents the mean \pm SEM of triplicate wells.

Zn SOD probe. There were no bands observed in the control or treatment groups at any time point likely due to low mRNA abundance. The same probe has been used to quantify Cu/Zn SOD expression in erythroblasts and positive signals were detected (results not shown).

3.2. Effect of other dietary flavonoids on MT and CAT mRNA levels in Caco-2 cells

Caco-2 cells were incubated with various flavonoids, including isoflavones (daidzein and biochanin A), flavonols (quercetin and kaempferol), and flavanols (catechin, epigallocatechin, and epigallocatechin

gallate) for 24 h at the concentration of 100 μ M. Results of Northern blot with the hMT IIA probe are shown in Fig. 2. Isoflavones biochanin A and daidzein increased the MT mRNA level by 15 and 5 fold, respectively. Kaempferol treatment also led to MT induction to a lesser degree. Catechin, epigallocatechin, or epigallocatechin gallate showed little effect on the MT mRNA level. Similar Northern blot results were observed when the total RNA from flavonoid-treated cells was purified using oligo-dT column to obtain mRNA (results not shown). The CAT mRNA level was not affected by any of the flavonoid treatments compared to the control (Fig. 2). Again, no bands for Cu/Zn SOD mRNA were detected.

3.3. Effect of dietary flavonoids on Cu/Zn SOD and CAT activities in Caco-2 cells

The expressions of CAT and Cu/Zn SOD in treated Caco-2 cells were further characterized at the protein level. CAT activities have been shown to change in the absence of mRNA changes [27]. We used the gel activity assay to measure CAT activities in Caco-2 cells treated with flavonoids and the results are shown in Fig. 3. Compared to the standard enzyme, the control sample had a band intensity of CAT around 2 units on the gel (Fig. 3, top). We calculated the CAT specific activity in Caco-2 cells to be around 15 units/mg protein. Neither tested flavonoids nor

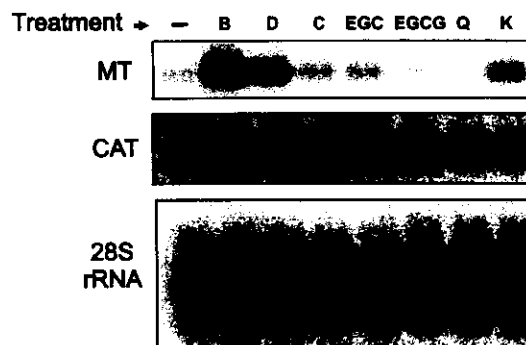


Fig. 2. Effect of biochanin A (B), daidzein (D), catechin (C), epigallocatechin (EGC), epigallocatechin gallate (EGCG), quercetin (Q), and kaempferol (K) on the levels of MT and CAT mRNA in Caco-2 cells. Caco-2 cells were treated with 100 μ M various flavonoids for 24 h. Northern blot was performed with radiolabeled MT IIA, CAT and 28S rRNA probes. Representative lanes of the triplicate are shown. -, control cells.

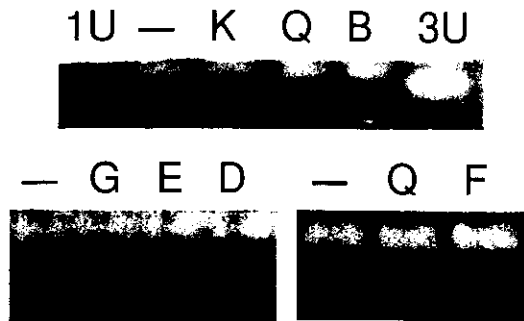


Fig. 3. Effect of kaempferol (K), quercetin (Q), biochanin A (B), genistein (G), daidzein (D), flavone (F), and 17β -estradiol (E) on CAT activity in Caco-2 cells. Caco-2 cells were treated with flavonoids and estradiol at $100\ \mu\text{M}$ for 48 h. Total protein was separated on native polyacrylamide gels and stained for CAT activity. CAT appeared as a single band close to the top of the resolving gel. There were no other bands on the gel. Representative lanes of the triplicate are shown. Human erythrocyte CAT (1 and 3 units) were run as the standards. –, control cells. The upper gel had $135\ \mu\text{g}$ of total protein per lane.

17β -estradiol changed CAT activity significantly in Caco-2 cells compared to the control. Results of the gel activity assay for Cu/Zn SOD are shown in Fig. 4. Compared to the standard enzyme on the gel, the amount of Cu/Zn SOD in the control sample on the gel was estimated to be approximately 1 unit (Fig. 4, top). We calculated the Cu/Zn SOD activity in Caco-2 cells to be around 5 units/mg protein. Neither tested flavonoids nor 17β -estradiol caused any significant changes in Cu/Zn SOD activity compared to the control. Mn SOD band should appear above the Cu/Zn SOD band [26] and we have observed MnSOD using rat liver and kidney lysates as well as purified protein (results not shown). Mn SOD was not observed in the control or treatment groups (Fig. 4). Our inability to detect Mn SOD is consistent with the known low abundance of this isoform in intestinal cells compared to Cu/Zn SOD [28].

4. Discussion

Reactive oxygen species can induce DNA damage and DNA damage has been implicated as an important factor for cancer development [29,30]. Antioxidant defense system protects the cells from these damages [31]. One goal of this study was to quantify antiox-

idant protein levels in human intestinal cells. Caco-2 cells, a widely-used model of human enterocytes, were shown by spectrophotometric assays to contain relatively low levels of CAT (6 ± 0.5 units/mg) and total SOD (6 ± 0.5 units/mg), which represented only 2 and 13% of those values for human liver [32]. Our studies, using the gel activity assay, also showed that Caco-2 cells contained low levels of CAT (≈ 15 units/mg) (Fig. 3) and Cu/Zn SOD (≈ 5 units/mg) (Fig. 4). Low Cu/Zn SOD activity is consistent with our inability to detect Cu/Zn SOD mRNA by Northern blot analysis.

Dietary flavonoids are free radical scavengers [6–9] but their regulatory roles on antioxidant proteins remain to be elucidated. We have reported an induction of antioxidant protein MT by genistein in a time-dependent fashion with a maximal increase observed at 48 h [13]. Here, a time-dependent increase in the MT mRNA level upon genistein treatment was observed (Fig. 1). This is consistent with the knowledge that MT is mainly regulated at the transcriptional level [33]. In addition to genistein, the MT protein level was significantly affected by biochanin A and kaempferol treatment in our previous study [12]. Here, biochanin A, daidzein, and kaempferol treat-

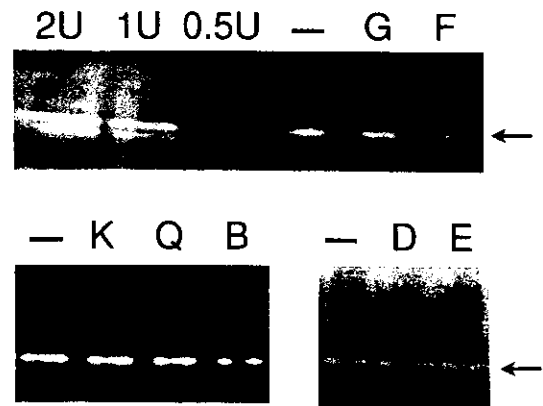


Fig. 4. Effect of genistein (G), flavone (F), kaempferol (K), quercetin (Q), biochanin A (B), daidzein (D), and 17β -estradiol (E) on SOD activity in Caco-2 cells. Caco-2 cells were treated with flavonoids and 17β -estradiol at $100\ \mu\text{M}$ for 48 h. Total protein was separated on native polyacrylamide gels and stained for SOD activity. There were no other bands on the gel besides the bands shown in the figure. Representative lanes of the triplicate are shown. Human erythrocyte SOD (0.5, 1 and 2 units) were run as standards. –, control cells. The upper gel had $200\ \mu\text{g}$ of total protein per lane.

ments also increased MT mRNA level (Fig. 2). The effects of these flavonoids on MT are thus likely mediated through transcriptional changes as well. Biochanin A was shown to be metabolized to genistein in breast tumor cells [34] and its observed MT-induction activity may also be due to a partial conversion of biochanin A to genistein in Caco-2 cells. In our previous study, catechin treatment did not affect the MT protein level [12]. Not surprisingly, all three members of the catechin family had little effect on MT mRNA level here (Fig. 2).

There seems to be a structural requirement of flavonoids for inducing MT. This structural specificity is likely to be independent of the free radical scavenging activity. Flavanols including catechin, epigallocatechin, and epigallocatechin gallate were known to have strong free-radical scavenging activities [35]. However, flavanols did not change the level of MT in our studies (Fig. 2) [12]. Although quercetin is known as a potent free-radical scavenger [7,36], it appears to decrease MT (Fig. 2) [12,13]. Interestingly, quercetin is structurally different from MT-inducing kaempferol by only one OH at 3'-position. In comparison, isoflavones especially biochanin A were found to be weak chemical chelators of free radicals [1,37,38] but they induce MT (Figs. 1 and 2) [12,13]. The apparent separation of free radical scavenging and MT protein induction activities of flavonoids have biological significance. To conclude on the net antioxidant potency of flavonoids, both of these properties should be taken into consideration.

Our finding of a lack of response in the Cu/Zn SOD activity to genistein treatment is consistent with those of Cai and Wei in their mice study [11]. In their study, however, genistein feeding gave rise to a 25% increase in the CAT activity of the small intestine based on the spectrophotometric assay. Our more specific gel activity assay did not detect such change. The CAT induction, even if present, is much smaller compared to the observed changes in MT in our cells. It appears that MT, compared to SOD and CAT, may be a more prominent mediator of the antioxidant activity of flavonoids in human intestinal cells. Future animal studies are needed to determine whether dietary feeding of genistein or other flavonoid can also increase intestinal MT levels as observed in Caco-2 cell studies.

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