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Metallothionein content and zinc status in various tissues of rats treated with iodoacetic acid and zinc

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Abstract. Metallothionein (MT) content was determined in 11 tissues of saline-treated rats, and 18 h after single IP injections of iodoacetic acid (IA, 15 and 30 mg/kg) or zinc sulfate (20 mg zinc/kg). Zinc increased MT content in eight tissues, being most effective in pancreas, liver, and small intestine. After IA, MT increased in liver, muscle, pancreas, and thymus, the highest amount being in liver. Increased levels of total tissue zinc were observed in those tissues which clearly responded to the treatments with elevated MT levels, such as liver, kidney, small intestine, and pancreas. MT induction by zinc in these tissues was associated with an accumulation of zinc not bound to MT. In the IA-treated rats non-MT zinc accumulated in liver and pancreas.

It is concluded that induction of MT by zinc and in particular by organic chemicals such as IA without exogenously supplied metals interferes with zinc homeostasis and the physiological role of MT in liver and extrahepatic tissues of the rat.

Key words: Metallothionein – Zinc status – Iodoacetic acid – Rat tissues

Introduction

Metallothionein (MT) is a unique low molecular weight protein with high cysteine and metal content (Kägi and Nordberg, 1979; Foulkes 1982). The protein binds both essential (Cu, Zn) and non-essential metals (Cd, Hg, Ag), and may therefore play an important role in the pharmacokinetics and toxicity of these metals (Suzuki et al. 1983; Cherian and Goyer 1978). Elevated levels of MT have been reported in liver in response to metals like zinc or cadmium (Eaton et al. 1980; Durnam and Palmiter 1981), various stresses, and organic chemicals like carbon tetrachloride, bromobenzene and iodoacetic acid (Oh et al. 1978; Brady 1981; Kotsonis and Klaassen 1979).

More recently, MT has been detected in a number of extrahepatic tissues of untreated rats and after treatment with metals like zinc and cadmium (Onosaka and Cherian 1982b; Onosaka and Cherian 1981).

There is growing evidence that MT accumulation proceeds with elevated concentrations of thionein-mRNA and is regulated at the level of gene transcription (Cousins 1982). Recent studies indicate that inducing metals or hormones interact with a MT gene promoter, thereby regulating the rate of MT synthesis (Durnam and Palmiter, 1981; Karin et al. 1981).

The mechanism of MT induction by electrophilic agents is as yet unclear. It has been proposed that electrophilic chemicals might liberate zinc from various molecules (Kotsonis and Klaassen 1979) or increase zinc uptake by the liver. Increased zinc levels would then lead to MT induction (Webb and Cain 1982).

Since relatively few data are available on endogenous MT levels and MT induction by chemicals, we determined MT accumulation in various rat tissues after single intraperitoneal injections of zinc sulfate and iodoacetic acid. Furthermore, we studied the zinc status in those tissues which clearly responded to IA or zinc with elevated levels of MT.

Material and methods

Chemicals. Sephadex G-75 was obtained from Pharmacia, Freiburg, FRG and chromatographic equipment from LKB, Broma, Sweden. Bovine hemoglobin, iodoacetic acid (IA), and 5,5′-dithio-bis-(2-nitrobenzoic acid) were purchased from Sigma, Taufkirchen, FRG. All other chemicals were obtained from Merck, Darmstadt, FRG and were of analytical grade.

Animals and treatments. Male Wistar rats (inbred strain Neuherberg) weighing 180–200 g were fed a standard laboratory diet (Altromin, Lage, FRG) as required and had free access to tap water. The zinc content of the diet was 75 mg/kg. Rats were divided in four groups of three animals each. Control animals received saline (2 ml/kg, IP); animals of the other groups reveived either IA (15 or 30 mg/kg in saline, IP) or zinc sulfate (20 mg zinc/kg in saline, IP). Eighteen hours after injections, rats were decapitated under light ether anesthesia. Organs were removed, immediately frozen and stored at −80° C. For measurements organs were homogenized in nine volumes of ice cold TRIS-HCl buffer (30 mM, pH 7.4).

Gel chromatography. Hepatic cytosol (100,000 *g* supernatant) referring to 0.3 g wet weight of liver was applied to a column (1.6 × 84 cm) packed with Sephadex G-75 and equilibrated with TRIS-HCl buffer (30 mM, pH 7.4). Fractions of 5 ml were collected at a flow rate of 18 ml/h. All chromatographic procedures were accomplished at 4° C. Fractions were analysed for zinc and mercapto groups either before or after

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precipitation of heat labile proteins by heating for 3 min at 100° C and centrifugation for 5 min at 8,000 *g*.

Assays. Zinc and cadmium were detected by atomic emission spectroscopy with inductively coupled plasma excitation (ICP-AES) using a JY 38 P sequence spectrometer from Instruments SA (Schramel et al. 1982). Tissues homogenates were analysed after wet ashing with nitric acid at 140° C (Schramel et al. 1980).

For routine analysis, MT was determined in 18,000 *g* supernatants of tissue homogenates (S18) by an assay which is based on the cadmium saturation method of Onosaka and Cherian (1982a). Samples of S18 were diluted with TRIS-HCl buffer (30 mM, pH 7.4) to 1.1 ml and incubated at room temperature for 5 min with cadmium chloride (1,000 ng Cd). To precipitate non-MT-bound cadmium, hemoglobin (100 µl of a 4% solution, w/v) was added and the samples were heated for 3 min at 100° C and centrifuged for 5 min at 8,000 *g*. Addition of hemoglobin, heating, and centrifugation of the samples was repeated three times. From the Cd content of the resulting clear supernatant MT was calculated according to a molecular weight of 6,600 and the definite molar ratio of 7 moles Cd/mole of MT (Winge and Miklossy 1982).

Test results were corrected from a small amount of non-hemoglobin-precipitable Cd of 2 ng Cd/ml. This experimental blank value could not be reduced either by increasing hemoglobin or by decreasing Cd concentrations (results not shown). Additionally, interaction of Cd with low molecular weight SH-compounds like glutathione (GSH) will increase the value of non hemoglobin-precipitable Cd (Eaton and Toal 1982). GSH-containing samples, therefore, should be properly diluted so as not to exceed a GSH concentration of 0.5 mM in the test. For GSH-rich tissues like mouse or rat liver a dilution factor of 20 will meet this requirement.

Due to high affinity for MT, copper will lead to an underestimation of MT by the Cd saturation method. Under normal conditions, however, this limitation of the assay is considered to be negligible, since zinc is the predominant metal in MT (Kägi and Nordberg 1979). Furthermore, neither treatment with IA nor zinc led to significant changes in the copper content of the various tissues (data not shown).

The assay showed a linear relationship between purified zinc-MT and measured Cd up to 8 µg MT, corresponding to 870 ng Cd/ml.

As a control, MT was also measured in the chromatographically separated tissue supernatants by metal and mercapto group analysis. Mercapto groups were determined spectrophotometrically at 405 nm with 5,5′-dithio-*bis*-(2-nitrobenzoic acid) according to Ellman (1959) and Sedlak and Lindsay (1968) using a molar extinction coefficient of 13,940 (cm^2/mol). Maximal and stable colour formation was obtained at 120 min for MT (fraction 23–29) and glutathione (fraction 31–40) containing fractions. The level of statistical significance was analysed by means of a student *t*-test evaluation.

Results

MT determination by G-75 chromatography with metal and thiolate group detection and the Cd-saturation method

Figure 1 shows the G-75 elution profiles of hepatic cytosol of IA-treated rats as detected by zinc- and thiolate-group analysis (upper and lower panel, respectively). Heating of the samples for 3 min at 100° C (dotted lines) improved the resolution of the MT peak by lowering metal and thiolate content of the high molecular weight fractions. Assuming 7 mol metal and 21 mol thiolate groups/mol of MT, MT quantitation by zinc analysis revealed 11% lower values than those obtained via mercapto groups.

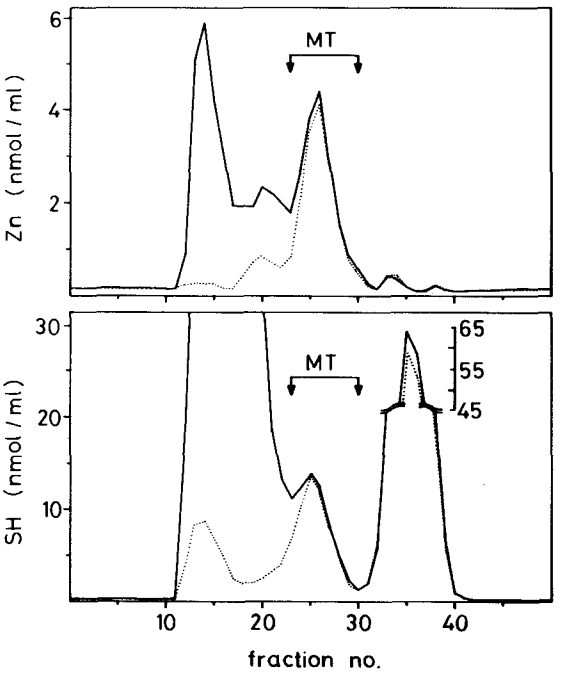

Fig. 1. Sephadex G-75 chromatography of hepatic 100,000 *g* supernatant of IA (30 mg/kg)-treated rats (three animals pooled). Zinc (*Zn*) and mercapto (*SH*) groups were determined either before (*solid lines*) or after (*dotted lines*) precipitation of heat labile proteins by heating for 3 min at 100° C and centrifugation for 5 min at 8,000 *g*. Position of metallothionein (*MT*) is indicated by *arrows*

For the determination of basal MT values, particularly in extrahepatic tissues, the more sensitive Cd-saturation assay was used. Values obtained by this method were identical as compared to those determined by G-75 chromatography with thiolate group analysis and were up to 20% higher than those obtained by zinc analysis (data not shown).

MT levels of rat tissues after IA and zinc treatment

MT content was measured by the Cd-saturation method in 11 tissues of control rats and 18 h after single IP injections of IA (15 and 30 mg/kg body weight) or zinc sulfate (20 mg Zn/kg). MT content of control animals varied between 2 µg/g tissue wet wt in lung and muscle and 52 µg/g tissue wet wt in kidney. IA treatment caused a marked increase in the MT content of liver and pancreas, whereas minor increases were found in muscle and thymus occuring at the higher dose of IA only. Injection of zinc sulfate increased MT contents in heart, kidney, liver, lung, pancreas, small intestine, spleen, and stomach being most effective in pancreas, liver, and small intestine (Table 1).

Zinc status of rat tissues after IA and zinc treatment

Total tissue zinc content and zinc distribution in MT and non-MT fractions were determined in liver, kidney, small intestine, and pancreas. Treatment with zinc increased total tissue zinc levels in all four tissues, being most effective in liver and pancreas. Treatment with IA increased total tissue zinc in liver and pancreas only. Elevated contents of non-MT bound

Table 1. Metallothionein content of normal rat tissues and after treatment with iodoacetic acid or zinc sulfate

Tissue	Metallothionein (μg/g tissue wet wt)			
	Control (saline)	Iodoacetic acid		Zinc sulfate (20 mg Zn/kg)
		(15 mg/kg)	(30 mg/kg)	
Brain	38 ± 8[a]	25 ± 3	28 ± 6	39 ± 3
Heart	4 ± 1	5 ± 2	6 ± 2	17 ± 1*
Kidney	52 ± 8	38 ± 4	44 ± 12	253 ± 68*
Liver	9 ± 2	204 ± 12*	232 ± 8*	804 ± 113*
Lung	2 ± 1	3 ± 1	4 ± 1	5 ± 1*
Muscle	2 ± 1	2 ± 1	6 ± 1*	3 ± 1
Pancreas	11 ± 1	19 ± 3*	52 ± 13*	1,116 ± 251*
Small intestine	13 ± 1	9 ± 3	16 ± 2	356 ± 62*
Spleen	4 ± 1	3 ± 1	5 ± 2	12 ± 4*
Stomach	7 ± 1	7 ± 3	9 ± 2	32 ± 7*
Thymus	16 ± 2	15 ± 2	27 ± 4*	19 ± 2

[a] Mean ± SD of three rats
* Significant versus control ($P < 0.01$)

Table 2. Zinc status of normal rat liver, kidney, small intestine, and pancreas and after treatment with iodoacetic acid or zinc sulfate

Tissue		Zinc (μg/g tissue wet wt)			
		Control	Iodoacetic acid		Zinc sulfate (20 mg Zn/kg)
			(15 mg/kg)	(30 mg/kg)	
Liver	Total	29 ± 2[a]	50 ± 1*	56 ± 6	102 ± 14*
	MT-bound[b]	0.6 ± 0.2	14.2 ± 0.9*	15.8 ± 1.2*	55.8 ± 7.8*
	Total-(MT-bound)	28 ± 2	36 ± 1*	40 ± 5	46 ± 7*
Kidney	Total	23 ± 2	28 ± 5	25 ± 1	46 ± 6*
	MT-bound	3.6 ± 0.6	2.6 ± 0.2	3.1 ± 0.8	17.5 ± 4.7*
	Total-(MT-bound)	20 ± 3	25 ± 4	22 ± 2	28 ± 4*
Small intestine	Total	21 ± 4	20 ± 2	22 ± 1	60 ± 25*
	MT-bound	0.9 ± 0.5	0.6 ± 0.3	1.1 ± 0.1	24.7 ± 4.3*
	Total-(MT-bound)	20 ± 4	19 ± 1	21 ± 1	35 ± 7*
Pancreas	Total	21 ± 1	29 ± 2*	30 ± 5*	215 ± 85*
	MT-bound	0.7 ± 0.1	1.3 ± 0.2*	3.6 ± 1.0*	77.4 ± 17.4*
	Total-(MT-bound)	21 ± 1	27 ± 2*	26 ± 4**	132 ± 82**

[a] Mean ± SD of three rats; * significant versus control ($P < 0.01$); ** significant versus control ($P < 0.05$)
[b] Calculated from the MT values of Table 1

zinc were observed in all tissues responding to either treatment with increased MT and total tissue zinc levels (Table 2).

Discussion

MT was detected in all tissues investigated. As compared to MT levels observed in Sprague-Dawley rats (Onosaka and Cherian 1982b; Onosaka and Cherian 1981), higher or identical levels were found in brain and kidney but lower levels in most other tissues.

MT content was significantly increased in eight tissues of rats after IP injection of 20 mg zinc/kg as zinc sulfate. Maximum increase was found in pancreas followed by liver, kidney, and small intestine. Similar responses of MT induction have been reported in Sprague-Dawley rats after multiple SC injections of cadmium chloride (Onosaka and Cherian 1981) and higher doses of zinc sulfate (40–140 mg/kg; Onosaka and Cherian 1982b).

After IA treatment, elevated MT levels were observed in liver and pancreas and to a minor extent in muscle and thymus at the higher dose of IA. Notably, the latter tissues did not show MT induction after zinc injection. A similar increase in the content of hepatic MT after IA treatment has been reported by Kotsonis and Klaassen (1979). The biological significance of the MT increase in these particular tissues is unclear. Elevated levels of MT can be considered to reflect de novo MT synthesis in the tissues, because an intracellular protein such as MT is not supposed to be transferred from one organ to another during the short experimental period of 18 h (Onosaka and Cherian 1982b).

Since zinc was discussed to be associated with MT synthesis even without an exogenous supply of this metal

(Oh et al. 1978; Webb and Cain 1982), distribution of zinc was investigated in liver, pancreas, kidney, and small intestine.

After zinc pretreatment, zinc contents in all the four tissues were increased. This zinc accumulation exceeded that associated with the increased MT levels. Similarly, pretreatment with IA led to an increase of total tissue zinc in liver and pancreas which exceeded the zinc increase due to MT induction. As a consequence, all four tissues showing MT induction by either treatment showed an accumulation of zinc not bound to MT. Total zinc contents of the tissues showing no MT induction were not significantly different from the controls (data not shown).

The mechanism regulating tissue zinc concentrations is poorly understood. After zinc pretreatment, MT induction may be a direct response to elevated intracellular zinc concentrations. Accordingly, a direct positive correlation between the deposition of zinc and MT was described in tissues of zinc-injected rats (Onosaka and Cherian 1982b). The accumulation of zinc not bound to MT might then be explained by a lower rate of MT synthesis as compared to the rate of cellular zinc uptake. At least for liver and pancreas high rates of zinc uptake appear reasonable to assume since overall zinc balance of these organs seems to be regulated via biliary and pancreatic zinc secretion (Weigand and Kirchgessner 1978; DiSilvestro and Cousins 1983).

Nevertheless, the close relationship between MT content and zinc status becomes evident by the fact that the level of total tissues zinc and non-MT zinc also increases in tissues responding to IA with elevated MT levels. However, the question whether zinc preceeds or follows MT induction by chemicals and the reason for the accumulation of non-MT-bound zinc after IA administration remains unclear. Whereas similar increases in hepatic zinc content have been described after MT induction by an acrylic acid derivative (Giroux and Lachmann 1984), no change in hepatic zinc was observed after MT induction by bacterial endotoxin and carbon tetrachloride (Durnam et al. 1984; Oh et al. 1978). Carbon tetrachloride, however, caused a marked intracellular zinc shift from high molecular weight proteins to metallothionein (unpublished results).

Recently, an explanation for a zinc-independent induction of MT has been given by Durnam et al. (1984) and Durnam and Palmiter (1984). In addition to metal- and glucocorticoid-mediated MT gene promotor sequences, the authors discuss a third promoter sequence mediating MT induction by chemicals such as bacterial endotoxin.

Irrespective of the precise mechanism of induction, MT induction by chemicals in the absence of exogenously supplied metals will interfere with the homeostasis of zinc and toxic metals which are bound to MT. Induction of MT by chemicals, therefore, is considered to be a useful tool to investigate metal homeostasis and the physiological role of MT. In view of recent findings of increased resistance towards IA of MT-rich cadmium resistant cells (Durnam and Palmiter 1984) such studies may be particularly helpful in clarifying the role of MT in binding and detoxication of electrophilic agents (Cagen and Klaassen 1979; Cagen and Klaassen 1980).

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