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INDUCTION OF RENAL METALLOTHIONEIN SYNTHESIS BY PARENTERAL CADMIUM–THIONEIN IN RATS

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Abstract—Cadmium–thionein (Cd–Th) was isolated from livers of rats repeatedly treated with small doses of cadmium chloride. A major portion (55 per cent) of intraperitoneally injected Cd–Th was deposited in the kidney followed by renal damage. Subcellular fractionation of kidney homogenate at 1, 4, 24 and 48 hr after Cd–Th injection revealed that only less than 7 per cent of the cadmium was accumulated in the lysosomal fraction and more than 50 per cent of cadmium was recovered from the cytosol. Injection of [^{35}S]cysteine into rats pre-injected with sublethal doses of Cd–Th resulted in the incorporation of radioactivity into newly synthesized thionein in the kidney between 4 and 24 hr. A major portion of the renal cadmium was bound to metallothionein at all time intervals studied. These studies demonstrated that the Cd–Th complex in the processes of entering the renal cells intact produces cell injury. The thionein moiety of the injected Cd–Th is degraded and resynthesized in the kidney, while nearly all of the metal (> 90 per cent) is always bound to thionein. The role of the lysosomal system in the renal toxicity of Cd–Th is minimal.

Previous studies [1–4] have indicated a paradoxical role of cadmium-induced metallothionein in the renal toxic effects of cadmium. Though cadmium–thionein (Cd–Th) can protect target organs from the acute toxic effects of cadmium [5] and probably other heavy metals like mercury [6], it seems to be directly involved in the manifestation of the pathogenesis of cadmium-induced nephropathy on chronic cadmium exposure. When administered parenterally, Cd–Th is a potent nephrotoxin and its early sites of action are on cell membrane and mitochondria [1]. The mechanism by which Cd–Th causes necrosis to the renal tubular cells is not clearly understood. We have recently postulated that the Cd–Th complex itself may cause cell injury by either uncontrolled pinocytosis or a direct effect on energy metabolism in mitochondria [1]. It was suggested [4, 7], however, that the toxic effects of Cd–Th are not due to the Cd–Th complex itself, but through the Cd^{2+} ions released from this complex in renal cells.

Cherian and Shaikh [8] have shown that intravenously injected Cd–Th reaches the kidney intact and within 3 hr the protein moiety of this metalloprotein may be partially degraded, while most of the cadmium in the kidney is bound to metallothionein. The present study deals with the form of cadmium in the renal cells after the injection of Cd–Th and also the induced synthesis of this protein by renal cells in response to the Cd–Th complex.

MATERIALS AND METHODS

Sprague–Dawley male rats weighing 200–250 g were used in all experiments. Cadmium–metallothionein was isolated from rat liver after repeated injection of $CdCl_2$ (0.6 mg Cd/kg) daily for 5 days by the method described elsewhere [9]. The Cd–Th was further purified by chromatography on DEAE Sephadex A-25 columns using a gradient of 10–250 mM Tris–HCl buffer, pH 8.6 [10]. Two cadmium-binding proteins were isolated by this method. The major protein which was eluted out from the DEAE Sephadex column at an ionic strength of 150 mM was freeze dried and injected into rats in these experiments. Cadmium was estimated by an atomic absorption spectrophotometer using a graphite furnace. The instrumental parameters used for atomic absorption measurements were: analytic line, 2288; slit width 4; spectral band pass 23 A; hollow cathode cadmium lamp current, 9 mA. L[^{35}S]cysteine hydrochloride (78.9 mCi/m-moles) was purchased from the Amersham Searle Corp., Oakville, Ontario, Canada.

Six rats were injected i.p. with a sublethal dose of 0.3 mg Cd/kg as Cd–Th and 1 hr later with 20 μCi [^{35}S]cysteine. Rats were kept individually in metabolic cages and fed a commercial laboratory chow and tap water *ad lib.* Two rats were sacrificed at 4, 24 and 48 hr after Cd–Th injection. The kidneys were removed and a 20% homogenate was prepared in cold 0.25 M sucrose in TKM Tris–KCl–$MgCl_2$ buffer, pH 8.6. In certain experiments, kidney supernatants were heated in a water bath at 80° for 2 min and centrifuged at 13,000 *g* for 10 min [9]. The heated supernatant fractions were further fractionated on Sephadex G-75 columns. One-ml fractions were collected, the elution was monitored at 254 nm and 278 nm, and fractions were analyzed for cadmium. The ^{35}S-radioactivity in the fractions was measured in a Packard liquid scintillation spectrometer with an efficiency greater than 80 per cent. Liquifluor (NEN) in toluene containing 30% Triton X-100 was used as the scintillation fluid.

In a second set of experiments to study the subcellular distribution of renal cadmium, twelve rats were injected i.p. with ^{109}Cd-labeled Cd–Th (0.3 mg Cd/kg). ^{109}Cd–Th was prepared from rat liver

after repeated injection of $^{109}CdCl_2$, as described earlier [8]. Three rats were sacrificed at 1, 4, 24 and 48 hr after the ^{109}Cd–Th injection and kidneys and livers were removed. One kidney and a portion of liver (about 0.5 g) were counted for ^{109}Cd radioactivity immediately and the total radioactivity in these organs was calculated. Since a major portion of the injected radioactive cadmium (about 50 per cent) was accumulated in kidney, a 20 per cent kidney homogenate was prepared in 0.25 M sucrose in TKM buffer, pH 8.6, and fractionated into crude nuclei (600 *g* for 10 min), crude mitochondria (8,000 *g* for 10 min), lysosomal (30,000 *g* for 15 min), microsomal (105,000 *g* for 1 hr) and cytosol (supernatant) fractions by the method of Sabbioni and Marafante [11]. Samples (1 ml) of homogenate and each fraction were counted for ^{109}Cd radioactivity.

RESULTS

When rats were injected with isolated Cd–Th, only a small portion of the cadmium was accumulated in the liver at various time intervals. A major portion of cadmium from Cd–Th was deposited in the kidney (Table 1). On subcellular fractionation of the kidney, more than 50 per cent of cadmium was associated with the supernatant (cytosol) fraction. If cadmium bound to thionein was taken up by lysosomes, one would have expected increased accumulation of cadmium in this fraction with time. Our results show that there is no increase of cadmium in lysosomes even at 48 hr after the injection of Cd–Th. An increased number of lysosomal bodies in proximal tubular lining cells 7 days after Cd–Th injection was reported by Fowler and Nordberg [12]. This could be a secondary effect of the renal cell injury rather than a direct effect of Cd–Th because the cell necrosis due to Cd–Th occurs within 24 hr [11].

The synthesis of renal metallothionein in response to the injected Cd–Th complex was studied by measuring the incorporation of [^{35}S]cysteine into the thionein moiety at different time intervals after the injection of non-radioactive Cd–Th. As shown in Fig. 1, there was marked synthesis of thionein as measured by the incorporation of [^{35}S]cysteine, most of which took place between 4 and 24 hr after Cd–Th injection. In contrast, there was little incorporation of [^{35}S]cysteine into 10,000 mol. wt protein fractions in a control rat during 48 hr. Analysis

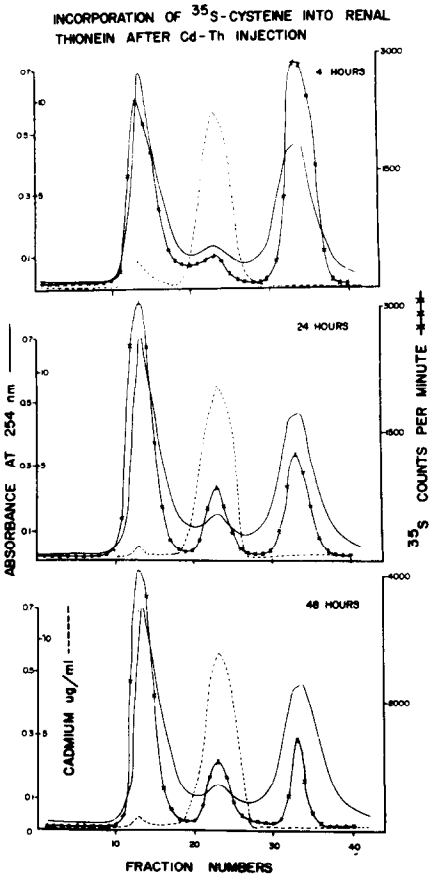

Fig. 1. Sephadex G-75 gel filtration of kidney supernatant (105,000 *g* for 1 hr) fractions from rats injected with cadmium–thionein (Cd–Th) and [^{35}S]cysteine. Rats were sacrificed at 4hr (upper panel), 24 hr (middle panel) and 48 hr (lower panel) after injection of Cd–Th. Metallothionein was eluted in fractions 22–25.

Table 1. Distribution of cadmium in organs and subcellular fractions of kidney at different time intervals after Cd–Th injection*

	Distribution of cadmium			
	Time after Cd–Th injection (hr)			
	1	4	24	48
Organs	(Per cent of administered dose)			
Liver	14.5	20.7	24.0	22.4
Kidney	37.0	49.0	56.0	43.3
Subcellular fractions of kidney	(Per cent of Total homogenate)			
Nuclei (700 *g* pellet)	22.4	20.4	17.8	18.7
Mitochondria (900 *g* pellet)	7.0	7.5	7.1	6.9
Lysosomes (13,000 *g* pellet)	6.6	6.4	5.9	5.3
Microsomes (105,000 *g* pellet)	5.8	4.9	4.4	4.3
Cytosol (105,000 *g* supernatant)	47.8	52.3	60.3	56.2

* Rats were injected i.p. with 0.3 mg Cd as cadmium–thionein (Cd–Th) and sacrificed at 1, 4, 24 and 48 h. Kidneys were homogenized and subcellular fractions were prepared as mentioned in Materials and Methods [11]. The results are mean values of three different experiments.

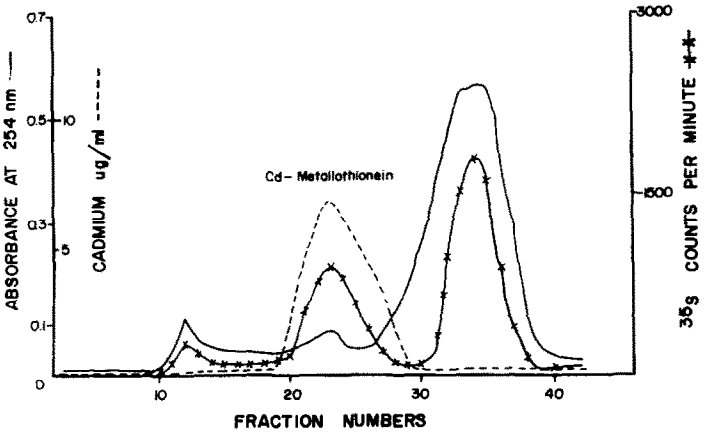

Fig. 2. Gel filtration of heated kidney supernatant from rats injected with Cd–Th and [^{35}S]cysteine. The supernatant (105,000 *g* for 1 hr) was heated at 80° for 2 min in a water bath and centrifuged at 13,000 *g* for 10 min.

of cadmium in the Sephadex eluates of the kidney cytosols at different time intervals reveals that more than 90 per cent of the total cadmium is bound to a 10,000 mol. wt protein moiety. These results show that most of the injected Cd–Th enters the cell as a complex, and the cadmium remains bound to thionein in the kidney.

Figure 2 shows the Sephadex G-75 elution profile obtained from a heated renal supernatant fraction of a rat injected with Cd–Th and [^{35}S]cysteine about 48 hr earlier. Most of the newly synthesized [^{35}S]cysteine containing 10,000 mol. wt proteins is heat stable. This protein peak also has a high absorption at 254 nm. These are considered to be characteristic properties of Cd–Th [9, 13].

DISCUSSION

The results presented in this report suggest the selective deposition of the injected Cd–Th complex in the kidney and its presence in the soluble fraction of the renal cytoplasm. It also shows that Cd–Th can induce the synthesis of new metallothionein in the kidney when injected in sublethal doses. Studies on the intravenous injection of Cd–Th labeled either with ^{109}Cd or [^{14}C]cysteine showed that Cd–Th can reach the kidney cells intact and can be filtered into urine without any major degradation of the metal–protein complex [8]. It could also be reabsorbed by renal tubular lining cells [14]. Cd–Th can enter the kidney cells by pinocytosis and may rupture cell membranes during this process. The irreversible mitochondrial damage was also observed morphologically and the reason for cell death may be due to a direct effect on energy metabolism [11].

However, in sublethal doses of Cd–Th, kidney cells can synthesize new metallothionein in response to the Cd–Th complex which has entered renal cells. This suggests that the protein moiety of this metalloprotein is turning over while the metal binds with the newly synthesized thionein. These results do not support the notion that Cd–Th is degraded to Cd^{2+} which can cause renal toxic effects. It is difficult to identify free Cd^{2+} in the cell because of the presence of metal-binding intracellular ligands. If Cd^{2+} ions are released from Cd–Th, they can also be formed from $CdCl_2$ and other cadmium salts in the cell. Since Cd–Th, unlike other cadmium salts, is a potent nephrotoxin after a single injection, there are factors other than the formation of Cd^{2+} ions involved in its acute renal toxicity.

Even though the mechanism of the nephrotoxicity of Cd–Th is not understood clearly, the disruption of the cell membrane and the mitochondrial injury can account for the Cd–Th-induced necrosis of renal tubular lining cells. These cellular changes can be observed within 24 hr, prior to the necrosis of the cell. In chronic cadmium exposure studies, we have recently shown (M. G. Cherian *et al.*, unpublished data) that typical symptoms of cadmium toxicity will appear only if the Cd–Th is present extracellularly in biological fluids. These results are in agreement with our hypothesis [1] that the presence of the Cd–Th complex in circulating plasma or in glomerular filtrate is a prerequisite for cadmium toxicity.

Zinc–thionein which is similar to Cd–Th in most of its physical and chemical properties is different from Cd–Th metabolically and in its toxicological properties. Zinc–thionein has been shown to be non-toxic [2, 4] when administered parenterally. The major reason for the differences in the toxicological properties of zinc–thionein and cadmium–thionein is due to the difference in their metabolism. A recent study [15] shows that zinc from this metalloprotein can be metabolized and reutilized easily. On the other hand, cadmium introduced as Cd–Th always stays bound to thionein in renal cell and is not transferred to other protein fractions in the cytoplasm.

The synthesis of Cd–Th in the kidney cells in response to sublethal doses of Cd–Th may also be important for the repair process of the cell. Metallothionein-like proteins can be synthesized by the kidney in response to cadmium chloride, mercuric chloride [16] and mercury vapor [17]. If renal cells can synthesize metallothionein and store it intracellularly, it can protect the cell from the toxic effects of certain heavy metals. Thus, metallothionein is not present normally in biological fluids [18]

and does not have any transport function for cadmium from liver to kidney. However, if Cd–Th is excreted or leaks out of the cell under certain conditions, the reabsorption of this metal–protein complex can cause cell injury.

The present results suggest that Cd–Th enters the renal cells intact and that the metal stays bound to metallothionein, though there is a continuous turnover of the protein moiety. Protein synthesis does continue in the kidney provided the Cd–Th-induced cell injury is not lethal.

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REFERENCES

1. M. G. Cherian, R. A. Goyer and L. Delaquerriere-Richardson, *Toxic appl. Pharmac.* **38**, 399 (1976).
2. M. G. Cherian, T. J. Kirby and R. A. Goyer, *Fedn Proc.* **35**, 503 (1976).
3. G. F. Nordberg, R. A. Goyer and M. Nordberg, *Archs Path.* **99**, 193 (1975).
4. M. Webb and A. T. Etienna, *Biochem. Pharmac.* **26**, 25 (1977).
5. G. F. Nordberg, *Envir. Physiol. Biochem.* **1**, 171 (1971).
6. L. Magos, M. Webb and W. H. Butler, *Br. J. exp. Path.* **55**, 589 (1974).
7. S. R. C. Raghavan and H. C. Gonick, *Clin. Res.* **24**, 162A (1976).
8. M. G. Cherian and Z. A. Shaikh, *Biochem. biophys. Res. Commun.* **65**, 863 (1975).
9. M. G. Cherian, *Biochem. biophys. Res. Commun.* **61**, 920 (1974).
10. Z. A. Shaikh and O. J. Lucis, *Experientia* **27**, 1024 (1971).
11. E. Sabbioni and E. Marafante, *Chem. Biol. Interact.* **15**, 1 (1976).
12. B. A. Fowler and G. F. Nordberg, *Proc. Int. Conf. Heavy Metals in the Environment*, Toronto, Canada, B52, University Press, Toronto (1975).
13. P. Pulido, J. H. R. Kagi and B. L. Vallee, *Biochemistry* **5**, 1768 (1966).
14. K. Nomiyamma, T. Taguchi and E. C. Foulkes, *Fedn Proc.* **35**, 849 (1976).
15. M. G. Cherian, *J. Nutr.* **107**, 965 (1977).
16. J. K. Piotrowski, B. Trojanowska, J. M. Wisniewska-Knypl and W. Bolanowska, *Toxic. appl. Pharmac.* **27**, 11 (1974).
17. M. G. Cherian and T. W. Clarkson, *Chem. Biol. Interact.* **12**, 109 (1976).
18. M. G. Cherian, *J. Toxic. envir. Hlth* **2**, 955 (1977).