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Dietary Flavonoids Interact with Trace Metals and Affect Metallothionein Level in Human Intestinal Cells

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ABSTRACT

Flavonoids are natural compounds found in food items of plant origin. The study examined systematically the interaction of structurally diverse dietary flavonoids with trace metal ions and the potential impact of dietary flavonoids on the function of intestinal cells. Spectrum analysis was first performed to determine flavonoid–metal interaction in the buffer. Among the flavonoids tested, genistein, biochanin-A, naringin, and naringenin did not interact with any metal ions tested. Members of the flavonol family, quercetin, rutin, kaempferol, flavanol, and catechin, were found to interact with Cu(II) and Fe(III). On prolonged exposure, quercetin also interacted with Mn(II). Quercetin at 1:1 ratio to Cu(II) completely blocked the Cu-dependent color formation from hematoxylin. When quercetin was added to the growth medium of cultured human intestinal cells, Caco-2, the level of metal binding antioxidant protein, metallothionein, decreased. The effect of quercetin on metallothionein was dose- and time-dependent. Genistein and biochanin A, on the contrary, increased the level of metallothionein. The interaction between dietary flavonoids and trace minerals and the effect of flavonoids on metallothionein level imply that flavonoids may affect metal homeostasis and cellular oxidative status in a structure-specific fashion.

Index Entries: Flavonoids; quercetin; genistein; copper; zinc; metallothionein; intestinal cells; human.

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INTRODUCTION

Flavonoids represent a family of plant-derived compounds found in many food items (Fig. 1). Because of the differences in structural features and dietary distribution, they can be further subdivided into several groups. Flavonols, quercetin, rutin, and kaempferol, are present in common vegetables and fruits at a concentration as high as 400 mg/kg *(1)*. Isoflavones, genistein, daidzein, and biochanin-A, are found mainly in the soybean and food items derived from soybeans at a concentration between 100 and 1000 mg/kg *(2–4)*. Flavanols, catechin and related compounds, are abundant in tea *(5)*. Flavanones, naringenin and related compounds, are present mainly in citrus fruits *(5)*. Overall, the total daily consumption of flavonoids could be over 1 g *(5)*. These compounds are known to be biologically active and interact with many substrates of the biological system (reviewed in *6,7*). The flavonoid–metal interaction is the main focus of this article.

Members of the flavonol group were shown to interact directly with trace elements. Quercetin and rutin were found to reduce Fenton reaction-induced lipid peroxidation by chelation of ferrous ion *(8)*. The iron–rutin complex was determined to have a 1:2 stoichiometry based on the spectrum analysis. In a different approach, quercetin and catechin were both shown to increase iron efflux from iron-loaded hepatocytes presumably by iron chelation in the incubation medium *(9)*. An interaction was also found between flavonol (quercetin, rutin) and copper *(10,11)*. The flavonoid–metal interaction was not reported for other flavonoids with different structural features, and the interactions with some other metals were also not evaluated. Thus, one objective of this article is to evaluate systematically the presence of a direct metal–flavonoids interaction among several classes of common dietary flavonoids and trace elements.

We are also interested in the role of these dietary flavonoids in the expression of metal binding proteins. Limited biological studies have been performed on this aspect and most of them have focused on catechin. Consumption of green or black tea as the sole source of fluids or including catechin in the diet did not consistently change the iron status and hematological parameter of rats *(12,13)*. Owing to the nature of dietary treatment, it is impossible to distinguish between the contribution of flavonoids and other components of the tea on the observed effect. The level of copper-dependent plasma protein, ceruloplasmin, was not changed by tea drinking in the animal study *(12,13)* or by an im injection of catechin as performed in a separate study *(14)*. Catechin was shown to inhibit the activity of copper-dependent lysyl oxidase in cell lysate prepared from chicken aortic tissue *(14)*.

To elucidate further the biological significance of flavonoids on trace mineral status, we evaluated the effect of flavonoids on the level of metallothionein in the intestinal cells. Metallothionein is a cysteine-rich small protein and is known to bind to varieties of metals, especially copper and

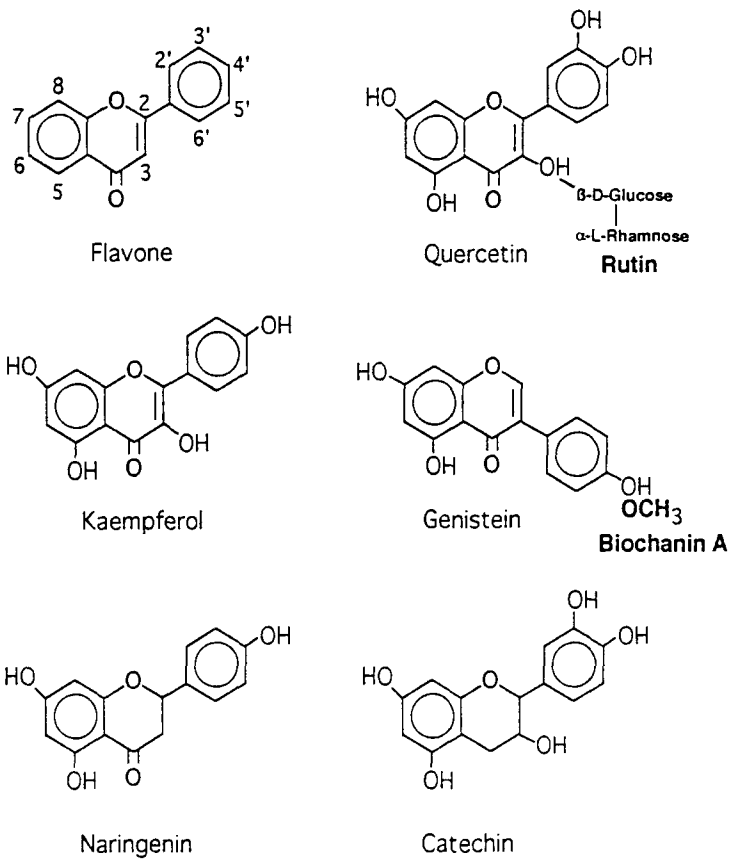

Fig. 1. Structure of various flavonoids.

zinc *(15,16)*. These two metals also affect the expression of metallothionein *(17–19)*. The biological function of metallothionein includes heavy metal detoxification *(20,21)*, intracellular metal transfer *(22–24)*, and cellular antioxidant *(25–27)*. Flavonoids that affect metallothionein expression could thus have secondary effects on metal status and other cellular functions. Human intestinal cells were chosen as the model system for the study because of the exposure of these cells under physiological conditions to a considerable amount of flavonoids from dietary intake.

MATERIALS AND METHODS

Spectrum Analysis of Flavonoids

UV/Vis spectra of various flavonoids were determined by a Beckman DU-65 Spectrophotometer at 750 nm/min of scanning. The spectra of flavonoids in the presence of various trace elements were also determined under the same condition. Dulbecco's phosphate-buffered saline (D-PBS), pH 7.2, was used as the buffer in the spectrum analysis. Some

analyses were repeated with Hank's Balanced Salt Solution, pH 7.4, and the results were identical. Flavonoids used were all of reagent-grade with >95% purity. $FeCl_2$, $FeCl_3$, $MnCl_2$ and $CuSO_4$ were used in the analysis as sources of metal ions and were also all of reagent-grade. Flavonoids solutions (dissolved in 100% ethanol) and metal solutions were prepared freshly for each reaction. Based on the previously reported 2:1 stoichiometry between flavonoids and metal *(8)*, the concentration of flavonoids was set at 10 μ*M* and that of metal was at 5 μ*M* for the spectrum analysis. All incubations were carried out at room temperature in the dark to prevent any light-induced reaction. The spectrum analysis was carried out in duplicate, and the results were identical.

Hematoxylin-Cu(II) Reaction

Hematoxylin was known to react with Cu(II) *(28)*. The reaction led to the formation of a complex, and the absorption peak was identified at 750 nm (S.-M. Kuo, unpublished observation). This property was used to test in vitro the ability of flavonoids to react with Cu(II). Absorption at 750 nm was measured in 10-s intervals at room temperature by a Beckman DU-65 Spectrophotometer. The reaction mixture included 50 μ*M* hematoxylin and 10 μ*M* $CuSO_4$ in the absence or presence of various concentrations of flavonoids. All reagents with the exception of hematoxylin stock solution were prepared freshly for the reactions, and D-PBS, pH 7.2, was used as the reaction buffer. Hematoxylin stock solution prepared in 100% ethanol was found to be stable at –20°C for several months.

Cell Culture and Treatment

Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD), and passages 21–33 were used for the experiments. These cells have been established as a model of human enterocytes *(29,30)* and were cultivated in high-glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS as described before *(31)*. Cells were grown on six-well plates at a seeding density of $2 \times 10^4/cm^2$. The growth media were changed at the third, fifth, and seventh days postseeding. The cells reached confluence around the sixth day. Dome formation can be observed after confluence reflecting epithelial cell differentiation. In the preliminary studies, we have observed a differentiation-dependent increase in cellular metallothionein activity (P. Leavitt and S.-M. Kuo, unpublished observation). To mimic the condition of mature enterocytes, all flavonoid treatments were carried out on the seventh day after the medium change. The cells were harvested on the ninth day (48 h of treatment). Flavonoids were dissolved in 100% ethanol and sterile-filtered as the stock solution. They were added to the growth medium with a final ethanol concentration of <0.8%. Ethanol at the concentration used did not affect the cell growth or metallothionein level.

Nevertheless, ethanol was routinely included in the control wells. Also, the concentration of ethanol in all wells of a set of experiment was maintained the same independent of the concentration of flavonoids.

Assay of Metallothionein

Metallothionein was measured by ^{109}Cd-binding assay following previously described procedures *(32,33)* with modifications. To harvest the cells from the six wells, cell monolayers were rinsed with D-PBS three times after the medium removal and scraped into 10 m*M* Tris buffer, pH 8.0 g with 1 μg/mL Cadmium. The cells were lysed by three cycles of freeze–thawing and a brief sonication. The lysate was then centrifuged at 8000*g* for 10 min, and the supernatant was used for assay immediately. The supernatant was found to be stable when frozen at –80°C for 1 wk. To avoid adherence of ^{109}Cd to the glass surface, plastic tubes and vials were used throughout the assay. The amount of metallothionein (MT) was calculated based on 7 cadmium per metallothionein. Triplicate wells were used for the determination of metallothionein, and duplicate assays were performed for each well.

Data Analysis

Statistical analysis was performed using Student's *t*-test or one-way analysis of variance (ANOVA). *Post hoc* multiple comparisons were made as indicated in the legends of Tables 4 and 5, and Figs. 4 and 5. *P*-value < 0.05 was considered statistically significant.

RESULTS

Flavonoid–Metal Interaction

If flavonoids indeed react with metals, the absorption spectrum of flavonoids would change after the addition of metal ions in the solution. The results in Table 1 were compiled from studies of eight different flavonoids (at 10 μ*M*) with Cu(II) (at 5 μ*M*). The concentrations of flavonoids and Cu(II) were chosen because previous studies had shown that rutin interacted with Cu(II) at 2:1 ratio in the phosphate buffer *(8)*. Of the flavonoids tested, flavone and members of the isoflavone and flavanone group by themselves did not show any changes in spectra after overnight incubation. In the presence of Cu(II), there were also no changes in the spectra at either time-point. Flavonol (quercetin, rutin, kaempferol) and flavanol (catechin) were found to react with Cu(II). The extent of absorption spectrum changes following the addition of Cu(II) was in the order of quercetin > kaempferol, catechin > rutin. Spectra of quercetin in the absence and presence of Cu(II) are shown in Fig. 2. The spectrum of quercetin changed immediately on the addition

Table 1
Changes of UV/Vis Absorption Spectra of Flavonoid and Cu(II)–Flavonoid Solutions in Phosphate Buffer[a]

Compound	Class	-OH position	no Cu(II)	Cu(II) added	
			Spectrum change with time [b]	Spectrum change at 0 hour [c]	Spectrum change 20 hr later [d]
Flavone	Flavone	none	-	-	-
Genistein	Isoflavone	5,7,4'	-	-	-
Biochanin A	Isoflavone	5,7,4'-OCH_3	-	-	-
Kaempferol	Flavonol	3,5,7,4'	-/+	+	+
Quercetin	Flavonol	3,5,7,3'4'	+	+	+
Rutin	Flavonol	3-O-Ru, 5,7,3',4'	-/+	+	+
Naringenin	Flavanone	5,7,4'	-	-	-
Catechin	Flavanol	3,5,7,3'4'	+	+	+

[a]D-PBS containing 10 μ*M* flavonoid only or 10 μ*M* flavonoid plus 5 μ*M* Cu(II) was analyzed by spectraphotometer at 0 h and after 20-h incubation at 25°C.
[b]Spectra of flavonoids at 0 h and after 20-h incubation were compared.
[c]Spectra of Cu(II)–flavonoid solution and flavonoid alone at 0 h were compared.
[d]Spectra of Cu(II)–flavonoid solution and flavonoid alone after 20-h incubation were compared.
+Changes in the peak position were observed.
–No changes in peaks were observed.
–/+Changes in the peak height were observed, but not the peak position.

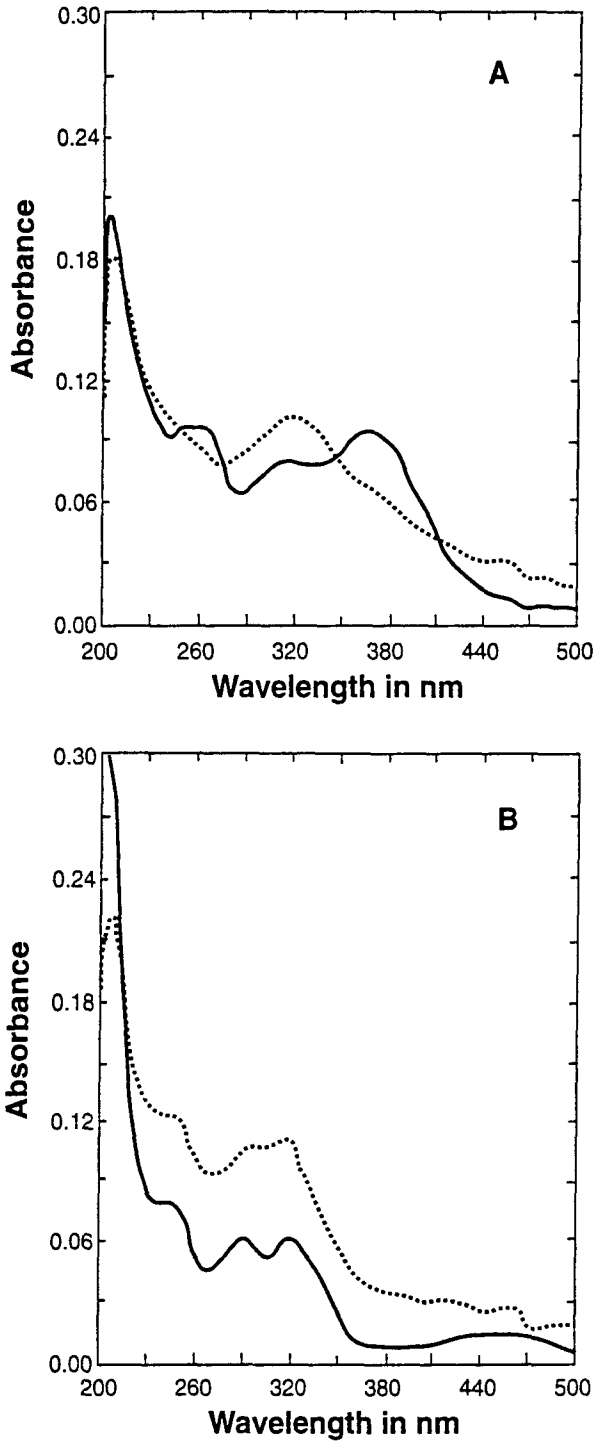

Fig. 2. UV/Vis spectrum of flavonoids in the absence and presence of Cu(II). **(A)** Quercetin with (__) and without Cu(II) (.....) immediately after preparation. **(B)** Quercetin with (__) and without Cu(II) (.....) after overnight incubation.

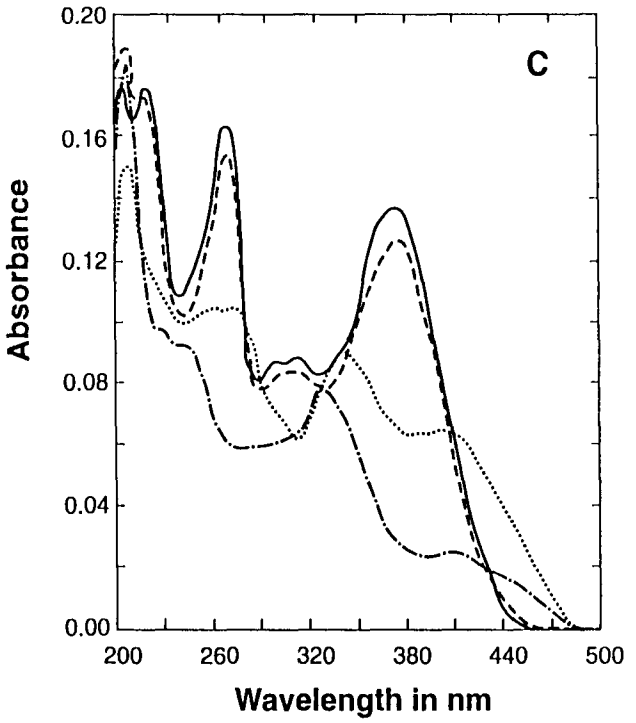

Fig. 2. *(continued)* **(C)** The spectra of kaempferol. The absorption spectrum of kaempferol was monitored in the absence of Cu(II) right after preparation (___) and after overnight incubation in the dark (_ _). The absorption spectrum of kaempferol in the presence of Cu(II) was also measured right after preparation (.....) and after overnight incubation in the dark (_ . _). The concentration of flavonoids was 10 μ*M* and that of Cu(II) was 5 μ*M* for all experiments.

of Cu(II) (Fig. 2A). The rapid quercetin-Cu(II) reaction probably reflected a direct Cu(II) chelation by quercetin. On prolonged incubation of quercetin in the buffer, the spectrum of quercetin itself changed because of its instability (comparing Fig. 2A with B). The spectra of quercetin with and without Cu(II) were also different after overnight incubation (Fig. 2B).

Rutin and kaempferol were found to be more stable compared to quercetin, since their spectra exhibited little changes after overnight incubation (Table 1). Nevertheless, on the addition of Cu(II), spectral changes can be observed for kaempferol, rutin, and catechin (Table 1). The Cu(II)-induced spectral changes were observed at both time-points, i.e. immediately after the addition and after overnight incubation. The results of kaempferol study are shown in Fig. 2C. Although kaempferol by itself showed little change of spectrum after overnight incubation, in the presence of Cu(II), the absorption spectrum changed after overnight incubation.

The interactions of flavone, genistein, quercetin, and rutin with other metal ions were also studied, and the results are summarized in

Table 2
Effects of Metal Ions on UV/Vis Absorption Spectra of Flavonoids[a]

Metal added	Fe(II)	Fe(III)	Mn(II)	Zn(II)	Ca(II)
Flavone	-[b]/-[c]	-/-	N.D.	-/-	N.D.
Genistein	-/-	-/-	-/-	-/-	N.D.
Quercetin	+/+	+/+	-/+	-/-	-/-
Rutin	+/+	+/+	N.D.	-/-	-/-

[a]D-PBS containing 10 μ*M* flavonoid only or 10 μ*M* flavonoid plus 5 μ*M* metal ion was analyzed by spectraphotometer at 0 h and after 20-h incubation at 25°C in the dark.

[b]Spectra of metal–flavonoid solution and flavonoid alone at 0 h were compared.

[c]Spectra of metal–flavonoid solution and flavonoid alone after 20-h incubation were compared.

+Changes in the peak position were observed.

-No changes in peaks were observed.

N.D., Not determined.

Table 2. Similar to the observation on Cu(II), quercetin and rutin, but not flavone and genistein, displayed spectral changes after the addition of Fe(II), Fe(III), or Mn(II). The spectrum of quercetin in the absence and presence of Fe(II), Fe (III), and Mn (II) is shown in Fig. 3. The spectrum of quercetin changed with the addition of Fe(II) and Fe (III) immediately (Fig. 3A), and further spectrum changes were also evident after the overnight incubation (Fig. 3B). Mn(II) did induce spectrum changes for quercetin, but the effect was less and was only observed after overnight incubation (Fig. 3C). To a lesser extent, spectral changes of rutin were also observed after the addition of Fe(II) and Fe(III) (spectra not shown).

The three flavonoid-interacting metals identified all have redox potential. We have also studied the interaction between flavonoids and two metal ions that have no redox potential, Zn(II) or Ca(II). Overall, no changes in the absorption spectrum of flavonoids were observed after the addition of zinc or calcium ions. We have also performed the flavonoid-Zn(II) and flavonoid-Ca(II) interaction at 37°C. Higher incubation temperature accelerated the degradation of quercetin and made other flavonoids unstable, but no interaction with Zn or Ca was observed.

If flavonoids can chelate metals as proposed above at high enough affinity, they should be able to prevent the interaction of metal with other chemicals. Hematoxylin was known to interact with various trace elements *(28)*. Hematoxylin–Cu(II) interaction was found to lead to the appearance of a new absorption peak at 750 nm (S.-M. Kuo, unpublished observation). We used this assay as an alternative method to test the ability of flavonoids to chelate Cu(II) in aqueous solution, and the results are shown in Table 3. Quercetin (10 μ*M*) at 1:1 concen-

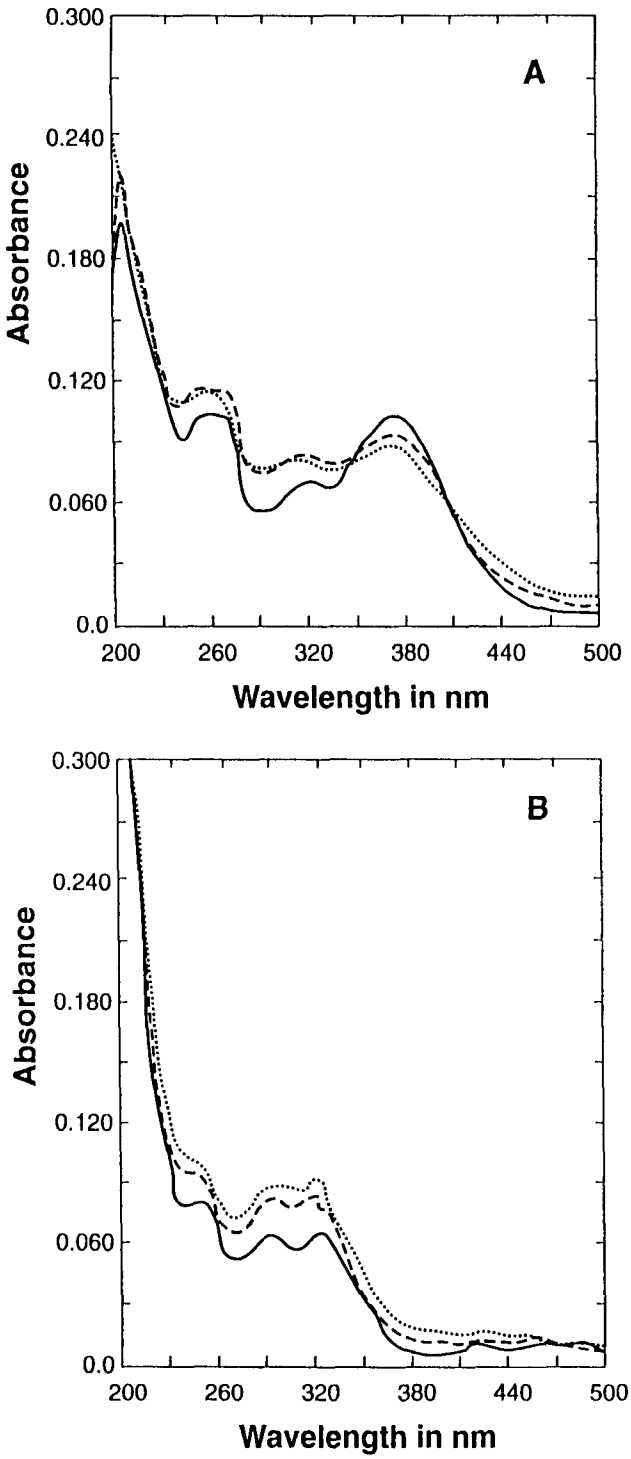

Fig. 3. UV/Vis spectrum of quercetin in the absence and presence of Fe(II), Fe(III), and Mn(II). **(A)** Quercetin (__) with Fe(II) (.....) and Fe(III) (_ _) right after preparation. **(B)** Quercetin (__) with Fe(II) (.....) and Fe(III) (_ _) after overnight incubation in the dark.

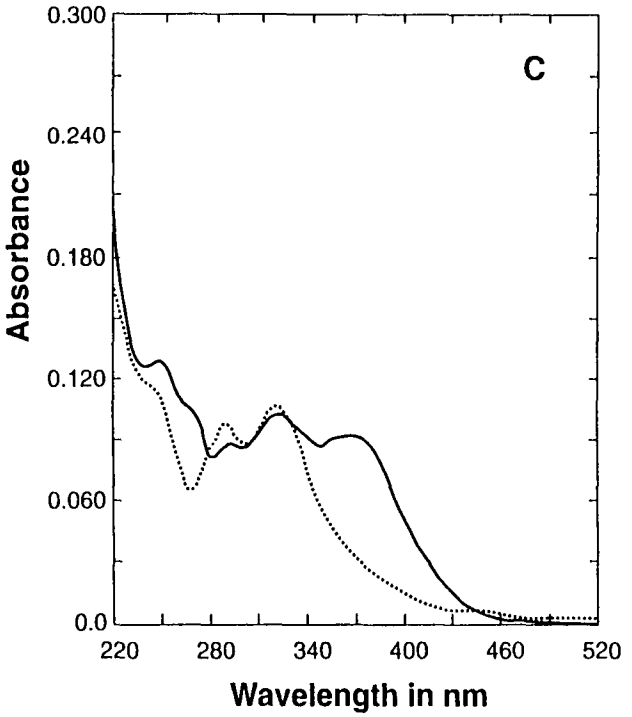

Fig. 3. *(continued)* **(C)** Quercetin (__) with Mn(II) (.....) after overnight incubation in the dark.

Table 3
Ability of Flavonoids to Inhibit Cu(II)–Hematoxylin Interaction in Phosphate Buffer[a]

FLAVONOIDS	10μM	30μM	50μM	100μM
		% of control ΔOD_{750}[b]		
Quercetin	0%	N.D.	N.D.	N.D.
Rutin	79.1±3.7%	51.0±1.8%	23.3±0.1%	1.4 ±1.3%
Genistein	N.D.	94.4±2.2%	89.5±3.0%	82.3±1.9%

[a]The absorption at 750 nm was measured at 10-s intervals. The reaction was carried out at 21°C in D-PBS containing 50 μ*M* hematoxylin and 10 μ*M* $CuSO_4$ in the absence or presence of various concentrations of flavonoids.

[b]The ability of flavonoids to inhibit the Cu(II)-induced color formation is expressed as % of control which was calculated by dividing ΔOD_{750}/min of the sample by that of the control. Results shown are mean ± SD of triplicate. Addition of ethanol (vehicle) to the reaction medium in the absence of flavonoids did not change ΔOD_{750}.

N.D., not determined.

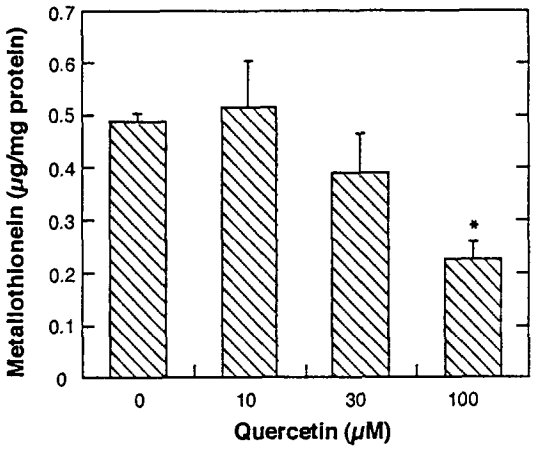

Fig. 4. Effect of different concentrations of quercetin on the level of metallothionein in human intestinal Caco-2 cells. Quercetin was added to the culture medium at various concentration, and cells were harvested after 48 h of incubation. Data shown are means ± SD of three or four wells. *Significant difference at $P < 0.05$ using post-hoc Bonferroni/Dunnett's multiple range test to compare to the control.

tration ratio to Cu(II) completely blocked the Cu(II)-induced absorbance increase at 750 nm. Quercetin could also arrest the color formation even if added at the same concentration ratio of 1:1 to Cu(II) after the Cu(II)-induced color reaction has started (results not shown). Rutin could also prevent the color formation, but was only effective at a higher concentration ratio (between 50 and 100 μ*M* of rutin to 10 μ*M* Cu[II]). The effect of genistein in blocking the color formation was found to be very limited even at a ratio of 10:1 (100 μ*M* genistein to 10 μ*M* Cu[II]).

Flavonoid and Metallothionein Level

In addition to direct metal chelation, flavonoids could also affect trace element homeostasis by affecting the expression of metal binding proteins. In this article, we studied the cellular level of metallothionein on 48 h of exposure to flavonoids. Confluent and differentiated human intestinal cells were used as the test system, because intestinal cells are expected to routinely be exposed to significant levels of flavonoids from food items ingested.

As shown in Fig. 4, when quercetin was included in the culture medium for 48 h, we observed a dose-dependent decrease in the level of metallothionein in intestinal cells as determined by ^{109}Cd binding assay. The effect started to appear at 30 μ*M* and was statistically significant at 100 μ*M* (47% of the control). The effect of quercetin was also found to be time-dependent (Fig. 5). Short-term incubation (1 h) did not lead to an

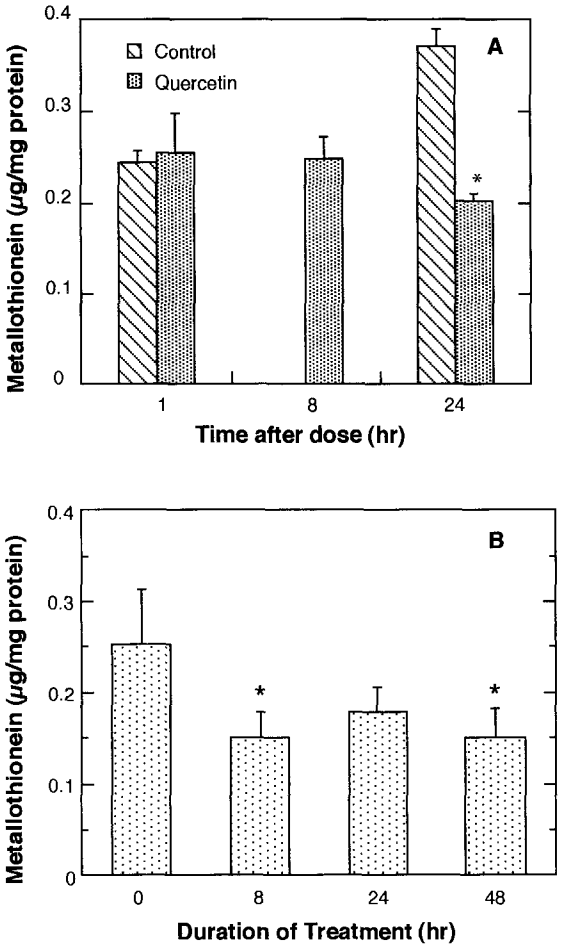

Fig. 5. Effect of treatment duration on the quercetin-induced decrease of metallothionein in human intestinal Caco-2 cells. **(A)** Quercetin was added at 100 µ*M* to a freshly changed growth medium at time 0, and cells were harvested at 1, 8, and 24 h after the quercetin addition. Control cells given ethanol were also harvested at 1 and 24 h. **(B)** Quercetin was added at 100 µ*M* to a freshly changed growth medium at time 0 and removed at 8 or 24 h after the addition (for the 8- and 24-h groups). Cells were then rinsed and replenished with fresh medium and returned to the incubator. All treated cells and control cells given ethanol at time 0 were harvested at 48 h. Data shown are means ± SD of three or four wells. *Significant difference at $P < 0.05$. (A) Student's *t*-test was used to compare between the control and the treatment group at each respective time-point. (B) Post-hoc Bonferroni/Dunnett's multiple-range test was used to compared to the control.

Table 4
Effect of Quercetin on the Upregulation of Metallothionein by Zinc in Human Intestinal Caco-2 Cells[a]

TREATMENT	METALLOTHIONEIN (μg/mg protein)[b]
Control	0.485± 0.016 a
100 μM Quercetin	0.226± 0.034 b
100 μM Zinc	4.478± 0.557 c
100 μM Quercetin + 100 μM Zinc	0.462± 0.063 a

[a]Zinc (100 μ*M*) was added to the culture medium in the presence or absence of 100 μ*M* quercetin. After 48 h of incubation, cells were harvested and used for the quantification of metallothionein.

[b]Data shown are means ± SD of three wells. a,b,c: significant difference at $P < 0.05$ based on post-hoc Games-Howell multiple-range test to take into consideration the large differences between means of samples.

immediate decrease in metallothionein level compared to the control (Fig. 5A). Direct addition of quercetin to the control cell lysate followed by 1 h of incubation also did not decrease metallothionein level (results not shown). The effect of quercetin on metallothionein level became significant after 24 h of treatment (54% of the control). The effect of quercetin persisted in the cells even after the removal of quercetin as shown in Fig. 5B. In this experiment, cells were all treated at the same time with 100 μ*M* quercetin, quercetin was then removed at different time-points, and all cells were harvested at 48 h. In a comparison between cells that were exposed to 100 μ*M* quercetin for 8 h only and for the entire 48 h, the levels of metallothionein were similarly decreased compared to the control.

Various metals, including copper and zinc, were shown to upregulate the expression of metallothionein *(17–19)*. Zinc treatment, as expected, was found to increase the level of metallothionein in human intestinal Caco-2 cells (Table 4). Interestingly, 100 μ*M* quercetin was able to prevent the induction of metallothionein by 100 μ*M* zinc in Caco-2 cells (Table 4).

The effects of other flavonoids on metallothionein level were studied, and the results are summarized in Table 5. Genistein, biochanin A, and kaempferol at 100 μ*M* all significantly increased the level of metallothionein. Flavone, rutin, and catechin were found not to affect the level of metallothionein.

Table 5
Effect of Various Flavonoids on the Level of Metallothionein in Human Intestinal Caco-2 Cell[a]

FLAVONOID	METALLOTHIONEIN (μg/mg protein)[b]
study 1	
Control	1.544±0.044
Flavone	1.386±0.054
Rutin	1.522±0.097
Genistein	3.804±0.190*
study 2	
Control	1.355± 0.035
Kaempferol	2.230± 0.113*
Catechin	1.130± 0.301
Biochanin A	1.805± 0.086*

[a]Individual flavonoid was included in the culture medium at a final concentration of 100 μ*M*. After 48 h of incubation, cells were harvested and used for the quantification of metallothionein.

[b]Data shown are means ± SD of three wells.

*Significant difference at $P < 0.05$ based on post-hoc Bonferroni/Dunnett's multiple range test compared to the control of each group.

DISCUSSION

Flavonoid–metal interaction was observed before, but a systemic analysis of structure–function relationship was not available. Because of the wide presence of flavonoids in the diet, the flavonoid–metal interaction could have profound impact on the trace element homeostasis in the body. In this study, we examined the four major classes of naturally present flavonoids, flavonol, flavanol, flavanone, and isoflavone.

The results from spectrum analysis here in general are consistent with the published observation from other groups. Quercetin, rutin, and catechin were all found to interact with Cu(II) or iron in other types of studies *(8–11)*, and they were found to interact with Cu(II), Fe (II), and Fe (III) in this study as judged by the observation of spectral changes (Tables 1 and 2 and Figs. 2 and 3). Quercetin and rutin also prevented Cu(II)–hematoxylin interaction (Table 3). Based on their ability to block completely the color formation, quercetin is at least 10 times more potent compared to rutin. The higher potency of quercetin–Cu(II) interaction in

this assay is consistent with the published result *(11)*. Based on the spectral analysis in this study, kaempferol also interacted with Cu(II) (Table 1 and Fig. 2C). Kaempferol has structure features similar to quercetin (Fig. 1) and thus is expected to react with metal ions similar to quercetin.

Information on interaction among flavone, isoflavone, flavanone, and trace elements was not available before, and in our studies, these compounds were found to be much more stable in nature and did not react with metals (Tables 1 and 2). Although genistein was found to prevented the Cu(II)-dependent color formation in the hematoxylin assay (Table 3), it was much less potent compared to quercetin and even rutin. Based on the concentration that exerted 20% inhibition, genistein is at least 10-fold less active compared to rutin (Table 3). Apparently, the flavonoid–metal interaction is structure-dependent. In comparing the structures among different flavonoids, we found that the 3-hydroxyl group is a consistent feature of all members of the flavonol and flavanol families (which react with trace elements). Rutin has a conjugated 3-hydroxyl, and the reactivity with metal was reduced compared to the structurally identical unconjugated quercetin.

Of all the flavonoids that were found to interact with Cu(II) and Fe(II) (or Fe[III]), the spectral changes were always observed immediately after the addition of the metal (Figs. 2 and 3). Thus, direct chelation by flavonoids at least represented one mechanism of interaction between flavonoid and metal ions. With prolonged incubation, there were likely other metal-induced changes in flavonoids. We have two sets of observation supporting this theory. The first set of observation is on the kaempferol–Cu(II) interaction. As shown in Fig. 2C, kaempferol itself showed little spectral changes in the absence of Cu(II) after overnight incubation in the buffer reflecting its relative stability. The addition of Cu(II) led to immediate spectral changes of kaempferol most likely reflecting chelation of Cu(II). Overnight incubation with Cu(II) led to more spectral changes of kaempferol that was not observed in the Cu-free sample. The overnight changes in the spectrum would have to be a consequence of Cu(II)-induced chemical reaction. The second set of observation supporting the presence of metal-induced chemical reaction of flavonoids comes from the study of quercetin and Mn(II). The addition of Mn(II) to quercetin solution did not induce immediate change of quercetin spectrum, but on overnight incubation, the spectrum of the quercetin sample with Mn(II) was different from that without Mn(II) (Fig. 3C). The time-dependent changes in quercetin structure supports the presence of the metal-induced reaction on prolonged incubation. Studies that have been performed previously on the metal–flavonoid interaction did not distinguish between the immediate and long-term effect *(8–11)*. Our observation reported here is, thus, new.

To elucidate further the biological significance of flavonoids on trace mineral status, we evaluated the effect of flavonoids on the level of metal

binding protein, metallothionein, in the Caco-2 cells. Metallothionein is a cysteine-rich small protein and is known to bind to varieties of metals, especially copper and zinc *(15,16)*. Flavonoids that affect metallothionein expression could thus have secondary effects on metal status. Flavonoids were found not to affect the expression of several metal-dependent proteins in previous animal studies as described in the Introduction *(12–14)*. Our study specifically examined the effect of flavonoid on the expression of metallothionein in human intestinal cells. As shown in Figs. 4 and 5, quercetin reduced the basal level of metallothionein in a dose- and time-dependent fashion. The reduction is not owing to an interference of quercetin with the ^{109}Cd binding assay, since a direct addition of quercetin into the control cell lysate did not affect the apparent metallothionein level. Also, the reduction probably was not a consequence of accelerated metallothionein degradation by metal stripping. Incubation of 100 μ*M* quercetin with intact cells (Fig. 5A) or cell lysate (results not shown) for 1 h did not change the level of metallothionein.

The level of metallothionein in varieties of cells is known to be induced by metals including zinc *(17–20)*. In Caco-2 cells, addition of zinc to the growth medium also led to an increase in the level of metallothionein (Table 4). Consistent with its effect on the basal expression of metallothionein, quercetin also decreased the Zn(II)-induced metallothionein expression in Caco-2 cells (Table 4).

In addition to quercetin, we also studied the effect of other flavonoids on the level of metallothionein in Caco-2 cells. Catechin and rutin did not affect the level of metallothionein under the same condition as used for quercetin (Table 5). Kaempferol and two isoflavonoids, genistein and biochanin-A, were actually found to increase the level of metallothionein under the same condition (Table 5). Thus, flavonoids seem to be capable of changing metallothionein level in two different directions. Although we have not identified the essential elements that control their ability to upregulate or downregulate metallothionein, it is possible that a particular flavonoid could have two opposing properties, and the observed effect is a net of the two. Flavonoids have been proposed to act as chemical antioxidants in various in vitro and in vivo studies *(34–37)*. Since metallothionein has been shown to have the properties of an antioxidant protein *(25–27)*, flavonoids that can induce metallothionein theoretically could also increase the cellular antioxidant potential secondarily. Again, this observation could have implications for the biological activity of flavonoids. Interestingly, genistein was shown to increase the activities of some other antioxidant enzymes in the small intestine of mice *(38)*. Future studies to understand the mechanisms of action of flavonoids are needed, since they will have implications on the physiological significance and potential toxicity of various structurally diverse dietary flavonoids.

These four groups of structural distinct flavonoids are also different in their dietary distribution as described in the Introduction. Their dif-

ferent properties as observed in this study could have implications on the physiological availability of trace elements from different food items that are rich in different flavonoids. Further tests of this possibility in the in vivo studies are needed.

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