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Metallothionein mRNA levels are influenced by dietary cyclodextrins in rats

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Abstract

The relationship between metallothionein mRNA levels and the amounts of copper and zinc in liver, kidney and small intestine by feeding dietary cyclodextrin was examined in growing Wistar rats. α-, β- or γ-cyclodextrin was fed at 50 g/kg of diet for a 7-days period (ad libitum). After feeding, the liver zinc of rats fed β-cyclodextrin was greater than those of rats fed the other three diets. Copper accumulated in kidney of rats fed α- or β-cyclodextrin. Copper content in the small intestine did not show any alterations among rats fed all kinds of diets. The cyclodextrin-supplemented diets were ineffective in zinc content in every organ. There was the greatest level of copper in serum of rats fed β-cyclodextrin, whereas the highest level of serum zinc was observed in rats fed γ-cyclodextrin diet. Northern blot analysis demonstrated that dietary β- and γ-cyclodextrins, but not α-cyclodextrin markedly increased the metallothionein mRNA in the liver, whereas small intestinal metallothionein mRNA levels were markedly decreased. Kidney metallothionien mRNA levels were raised appreciably by all dietary cyclodextrin intakes. Metallothionein gene expressions in liver, kidney and small intestine were not proportional to liver and serum copper or zinc levels in those tissues. These results suggest that regulation of the metallothionein mRNA levels may at least partly involved with the accumulation of metals as copper in liver and kidney of rats fed cyclodextrins.

Keywords: Cyclodextrin; Metallothionein mRNA; Copper; Zinc; Rat

1. Introduction

Cyclodextrins are cyclic oligosaccharides which have a polar surface and a hydrophobic cavity. Commercially available cyclodextrins having six, seven, or eight α-(1→4)-glucopyranose units are termed as α-, β-, or γ-cyclodextrin, respectively [1]. Cyclodextrins are widely used both in food and medical industries. β-Cyclodextrin shows a remarkable ability to form inclusion complexes with cholesterol and bile acids, together with fermentations in the large intestine [2,3]. α- and β-cyclodextrins are poorly hydrolyzed in the small intestine [4], but γ-cyclodextrin is rapidly and essentially completely digested [5,6]. It is now well established despite their unusual structure, they are fermented by the large intestinal microflora in experimental animals and in humans [2,3]. Fecal data in normal subjects demonstrate that β-cyclodextrin entering the colon is almost completely degraded by the flora. The absence of β-cyclodextrin in stools means that its ring structure has disappear, making α-(1→4)-linked glucose units available for fermentation [3]. However, the mechanism of absorption of intact β-cyclodextrin in the small intestine remains unclear. In our unpublished work, both α- and β-cyclodextrins led to particularly high acetic and propionic fermentations in the rat cecum, and lowered serum cholesterol in rats fed the cholesterol-free diet.

Metallothionein [7], a small cysteine-rich protein that tenaciously binds and exchanges specific metal ions, particularly copper and zinc, still lacks an unequivocally established biological function [8]. Metallothionein gene expression is regulated by homeostasis of essential minerals such as copper and zinc, and metallothionein mRNA levels in some tissues are a direct reflection of copper and zinc supplies [7,9]. In addition to metallothionein induction by metal ions, hepatic metallothionein synthesis is also under hormonal control [8,10,11]. This has fostered hypotheses that copper or zinc metabolism can be regulated by hormonal signals that affect metallothionein levels in tissues.

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Table 1
Composition of the diets

Ingredient	Basal (g/kg)	α-Cyclodextrin (g/kg)	β-Cyclodextrin (g/kg)	γ-Cyclodextrin (g/kg)
Casein[1]	250	250	250	250
Soybean oil[2]	70	70	70	70
Vitamin mixture[3] (AIN-93-VX)	10	10	10	10
Choline bitartrate[2]	2.5	2.5	2.5	2.5
Mineral mixture[3] (AIN-93G-MX)	35	35	35	35
Corn starch[4]	632.5	582.5	582.5	582.5
α-Cyclodextrin[5]	0	50	0	0
β-Cyclodextrin[5]	0	0	50	0
γ-Cyclodextrin[5]	0	0	0	50

[1] New Zealand Dairy Board, Wellington, New Zealand.
[2] Wako Pure Chemical Industries, Ltd., Osaka, Japan.
[3] P. G. Reeves, F. H. Neilsen, G. C. Fahey, AIN-93 Purifiled diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet, J. Nutr. 123 (1993) 1939–1951.
[4] Gelatinized; Chuo Shokuryo Co. Ltd., Inazawa, Aichi, Japan.
[5] Nihon Shokuhin Kako Co. Ltd., Tokyo, Japan.

Two major isoforms of metallothionein in the liver have been described in mammals, designated metallothionein-1 and metallothionein-2, that can be resolved by ion-exchange chromatography. Metallothionein-1 and metallothionein-2 are coordinately regulated in the rats and the proteins which are expressed in most organs are thought to be functionally equivalent [12]. In the present study, we tested to know the comparative effects on metallothionein gene expression in liver, kidney and small intestine in rats fed a diet supplemented with α-, β- or γ-cyclodextrin.

2. Methods and materials

2.1. Animals

This study complied with the Animal Experimental Guides according to the Committee of Experimental Animal Care at Hokkaido University. Male Wistar rats (Japan SLC Inc., Hamamatsu, Shizuoka, Japan) weighing about 75 g were housed individually in wire, stainless steel cages at a temperature 23 °C with a 12-hr cycle of light (8:00 AM-8:00 PM) and dark (8:00 PM-8:00 AM).

2.2. Diets

The compositions of the basal and experimental diets are described in Table 1. In the cyclodextrin diets, 50 g of α-, β- or γ-cyclodextrin (Nihon Shokuhin Kako Co. Ltd., Tokyo, Japan) per kg of diet was added to a basal diet at the expense of corn starch; 1) the basal diet, 2) α-cyclodextrin diet, 3) β-cyclodextrin diet, and 4) γ-cyclodextrin diet (Table 1). Rats were allowed free access to diets and distilled deionized water for 7 days. The body weight was measured daily. Residual and spilled portions of diet were carefully collected, dried and weighed, and the amount of food intake for all periods was corrected accordingly. At the end of the feeding period, the rats were killed by decapitation between 9:30 AM-10:30 AM. The liver, kidney and small intestine (jejunum, 10–20 cm from the pylori of the stomach) were immediately dissected, and weighed, and approximately 0.1 g of tissue from each sample was frozen in liquid nitrogen for RNA extraction and the rest was used to measure mineral contents. Blood was collected, and then serum was obtained by centrifugation at 3,000×*g* for 10 min and kept at −40°C until analysis.

2.3. Measurement of copper and zinc contents

An aliquot of each diet and freeze-dried tissues were prepared by wet-ashing with an acid mixture (16 N HNO_3 : 6 N $HClO_4$ = 3:1) [13]. The ashed sample was then diluted with 0.1 N HCl solution. Copper and zinc contents in each tissue and serum were estimated by the method of atomic absorption spectrophotometry (Atomic absorption spectrophotometer, HITACHI 170–50A, Hitachi, Tokyo, Japan) using certified reference standard [14].

2.4. Isolation of total RNA

Total RNA was isolated from the liver, kidney and small intestine by the Chomczynki and Sacchi method [15]. The tissue sample was homogenized with 1 ml of an Isogen solution (Nippon Gene Co. Ltd., Tokyo, Japan) in a homogenizer, and 0.2 ml of chloroform was added to the homogenate. The sample suspension was centrifuged at 20,000×*g* for 15 min at 4°C. The aqueous phase was transferred to a fresh tube, mixed with 0.5 ml of isopropanol, and centrifuged at 20,000×*g* for 10 min at 4°C. The resulting RNA pellet was dissolved in 1 ml of 75% ethanol, frozen for 30 min at −80°C and then reprecipitated at 20,000×*g* for 30 min at 4°C. The final RNA pellet was vacuum dried and

Table 2
Food intake, body weight gain and liver weight of rats fed a basal, and a diet supplemented with α-, β- or γ-cyclodextrin

	Basal	α-Cyclodextrin	β-Cyclodextrin	γ-Cyclodextrin
Initial body weight (g)	75.5 ± 1.9[1]	75.2 ± 1.3	75.3 ± 1.1	75.3 ± 0.9
Food intake (g/7 days)	84.6 ± 2.7[a]	82.7 ± 2.5[a]	73.4 ± 1.1[b]	84.0 ± 2.4[a]
Body weight gain (g/7 days)	48.6 ± 2.3	48.3 ± 2.8	45.8 ± 1.9	48.9 ± 2.1
Liver weight (g/kg of body wt)	43.9 ± 1.5	41.0 ± 0.8	41.1 ± 0.6	43.2 ± 1.0
Kidney weight (g/kg of body wt)	9.63 ± 0.20	9.28 ± 0.24	9.22 ± 0.28	9.05 ± 0.21

[1] Means ± SEM for six rats.
[a,b] Means within a horizontal line that do not share a common superscript letter were significantly different among groups ($P < 0.05$).

then dissolved in 50 μl of milli Q water. The concentration of RNA was measured from the absorbance at 260 nm (the ratio at 260/280 was between 1.6 and 1.9).

2.5. Extraction of mRNA

150 μg of total RNA was dissolved in 50 μl of milli Q water, and then 50 μl of 2×E solution [20 mM Tris buffer (pH 7.5) containing 2 mM EDTA and 0.2% SDS] and 100 μl of Oligotex were added. The mixture was left for 5 min at 65°C, before 20 μl of a 5 M NaCl solution were added. The solution was thoroughly mixed, incubated for 10 min at 37°C and then centrifuged at 20,000×*g* for 3 min at 4°C. The resulting precipitate was dissolved in 100 μl of a washing buffer and then centrifuged at 20,000×*g* for 3 min at 4°C. Milli Q water (100 μl) was added to suspend the precipite. The resulting solution was incubated for 5 min at 65°C and then centrifuged at 20,000×*g* for 3 min at 4°C. The resulting upper layer was mixed with 40 μl of a 2.5 M sodium acetate solution and 260 μl of 99.5% ethanol, and left for 30 min at −80°C. The mixture was then centrifuged at 20,000×*g* for 15 min at 4°C, before 500 μl of ice-cold 70% ethanol was added to the resulting precipitate and mixed. After centrifugation at 20,000×*g* for 15 min at 4°C, the precipitate was dried for 15 min. A Poly (A) RNA solution was obtained by adding 10 μl of milli Q water.

2.6. Northern blot analysis of metallothionein mRNA

The metallothionein mRNA levels were assessed by Northern blot analysis, using 3.5 μl of a poly (A) RNA solution extracted from each tissue. mRNA was denatured for 5 min at 65°C in a solution containing 5 μl of formamide, 1.5 μl of a 10×MOPS buffer (pH 7.0) and 2 μl of formaldehyde. mRNA was electrophoresed in a 1% agarose gel containing formaldehyde that had been prepared in a 1×MOPS buffer (pH 7.0), and electrophoresed for 90 min to 50 V essentially by the Thomas method [16]. After this electrophoresis, the RNA was transferred to a nylon membrane filter (Hybond-N^+) and cross-linked with a UV cross linker. Then, it was hybridized overnight at 68°C with cloned RNA probes complementary to each of the specific messenger RNAs from a reverse-transcription polymerase chain reaction (RT-PCR), as described in the next section. Membranes were washed three times with 2×citrate saline solution (SSC) (1×SSC = 0.15 mol sodium chloride/L · 15 mol sodium citrate/L)/0.1% SDS at room temperature for 5 min and then twice for 15 min with 0.1×SSC/0.1% SDS at 68°C. Afterwards, hybridization was made visible by exposing Fuji RX-U film and the bands were measured by densitometry. A similar procedure was done to study the β-actin mRNA expression.

2.7. Metallothionein-1 probe preparation

The metallothionein-1 cDNA fragment inserted between the SmaI and PstI sites of pUC118 plasmid was kindly provided by Dr. M. Kurasaki (Graduate School of Environmental Earth Science, Hokkaido University, Sapporo). pUC118 was digested with BglII and EcoRI, and then inserted between BamHI and EcoRI sites of pBluescript SKII + plasmid. pBluescript SKII+ plasmid was linearized with XbaI and used as a template for Digoxigenin-labeled RNA (DIG-RNA) probes. DIG-RNA probes were made by using DIG RNA Labeling Kit (Roche Molecular Biochemicals, France). The DIG-RNA probes used for metallothionein-1 and β-actin were used in the Northern blot hybridization [17].

2.8. Statistical analysis

Results are presented as means ± SEM, and significant differences among diet groups were determined by Duncan's multiple range test ($P < 0.05$) [18].

3. Results

3.1. Food intake, body weight gain, liver weight and kidney weight

Food intake was lower in the β-cyclodextrin group than in the basal group whereas, the addition of either α- or γ-cyclodextrin had no effect on food intake. Body weight gain was not different among the four groups. Liver and kidney weights were not significantly affected by dietary cyclodextrins (Table 2).

Table 3
Copper and zinc contents in liver, kidney, small intestine and serum, and metallothionein mRNA levels in each organ of rats fed a basal, and a diet supplemented with α-, β- or γ-cyclodextrin

	Basal	α-Cyclodextrin	β-Cyclodextrin	γ-Cyclodextrin
Liver				
Copper (μmol/g of dry wt)	0.258 ± 0.028[1b]	0.308 ± 0.038[ab]	0.371 ± 0.017[a]	0.336 ± 0.048[ab]
Zinc (μmol/g of dry wt)	1.18 ± 0.03	1.20 ± 0.02	1.19 ± 0.03	1.18 ± 0.02
Metallothionein mRNA (arbitrary unit)	100 ± 17[ab]	151 ± 16[b]	438 ± 75[a]	630 ± 83[a]
Kidney				
Copper (μmol/g of dry wt)	0.465 ± 0.027[b]	0.624 ± 0.062[a]	0.646 ± 0.055[a]	0.531 ± 0.021[ab]
Zinc (μmol/g of dry wt)	1.18 ± 0.13	1.21 ± 0.01	1.20 ± 0.03	1.20 ± 0.02
Metallothionein mRNA (arbitrary unit)	100 ± 9[b]	632 ± 205[a]	375 ± 27[a]	372 ± 39[a]
Small intestine				
Copper (μmol/g of dry wt)	0.159 ± 0.009	0.164 ± 0.017	0.152 ± 0.007	0.166 ± 0.004
Zinc (μmol/g of dry wt)	1.23 ± 0.02	1.21 ± 0.02	1.23 ± 0.02	1.21 ± 0.02
Metallothionein mRNA (arbitrary unit)	100 ± 27[a]	55.3 ± 17.9[ab]	28.3 ± 3.9[b]	36.5 ± 3.5[b]
Serum				
copper (μmol/L)	12.1 ± 1.01[b]	12.3 ± 0.7[b]	14.7 ± 0.2[a]	14.1 ± 0.2[a]
zinc (μmol/L)	198 ± 0.6[b]	21.7 ± 1.0[ab]	20.6 ± 0.6[b]	23.8 ± 0.8[a]

[1] Means ± SEM for six rats.
[2] The ratio of the metallothionein mRNA/β-actin mRNA intensities was used to evaluate the relative levels and is shown as a percentage of the level in the basal group.
[a,b] Means within a horizontal line that do not share a common superscript letter were significantly different among groups ($P < 0.05$).

3.2. Copper and zinc in organs

The copper and zinc contents in the liver, kidney and small intestine of rats are shown in Table 3. The copper contents of liver were significantly higher in the β-cyclodextrin group as compared to the basal group. The kidney copper contents in the α- and β-cyclodextrin groups were also higher than those in the basal group. The copper contents in the small intestine, and the contents of zinc in three organs were not significantly influenced by the cyclodextrin diets.

3.3. Serum copper and zinc

The concentrations of copper and zinc in the serum of rats fed the basal and the cyclodextrin diets are shown in Table 3. Serum copper was higher in the rats fed the β-cyclodextrin diet than that fed the basal or α-cyclodextrin diet. The concentration of zinc in serum of rats fed with the γ-cyclodextrin was higher than that of the rats fed with the basal or β-cyclodextrin diet.

3.4. Metallothionein mRNA levels

The effect of dietary cyclodextrins on metallothionein mRNA levels in the liver, kidney and small intestine is shown in Figure 1 and Table 3. Hepatic metallothionein mRNA levels in rats fed the β- or γ-cyclodextrin diet were higher than those in rats fed the basal or α-cyclodextrin diet. The kidney metallothionein mRNA levels for all cyclodextrin groups were higher than those from rats fed the basal diet. Small intestinal metallothionein mRNA levels in the β- or γ-cyclodextrin group were lower than those of the basal and α-cyclodextrin groups.

4. Discussion

Previously, we found that the induction of hepatic metallothionein mRNA levels in rats has been demonstrated to occur on β- or γ-cyclodextrin diet [19]. From the present results, this is the first time report concerning β- and γ-cyclodextrins that metallothionein mRNA levels were decreased in small intestine (Table 3). In addition, the expression of the kidney metallothionein gene is significantly stimulated by dietary α-, β- or γ-cyclodextrin. That is, metallothionein mRNA levels for the rats fed α-, β- or γ-cyclodextrin were 6.3-, 3.8- or 3.7-folds higher respectively, than that of the basal diet (Table 3). Four known isoforms of metallothionein exist in tissues. Metallothionein-1 and -2 isoforms have a ubiquitous tissue distribution with particular abundance in liver, kidney and intestine [17]. The amount of metallothionein-1 is more predominant in the tissues than metallothionein-2 [20]. We were not enable to absolutely differentiate metallothionein-1 and -2 mRNAs because Northern blot analysis of metallothionein mRNA carried out in this study was not adequate to estimate metallothionein-1 mRNA alone. As the sensitivity of this procedure is restricted, it will be essential to apply another more sensitive and specific method for accurately measuring the metallothionein-1 mRNA levels. Hence, the mechanism by which dietary cyclodextrins causes changes in metallothionein mRNA levels in some organs remains obscure. Cousins et al. [21] reported that metallothionein gene synthesis is also under hormonal control. Receiving earliest attention as regulators of metallothionein expression, hormones (e.g. glucocorticoids) are responsible in part for acting through intracellular signaling mechanisms to induce expression [11,12]. As coordinate hormonal control would

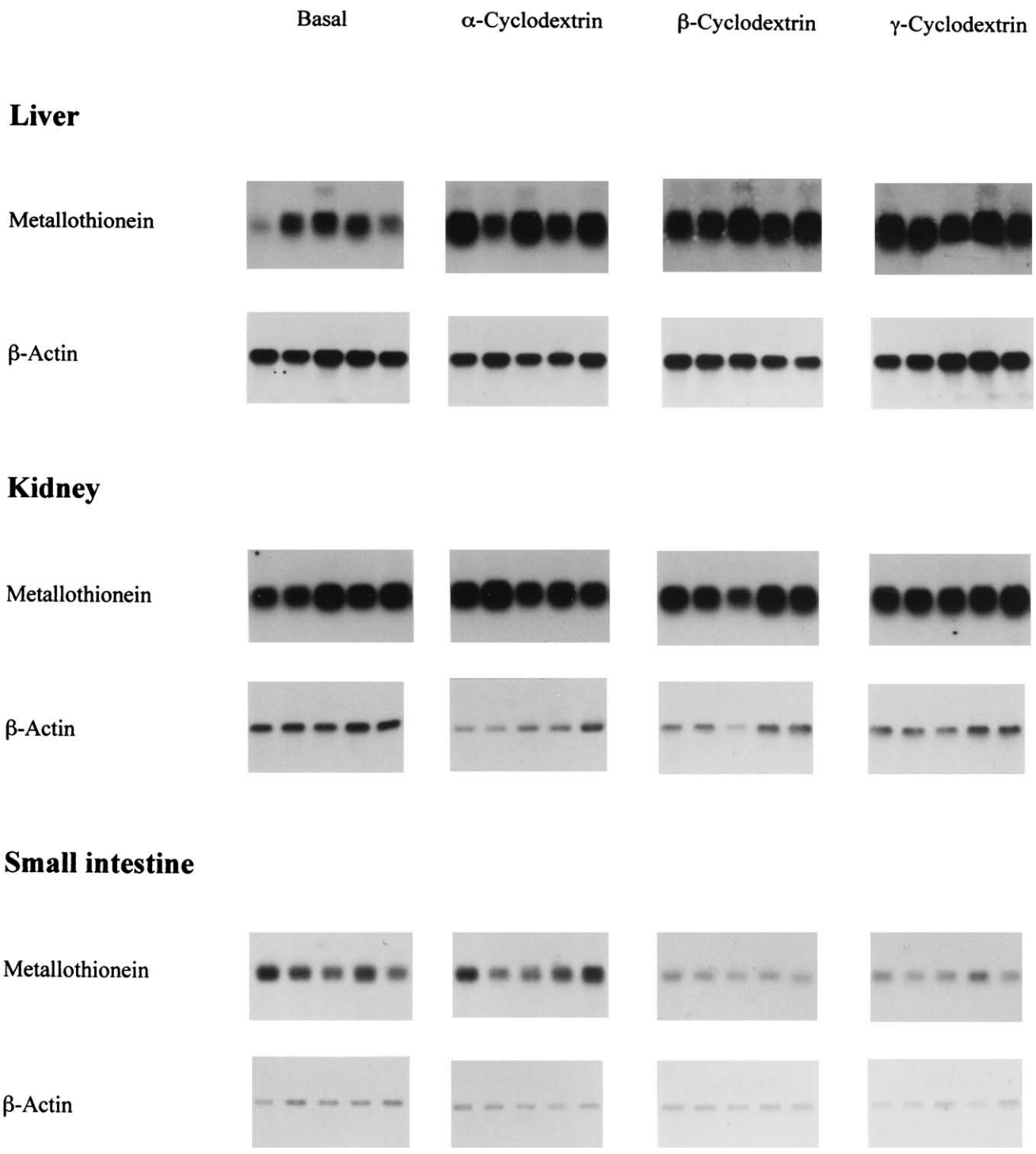

Figure 1. Agarose gel electrophoresis of liver, kidney and small intestinal metallothionein and β-actin mRNAs of rats fed the basal, α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), and γ-cyclodextrin (γ-CD).

be expected to cause an alteration in metallothionein mRNA levels. But the hormonal effect of oral administration of cyclodextrins has not yet been reported. Therefore, the future study will be designed to determine whether metallothionein mRNA levels were mediated by changes in serum corticosterone to assess the amelioration of this alteration by the feeding of α-, β- or γ-cyclodextrin. Also, the finding that metallothionein mRNA levels were largely elevated in certain organs when cyclodextrins were administered might be related to copper or zinc levels in nucleus. Moreover, determining metallothionein protein levels in the tissues may provide further information regarding the expression.

Related to the three-dimensional structure of cyclodextrins, they show a relatively hydrophobic cavity and hydro-

philic exterior, which enable them to form inclusion complexes with lipophilic components. These non-covalent inclusion complexes can improve the aqueous solubility, chemical and physical stability and thus the bio-availabity of the sequester molecule [1]. In recent work, Vanhaeckea et al. [22] observed the adjuvant effect of a derivative of the naturally occurring β-cyclodextrin, namely hydroxypropyl-β-cyclodextrin, as for thyroid hormones. Consequently, examination of cyclodextrin concentrations in each tissue may supply an explanation of the possible mechanisms in induction of tissue metallothionein mRNA.

As shown in Table 3, dietary β- or γ-cyclodextrin stimulates hepatic metallothionein mRNA levels in rats. Furthermore, it was found that kidney and small intestinal metallothionein mRNA levels were affected. We are attempting to elucidate the reason for the changes in metallothionein mRNA levels in some organs induced by dietary cyclodextrins. In certain tissues and cell types of normal rats, copper and zinc are predominant metals bound to metallothionein. Many reports suggested that metallothionein expression is related to copper or zinc accumulation in certain organs [8,12,17,23,24]. There was no relationship between metallothionein mRNA levels and copper or zinc, therefore, the candidate for increment of metallothionein mRNA levels might be in part due to the accumulation of copper or zinc in tissues for this study. Metallothionein expression is driven by a number of physical mediators through several response elements in the metallothionein promoter. Transcriptional regulation of the metallothionein gene by metals is conferred by metal response elements in the metallothionein promoter [25]. Because metal response elements are present within the promoter sequence of the metallothionein gene [25,26], it is likely that there is interaction between copper or zinc and a transcription factor, which then binds to this DNA sequence and initiates transcription. Although cyclodextrin intakes caused a little effect in the levels of copper and zinc in serum (Table 3), serum copper was the greatest in rats fed β-cyclodextrin, and serum zinc was the greatest in rats fed γ-cyclodextrin (Table 3). These results show that each dietary cyclodextrin might alter the absorption rate of copper or zinc from intestine.

In fact cyclodextrins can form inclusion complexes with a variety of molecule including metal ions. Since cyclodextrins have only OH groups, they are able to include metal ions at basic pH. In aqueous alkaline solution, the unprotonated 2- and 3-OH can coordinate copper (II) ions. Each copper (II) ion is bound to the cyclodextrins through a 2-OH and a 3-OH of an adjacent ring. Other similar metal complexes with manganese (II), zinc (II) and lead (II) have been described [27]. In the body of rats at normal condition, the pH is always maintained at neutral. Therefore, cyclodextrin molecules probably cannot form inclusion complex with transition metals as copper and zinc. In physical chemistry field, numerous reports related to cyclodextrins having transition metal center fixed at the cavity entrance has been found, whereas the studies aimed at this phenomenon in nutrition and/or biological system are relatively rare. Consequently, we cannot predict whether cyclodextrin can form inclusion complex with copper or zinc *in vivo*. However, criticizing from available data, we could not signify which is the direction of copper status because it has not yet been established whether ceruloplasmin used as an indication of copper status [28] is implicated in copper mobilization in rats fed cyclodextrins. Therefore, these metallothionein mRNA profiles of specific tissues might be due to the significant changes in nuclear copper or zinc levels.

In conclusion, we investigated the influence of dietary cyclodextrins on metallothionein gene expression and their effectivenesses in the amount of copper and zinc in various tissues. Our results demonstrate that β- and γ-cyclodextrins promoted hepatic metallothionein mRNA levels, but restrained metallothionein mRNA levels in kidney when compared with the action of basal or α-cyclodextrin diet. Further, the suppressive effect of metallothionein gene expression in the small intestine can be related to feeding rats with all kinds of cyclodextrin. The alteration in metallothionein gene expression may at least partly have been related with the results of the changes in copper concentrations in liver and kidney.

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