PREVENTION BY METALLOTHIONEIN OF CADMIUM-INDUCED INHIBITION OF VITAMIN D ACTIVATION REACTION IN KIDNEY

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1. Introduction

Several observations have indicated preferential accumulation of cadmium in the liver and kidney when animals were administered the metal by either peroral or parenteral route [1,2]. Most of the cadmium thus accumulated in the organs exists bound to a low molecular weight protein [3,4]. The cadmium binding protein has several characteristics which suggest the protein to be closely related to metallothionein originally detected in the horse kidney [5]. It is also remarkable that the protein appeared to be induced rapidly in the liver upon cadmium loads [6]. However, the biological significance of those observations has not been established yet.

It is generally accepted that vitamin D must be hydroxylated on C-25 position in the liver and subsequently on C-1 position in the kidney before it can function on bone and intestine [7].

We found that 1-hydroxylation reaction of 25-OH-D_3 in chick kidney mitochondria was completely inhibited in the presence of 0.1 mM of cadmium in vitro [8], but that the 1-hydroxylation reaction occurred in vivo even in the animals loaded with large amounts of oral cadmium [14,15].

In this study, we intend to clarify the significance of the discrepancy between the in vivo and the in vitro experiment. Evidence is shown that metallothionein in the kidney is involved in the prevention observed in vivo against the cadmium-induced inhibition of the vitamin D activation.

2. Materials and methods

2.1. *Animals and preparations of mitochondria*

One-day old white Leghorn cockerel chicks were maintained on a vitamin D deficient purified soy protein diet [9] containing 1.2% calcium for 2 weeks and then on the same vitamin D deficient diet containing 0.1% calcium for another 2 weeks. After the feeding for 4 weeks in total, the chicks were killed by decapitation. Kidney mitochondria free from heavy contamination of calcium were prepared as described earlier [8]. Mitochondrial protein was assayed by the method of Gornall et al. [10].

Abbreviations used: 25-OH-D_3, 25-hydroxycholecalciferol; 1,25-$(OH)_2$-D_3, 1,25-dihydroxycholecalciferol; 24,25-$(OH)_2$-D_3, 24,25-dihydroxycholecalciferol.

2.2. *Preparation and characterization of cadmium binding protein from kidney*

Cadmium chloride (3 mg/kg body weight) was injected subcutaneously to three male rabbits (3 kg) once a week for 3 weeks. Two months after the last injection the rabbits were killed, and the kidneys were removed, rinsed and homogenized in 60 ml of 0.05 M phosphate buffer, pH 7.0, in a chilled Waring blender. The homogenate was centrifuged at 100 000 *g* for 60 min at 4°C. The supernatant, adjusted to pH 8.6 by the addition of 1 N NaOH, was applied to a column of Sephadex G 75 (5 X 85 cm) equilibrated with 0.01 M Tris–HCl buffer, pH 8.6, containing 0.01% NaN_3. The elution was performed with the same buffer. The cadmium-containing protein, recovered in a single peak, concentrated by ultrafiltration on Diafilter UM-2 (Amicon) and desalted through a column of Sephadex G 25. The desalted material was further purified by a column of DEAE-Sephadex A 25 equilibrated with 0.05 M Tris–HCl buffer, pH 8.6. The column was eluted with a linear Tris–HCl gradient, obtained by running 1 litre of 0.25 M Tris–HCl buffer, pH 8.6, from a reservoir chamber into a mixing chamber containing 1 litre of 0.05 M Tris–HCl buffer, pH 8.6. The purified cadmium binding protein was hydrolyzed with 6 N HCl at 110°C for 24, 48 and 72 hr, and its amino acid composition was analyzed by means of an amino acid analyzer (JEOL 6AH).

2.3. *Determination of in vitro production of 1,25-(OH)$_2$-D$_3$ and 24,25-(OH)$_2$-D$_3$ from 25-OH-D$_3$*

In a 25 ml Erlenmeyer flask, mitochondria (7.0 mg of protein) were preincubated in air for 5 min at 30°C in 1 ml of a solution of Gray et al. [8,11], to which 0.2 mM of $CaCl_2$ and graded concentrations of $CdCl_2$ or the purified cadmium binding protein were added as indicated in the text. The reaction was started with the addition of 770 ng (0.14 μCc) of [26,27-^{3}H]-25-OH-D$_3$ (Amersham, Buckinghamshire, England), and stopped 20 min thereafter by the addition of 10 ml of a mixture of methanol and chloroform (2:1 v/v). Extraction was performed as reported by Gray et al. [11]. Chromatography of the extracts was carried out on a 1 X 30 cm column of Sephadex LH 20 using a solvent of 65% chloroform–35% hexane according to the method of Holick and DeLuca [12]. Amounts of each vitamin D$_3$ metabolite produced in vitro were calculated, as described earlier [8], from the radioactivities of the respective metabolites and the specific activity of the isotope.

Table 1
Amino acid composition of metallothionein isolated from the rabbit kidney

Amino acid	Observed residues/mole Time of hydrolysis				Calculated residues/ mole*
	24 hr	48 hr	72 hr	mean	
Aspartic acid	8.78	9.48	8.86	9.04	9
Threonine	4.42	4.38	4.26	4.46**	5
Serine	8.32	7.90	7.34	8.90**	9
Glutamic acid	4.12	4.14	4.12	4.12	4
Proline	7.22	6.30	6.50	6.68	7
Glycine	7.46	7.44	7.44	7.44	7
Alanine	(16)	(16)	(16)	(16)	16
Cysteic acid***				29.50	30
Methionine	1.64	1.62	1.52	1.60	2
Isoleucine	1.04	1.16	1.12	1.12	1
Lysine	12.28	12.60	12.26	12.38	12
Calculated molecular weight of thionein					10 795
Cadmium (μg/mg of metallothionein)				77.18	7
Zinc (μg/mg of metallothionein)				7.18	1

* Calculated on the basis of 16 residues of alanine/mole.
** Extrapolated to the value at zero hour.
*** Treated with performic acid.

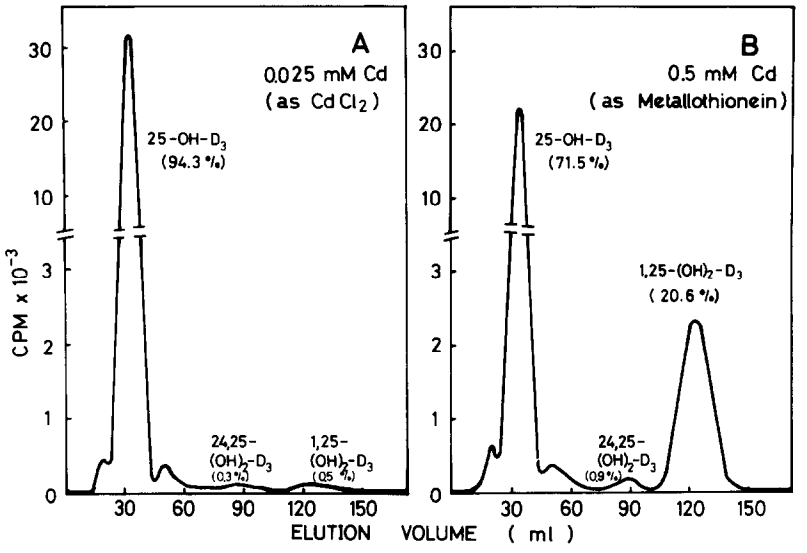

Fig. 1. Sephadex LH-20 chromatographic profiles of chloroform extracts of mitochondrial suspension incubated with 770 ng of [^{3}H]25-OH-D_3 in the presence of 0.025 mM $CdCl_2$ (A) or 0.5 mM of cadmium in the metallothionein (B). Columns were eluted using a solvent of 65% chloroform–35% hexane. Three ml fractions were collected automatically into liquid scintillation counting vials and evaporated to dryness under a stream of air. The radioactivity was determined using a toluene counting solution [12] and a Packard Model 3385 liquid scintillation spectrometer. The position of elution of vitamin D_3 metabolites was determined with standards of radioactive 25-OH-D_3, 1,25-$(OH)_2$-D_3 and 24,25-$(OH)_2$-D_3 as previously described [12].

3. Results

The cadmium-binding protein isolated from the rabbit kidney was unique in that it had an extremely high content of cysteine (calculated from the amounts of cysteic acid) and low content of aromatic amino acids (table 1). These features are similar to those of metallothioneins isolated from the rabbit, rat and human liver [3,4,13]. Therefore it is strongly indicated that the induced cadmium-binding protein in the kidney belongs to the group of metallothioneins which have been well characterized in the sample extracted from the liver. This protein, when one assumes the molecular weight as 10 000, contained 7 moles of cadmium and one mole of zinc per mole of apoprotein (table 1).

The 25-OH-D_3-1-hydroxylase activity in chick kidney mitochondria was assayed in vitro in the presence of 0.2 mM calcium, since in our experimental system the maximal activity was always obtained with this concentration of calcium [8]. The mitochondria produced as much as 16.8 ng of 1,25-$(OH)_2$-D_3 per 20 min per mg protein (fig. 2).

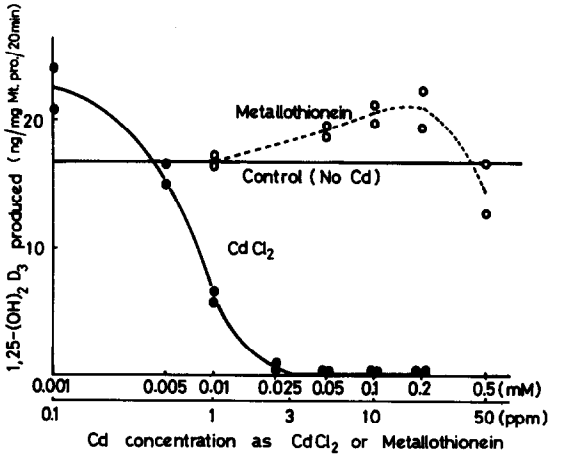

Fig. 2. Effects of cadmium of $CdCl_2$ and metallothionein on the 25-OH-D_3-1-hydroxylase activity in chick kidney mitochondria. Control value shows the amounts of 1,25-$(OH)_2$-D_3 produced in 20 min per mg of mitochondrial protein in the absence of cadmium. Note that as little as 0.025 mM of $CdCl_2$ inhibited the enzyme activity completely, while as much as 0.5 mM of cadmium in the metallothionein did not inhibit it.

The addition of 0.025 mM of $CdCl_2$ to the mitochondrial suspension inhibited the 1-hydroxylation almost completely. More than 94% of the radioactivity recovered was eluted as unchanged 25-OH-D_3 (fig. 1A).

On the contrary, the addition of the concentrations of the kidney cadmium-binding protein that contained from 0.01 to 0.5 mM (final concentration) of cadmium did not show any deleterious influence upon this enzyme reaction. Even when cadmium was introduced as the cadmium binding protein to a concentration as high as 0.5 mM, 1,25-$(OH)_2$-D_3 was produced at the rate of 14.4 ng/mg protein/20 min (fig. 1B and 2).

4. Discussion

The kidneys of the rats fed a diet containing 30 and 300 ppm of cadmium for 3 weeks, had 9.7 and 36.5 μg of cadmium per g of fresh weight [15]. These values are equivalent to about 0.09 and 0.3 mmoles per kilogram, respectively. The data shown in fig. 2 indicate that as little as 0.025 mM (2.8 ppm) of $CdCl_2$ in vitro inhibited completely the activity of the 25-OH-D_3-1-hydroxylase in chick kidney mitochondria. Therefore, it would be expected that the 1-hydroxylase activity in vivo could be completely abolished by the amount of cadmium accumulated in the kidney after the oral administration. Nevertheless, the activation reaction of 25-OH-D_3 proceeded in vivo without appreciable hindrance even in the rats fed the diet containing 30 and 300 ppm of the cadmium for 3 weeks [14,15].

This discrepancy of in vivo and in vitro effects of cadmium on the 1-hydroxylation led us to examine the possibility that the metallothionein, induced by cadmium, might exert a preventive effect against the cadmium toxicity. The characteristic features of the cadmium binding protein isolated from the rabbit kidney support the assumption that the protein belongs to a group of metallothionein. In fact, at least 90% of the cadmium accumulated in the kidney is found to be bound to the cadmium-binding protein [15]. In view of the present results that cadmium bound to the binding protein was inert to the 1-hydroxylation reaction, it seems reasonable to consider that the cadmium deposited in the kidney as metallothionein is practically without effect on vitamin D metabolism.

These data and discussion, though compiled from the experiments performed in three different species of animals, support strongly the suggestion that the metallothionein, induced by cadmium intake, is involved in the mechanism whereby cadmium toxicity is prevented in vivo. Though unsuccessful presently for technical reasons, the final conclusion will be obtained when the whole set of experiments are performed in a single species of animals.

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