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Subcellular distribution of metallothionein and cadmium in the liver and kidneys of bank voles (*Clethrionomys glareolus*) exposed to dietary cadmium

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Abstract

Metallothionein (MT) and cadmium (Cd) contents were determined in the subcellular fractions of the liver and kidneys of bank voles exposed for 6 weeks to elevated levels of dietary Cd-40 and 80 μg g^{-1} dry weight. Hepatic and renal MT was detected exclusively in the cytosol, while Cd was found in the cytosol (73–79% of the total content), nuclei (14–18%) and particulates (4–9%). The concentration of MT in the cytosol as well as Cd content in the particular subcellular fractions appeared to be a dose-dependent. The absence of MT in the nuclear and particulate fractions implied that Cd present in these compartments was not bound to the protein that is considered to provide protection against the toxic metal. Therefore, it is assumed that this component of intracellular Cd could be responsible for the histopathological changes that occurred in the liver (granuloma and focal hepatocyte swelling) and kidneys (focal degeneration of proximal tubules) of bank voles exposed to the higher level of dietary Cd.

Introduction

Metallothionein (MT) is a low molecular weight, thiol rich protein found in most animal cells. MT selectively binds heavy metal ions such as the essential trace elements zinc and copper, as well as the toxic elements cadmium (Cd) and mercury. Although the biological function of this protein is still a matter of discussion, its strong inducibility by heavy metals as well as increased production of MT on exposure to hormones, cytokines and many stress conditions have suggested its participation in protection from toxic metal ions, in metabolism of zinc and copper as well as in scavanging of free radicals (Cherian & Nordberg 1983; Kägi & Schäffer 1988; Bremner & Beattie 1990).

The studies carried out so far indicate that most of intracellular MT is localized primary in the cytoplasm. However, some immunohistochemical and biochemical studies have demonstrated that the protein can also be localized transiently or permanently in the cellular nuclei or other particulates (Riordan & Richards 1980; Panemangalore *et al.* 1983; Mehra & Bremner 1984; Tsujikawa *et al.* 1991; Kikuchi *et al.* 1993; Kondo *et al.* 1995; Sunderman *et al.* 1996). The mechanism responsible for the sequestration of MT by the nucleus is not known; it has been suggested that nuclear localization is promoted by increased intracellular MT levels, e.g. upon exposure to cadmium (Banerjee *et al.* 1982; Danielson *et al.* 1982; Tohyama *et al.* 1988; Breen *et al.* 1995) and by a process linked to cellular proliferation (Tsujikawa *et al.* 1991; Tohyama *et al.* 1993; Włostowski 1993). Likewise, the functional significance of nuclear localization of MT is not known; it is assumed that high levels of nuclear MT may protect critical sites inside the nucleus from oxidative stress (Chubatsu & Meneghini 1993), as well as from cisplatin and cadmium cytotoxicity (Kondo *et al.* 1995). However, immunohistochemical studies, although very important, have not provided any information about the amount of nuclear MT and its

capacity to bind and detoxify toxic elements such as cadmium.

A previous work from our laboratory has demonstrated that substantial amounts of cadmium accumulated in the liver of a free-living rodent, the bank vole, were associated with the nuclear fraction (Włostowski 1992). Likewise, others have shown appreciable amounts of nuclear cadmium in the liver and kidneys of rats and mice (Rummler *et al.* 1989; Liu *et al.* 1995). It is not known, however, whether the nuclear cadmium is bound to MT and thereby made nontoxic or is present as non-MT-bound metal, thereby exerting its toxic effects.

Therefore, the main purpose of the present work was to determine the amount and cadmium-binding capacity of MT in the subcellular fractions of the liver and kidneys of bank voles exposed subchronically to elevated levels of dietary Cd. Subcellular distribution of cadmium and histopathological examinations of both organs were also carried out.

Materials and methods

Animals and treatments

Male bank voles from our own laboratory stock were used throughout the study. One-month-old voles, weighing 11–13 g, were randomly allocated into three groups ($n = 25$ each) according to dietary Cd: (1) control, (2) Cd-40 and (3) Cd-80 μg g^{-1} dry weight. The animals were housed in groups of five in stainless-steel cages and kept for 6 weeks on a 12 h light/dark cycle in a room maintained at 18–20 °C and at 60–70% relative humidity. For 6 weeks, the bank voles received ad libitum distilled water and control (0.05–0.08 μg Cd g^{-1}) or Cd-containing wheat grains which are considered to be an adequate quality food for the bank vole (Włostowski *et al.* 1996). The grains contaminated with cadmium (soaked in $CdCl_2$ solution) were prepared prior to the experiment. Analysis of the grains revealed that actual levels of cadmium were between 90 and 95% of the intended level. In addition, an identical amount of apple containing 0.01 μg Cd g^{-1} wet weight was offered to all animals (3 g/vole/week) which ate it completely. Food intake was measured weekly.

Assays

At the end of the 6-week exposure period, bank voles were weighed and anesthetized with ether, and the liver and both kidneys were removed. Twenty animals from each group were used for biochemical analyses, whereas histopathological examinations were carried out on the remaining five bank voles. A small segment of both organs was removed and dried for moisture content which appeared to be similar in the three groups of animals. Due to a small mass the livers of two or the kidneys of five bank voles were pooled and used as one experimental sample. About one gram of the sample was transferred to 4.0 ml of chilled 0.25 M sucrose solution in 0.01 M Tris-HCl buffer (pH 7.4) (saturated with argon) and homogenized with Teflon pestle in glass homogenizer. Each homogenate was filtered through gauze and 0.5 ml was taken for cadmium determination. The remaining homogenate was then centrifuged at 4 °C ($900 \times g$, 10 min) and the resulting supernatant saved. The $900 \times g$ pellet was washed with 2.0 ml of the same sucrose solution and centrifuged ($900 \times g$, 10 min) twice. All three $900 \times g$ supernatants were combined and centrifuged at 4 °C ($100,000 \times g$, 60 min) using Beckman Model L ultracentrifuge. The $100,000 \times g$ pellet was washed with 2.0 ml of the sucrose solution and centrifuged ($100,000 \times g$, 60 min) and the two resulting $100,000 \times g$ supernatants were combined and considered as cytosol (cytoplasm). In this study, $900 \times g$ pellet was defined as nuclear fraction, whereas $100,000 \times g$ pellet composed of mitochondria, lysosomes and microsomes was defined as particulate fraction.

In order to extract MT from the nuclear and particulate fractions, the $900 \times g$ and $100,000 \times g$ pellets were further treated according to Mehra & Bremner (1984) by resuspending in 2.0 ml of 1% (v/v) 2-mercaptoethanol in H_2O. After freezing (with liquid nitrogen) and thawing three times, as well as leaving at 4 °C overnight, they were then centrifuged at $100,000 \times g$ for 30 min and their supernatants (2×0.1 ml) were used immediately for MT assay. The remaining supernatants and pellets were quantitatively transferred to glass tubes for subsequent cadmium determination. For the control purposes an appropriate amount of rabbit MT-1 (Sigma) was diluted in 2 ml of 1% 2-mercaptoethanol and further processed as described above. The recovery of the protein amounted to 95%.

Total content of MT protein in the cytosol, nuclei and particulates was determined by a silver-saturation method adapted from Scheuhammer & Cherian (1986), while Cd-binding capacity of MT was performed by using a cadmium-saturation method (Onosaka & Cherian 1982). Briefly, in a 1.5 ml

vial 0.1 ml of sample was incubated for 10 min at room temperature with 1.0 ml glycine buffer (0.5 M glycine-NaOH, pH 8.5), containing 20.0 μg Ag ml^{-1} as $AgNO_3$, and in parallel assay tube with Tris-HCl buffer (0.03 M, pH 7.8) containing 5.0 μg Cd ml^{-1} as $CdCl_2$. To remove non-MT-bound Ag or Cd, bovine hemoglobin (Sigma) (0.1 ml of a 5% solution in H_2O) was added and the sample was heated for 1.5 min at 100 °C, cooled and centrifuged for 5 min at $10,000 \times g$. Addition of hemoglobin, heating and centrifugation of the sample was repeated three or five times in the presence of 2-mercaptoethanol. The recovery of added rabbit MT-1 (Sigma) amounted to nearly 100% both in the presence and absence of 2-mercaptoethanol. From the Ag content in the resulting clear supernatant MT was calculated according to the molecular weight of 6600 and the definite molar ratio of 17 moles of Ag per mole of MT. The Cd content in the final supernatant of the parallel assay tube was defined as Cd-binding capacity of MT and expressed in μg Cd g^{-1} fresh tissue. In this work cadmium not bound to MT was defined as the difference between mean Cd concentration and mean Cd-binding capacity of MT in a given subcellular fraction.

The homogenate (0.5 ml) and the remaining material of the particular subcellular fractions were placed in glass tubes with 1.5 ml of concentrated nitric acid. After 20 h of sample digestion at room temperature, 72% perchloric acid (0.5 ml) was added and the mixture was heated at 100 °C for 3 h. Finally the temperature was raised to 150°C and digestion was continued for another 4 h. The residue (about 0.3 ml), after digestion, was made up with deionized water to 2.0 ml.

Cadmium and silver analyses were carried out by electrothermal atomic absorption spectrophotometry using AAS 3 Carl Zeiss instrument with an EA 3 furnace attachment. The conditions for Cd and Ag determinations were as follows: wavelenght 228.8 and 328.1 nm, drying at 110 °C for 15 s, ashing at 250 and 400 °C for 5 sec, and atomizing at 1200 and 1000 °C for 5 sec, respectively. Aldrich standard solutions and 0.015 N HNO_3 were used to prepare the standard curves. Before analysis all samples were appropriately diluted in 0.015 N HNO_3 . Samples of bovine liver 1577b (National Institute of Standards and Technology, Gaithersburg, MD) and CL-1 cabbage leaves (AGH, Poland) were also analyzed in an identical manner to check accuracy of the method. The recovery of the metals amounted to 90–93%.

A portion of the liver and whole kidney were fixed in 4% phosphate-buffered formalin, dehydrated in ethanol and xylene, embedded in paraffin, cut into 8-μm sections, and stained with hematoxylin and eosin for microscopic examination.

Data were reported as mean ± SD. The log-transformed values were analyzed by Student's *t*-test. Differences at $P < 0.05$ were considered statistically significant.

Results

The 6-week exposure to dietary Cd in amounts of 40 and 80 μg g^{-1} affected neither the consumption of food (2.0–2.5 g day^{-1}), nor the final body and organ weights of the bank vole. The mean body weights of the control, Cd-40 and Cd-80 bank voles were 16.1 ± 2.0, 15.8 ± 1.5 and 15.9 ± 2.1 g, respectively. The liver and kidneys weights were 800 ± 95 and 178 ± 10 mg, 790 ± 70 and 170 ± 11 mg, and 780 ± 75 and 168 ± 10 mg for the control, Cd-40 and Cd-80 bank voles, respectively.

As can be seen in Table 1 and 2, MT was detected only in the cytosol of the liver and kidneys; the hepatic and renal MT concentrations, as well as Cd-binding capacity of this protein were dose-dependent and similar in both organs. On the contrary, cadmium was found in all subcellular fractions examined (Tables 1 and 2). Although cadmium concentration in both organs appeared to be a dose-dependent, the percentage contribution of the particular subcellular fractions to the total cadmium content was similar in the bank voles fed diets containing 40 and 80 μg Cd g^{-1}. Most of the hepatic and renal cadmium was recovered in the cytosol (73–79%), whereas the contribution of the nuclear and particulate fractions amounted to 14–18 and 4–9%, respectively.

The absence of MT in the nuclear and particulate fractions implied that cadmium present in these compartments was not bound to MT. This component of intracellular cadmium rose significantly as total hepatic or renal Cd increased (Tables 1 and 2). Non-MT-bound cadmium associated with hepatic nuclei represented approximately 80% of the intracellular metal that was not bound to MT. In the kidneys also, the contribution of nuclear fraction to the total non-MT-bound cadmium was the highest, amounting to 67 and 50% in the Cd-40 and Cd-80 bank voles, respectively. In the latter case, cadmium ions not bound to MT appeared also in the cytosol since the concentration of cadmium

Table 1. Subcellular distribution of metallothionein (MT) and cadmium (Cd) in the liver of bank voles exposed for 6 weeks to dietary Cd[a]

Dietary Cd - μg g^{-1}	Subcellular fraction	MT (μg g^{-1} wet wt)	Cd-binding capacity of MT (μg Cd g^{-1} wet wt)	Cd (μg g^{-1} wet wt)	Cd not bound to MT[b] (μg g^{-1} wet wt)
Control	cytosol	17.0±3.8	1.09 ± 0.5	0.20± 0.07	–
	nuclei	ND[c]	nd[d]	nd	–
	particulates	ND	nd	nd	–
				(0.23 ± 0.09)	
Cd-40	cytosol	145 ± 25[e]	16.6 ± 2.9[e]	12.8 ± 2.2[e]	–
	nuclei	ND	nd	2.7 ± 0.4[e]	2.7
	particulates	ND	nd	0.9 ± 0.2[e]	0.9
				(17 ± 3[e])	
Cd-80	cytosol	274 ± 90[e,f]	33.7 ± 10.0[e,f]	32.2 ± 8.0[e,f]	–
	nuclei	ND	nd	7.6 ± 3.1[e,f]	7.6
	particulates	ND	nd	1.7 ± 0.8[ef]	1.7
				(42 ± 12[e,f])	

[a]Values represent the mean ± SD for 10 samples.The livers of two bank voles were pooled and used as one sample. In parentheses the tissue concentration of cadmium is presented.
[b]Difference from Cd concentration and Cd-binding capacity of MT.
[c]ND – not detected (below 1.0 μg g^{-1}).
[d]nd – not detected (below 0.1 μg g^{-1}).
[e]Significantly different from control ($P < 0.01$).
[f]Significantly different from Cd-40 ($P < 0.05$).

Table 2. Subcellular distribution of metallothionein (MT) and cadmium (Cd) in the kidneys of bank voles exposed for 6 weeks to dietary Cd[a]

Dietary Cd - μg g^{-1}	Subcellular fraction	MT (μg g^{-1} wet wt)	Cd-binding capacity of MT (μg Cd g^{-1} wet wt)	Cd (μg g^{-1}wet wt)	Cd not bound to MT[b] (μg g^{-1} wet wt)
Control	cytosol	21.5 ± 4.6	2.8 ± 0.7	0.33 ± 0.10	–
	nuclei	ND[c]	nd[d]	nd	–
	particulates	ND	nd	nd	–
				(0.35 ± 0.11)	–
Cd-40	cytosol	142 ± 28[e]	17.0 ± 3.1[e]	11.9 ± 2.0[e]	–
	nuclei	ND	nd	3.0 ± 0.6[e]	3.0
	particulates	ND	nd	1.5 ± 0.4[e]	1.5
				(16.5 ± 3[e])	
Cd-80	cytosol	235 ± 83[e,f]	26.4 ± 7.5[e,f]	29.2 ± 7.0[e,f]	2.8
	nuclei	ND	nd	5.3 ± 2.2[e,f]	5.3
	particulates	ND	nd	2.5 ± 0.8[e,f]	2.5
				(38 ± 10[e,f])	

[a]Values represent the mean ± SD for 4 samples. The kidneys of five bank voles were pooled and used as one sample. In parentheses the tissue concentration of cadmium is presented. [b]Difference from Cd concentration and Cd-binding capacity of MT. [c]ND – not detected (below 1.0 μg g^{-1}). [d]nd – not detected (below 0.1 μg g^{-1}). [e]Significantly different from control ($P < 0.01$). [f]Significantly different from Cd-40 ($P < 0.05$).

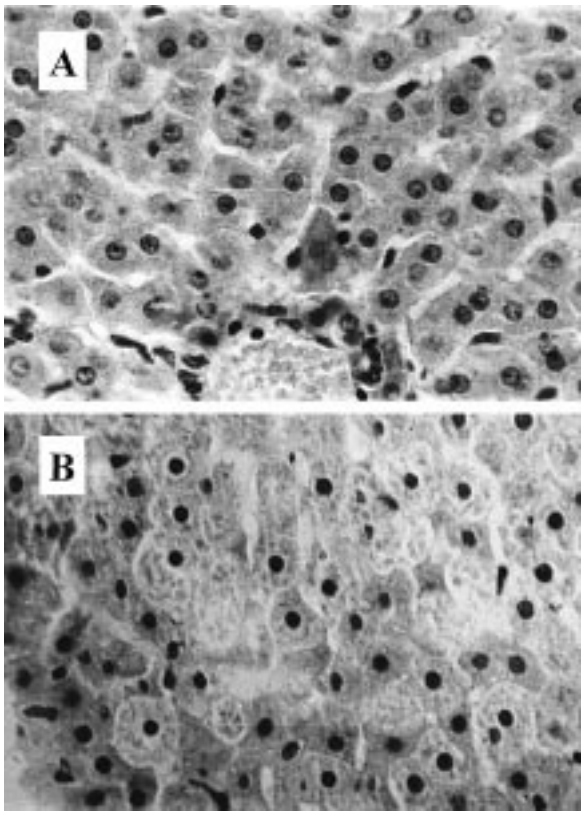

Figure 1. Representative photomicrographs of liver section from control bank voles (A) and bank voles fed for 6 weeks diet containing 80 μg Cd g^{-1} (B). A focal hepatocyte swelling and granuloma in the Cd-80 animals were observed. No changes in histological picture were seen in the bank voles fed diet containing 40 μg Cd g^{-1} (not shown). Hematoxylin-eosin staining, $\times$400.

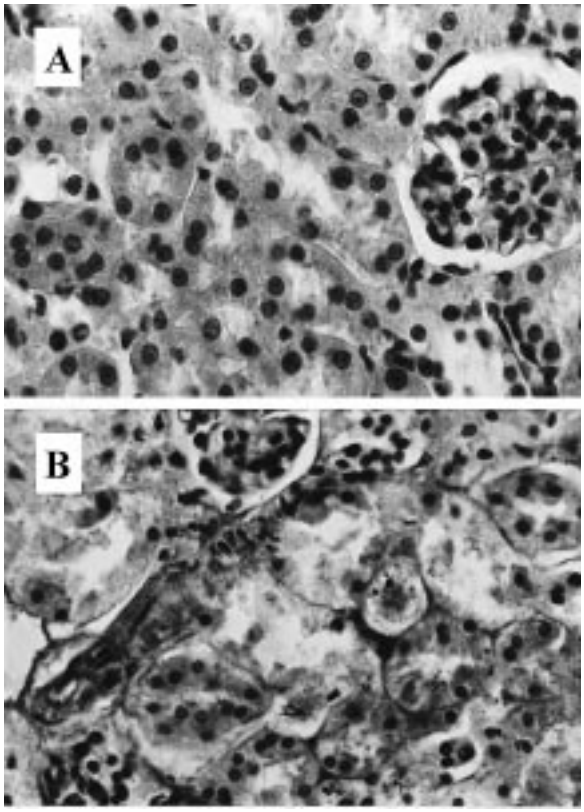

Figure 2. Representative photomicrographs of kidney section from control bank voles (A) and bank voles fed for 6 weeks diet containing 80 μg Cd g^{-1}(B). A focal degeneration of proximal tubular cells in the Cd-80 animals were observed. No changes in histological picture were seen in the bank voles fed diet containing 40 μg Cd g^{-1} (not shown). Hematoxylin-eosin staining, $\times$400.

in this fraction exceeded the Cd-binding capacity of MT (Table 2).

The histopathological examinations were performed on the liver and kidneys of five bank voles randomly selected in each group. In the liver and kidneys no histopathological changes were observed in the control and Cd-40 bank voles (Figures 1 and 2). However, all of five Cd-80 animals exhibited intracellular granuloma and focal hepatocyte swelling in the liver (Figure 1B), as well as a focal degeneration of proximal tubular cells in the kidneys (Figure 2B).

Discussion

The present work demonstrated the absence of MT in the nuclei and other subcellular particles in the liver and kidneys of bank voles after 6 weeks of the exposure to dietary Cd; instead the protein was localized exclusively in the cytoplasm. These data are consistent with some studies showing no MT immunostaining in the nuclei of cultured kidney cells treated with $CdCl_2$ (Zhang *et al.* 1995) as well as of the regenerating liver of Cd-injected rats (Margeli *et al.* 1994), but are opposite to those found by Banerjee *et al.* (1982), Danielson *et al.* (1982) and Tohyama *et al.* (1988) in the liver and kidneys of Cd-injected rats. The latter work has shown that the intensity of MT immunostaining in the nucleus increases with duration of the exposure and parallels a considerable increases in MT content in the cytoplasm (about 1000 μg g^{-1}), suggesting that MT could be transferred passively to the nuclei. Similar conclusion has been drawn by Breen at al. (1995) from the study performed on trophoblast cells. The concentration of hepatic and renal MT in the bank vole (Tables 1 and 2) was at least three times lower than that reported for Cd-injected rats. Therefore, it cannot be excluded that MT level in this case

was too low to be transferred efficiently to the nuclear compartment. Also, our results do not rule out the possibility that MT, due to its low molecular weight, was leached out through nuclear pores during preparation of the fraction (Panemangalore *et al.* 1983). However, even if this were the case the nuclear cadmium remained and was probably not bound to MT. It has to be noted finally that a recent study (Woo *et al.* 1996) has indicated an energy dependent subcellular retention system for MT that is cell type specific and independent of total MT content or cell cycle phase. This may account well for discrepancies concerning subcellular distribution of MT, especially its nuclear localization upon exposure to cadmium.

Observations made in the present study thus suggest that cadmium accumulated in the nuclear and particulate fractions is not bound to MT. It is generally accepted that MT provides a pool of binding sites to sequester cadmium, thereby decreasing its toxicity; the free ion is considered to be the probable toxic species (Goering & Klaassen 1983; Waalkes *et al.* 1985; Nomiyama & Nomiyama 1986; Goyer *et al.* 1989; Sudo *et al.* 1996). In addition, it has been shown that the accumulation of Cd especially in the cellular nuclei may be responsible for cell killing through breakdown of nuclear functions (Lin *et al.* 1995). Since most of the intracellular cadmium that was not bound to MT in the liver and kidneys of bank voles was concentrated in the nuclei (Tables 1 and 2), it is reasonable to assume that the nuclear cadmium could produce damages to the liver and kidneys (Figures 1 and 2).

The concentration of non-MT-bound cadmium in the liver and kidneys of bank voles, at which the histopathological changes occurred, amounted to about 10 μg g^{-1}. Similar value has been found in rats by Sudo *et al.* (1996), whereas others estimated the critical concentration on 35–40 μg g^{-1} (Nomiyama & Nomiyama 1986; Goyer *et al.* 1989). Although free cadmium ions can interact directly with various molecules inside the cell thereby causing an injury, our results do not exclude the possibility that dietary Cd could also affect indirectly the hepatic and renal functions. For example, dietary Cd has been shown to decrease intestinal iron absorption and its concentration in the liver and kidneys (Engstrom & Nordberg 1978; Chmielnicka & Cherian 1986; Groten *et al.* 1991). Likewise, our recent study (submitted) indicates that hepatic and renal iron decreases 5- and 2-fold respectively in bank voles fed diet containing 80 μg Cd g^{-1}. This in turn may cause disturbances in cellular respiration and ATP production. Thus, one may conclude that dietary Cd could exert its toxicity in the bank vole liver and kidneys not only directly but also indirectly through changes in iron metabolism, which might result in ATP deprivation and eventually cellular disintegration. The phenomenon, if so, may also account for the relatively low total level of renal cadmium at which the organ injury occurred in the bank vole -40 versus 200 μg Cd g^{-1} which is considered to be the critical concentration for kidney (Goyer *et al.* 1989). The assumption requires, however, verification in further study.

In conclusion, the data of the present work indicate that MT induced by cadmium in the liver and kidneys of bank voles is localized mainly in the cytoplasm. In contrast, substantial amounts of cadmium are also concentrated in the nuclei and particulates. This fraction of intracellular cadmium that is not bound to MT may be responsible for the histopathological changes occurring in both organs.

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