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Extents of Hepatic Zinc-thionein Induction in Mice given an Equimolar Dose of Various Heavy Metals

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Male ICR mice were injected intraperitoneally with various heavy metals (chromium, manganese, iron, cobalt, nickel, zinc, selenium, indium and lead) at an equimolar dose (150 μmol/kg body weight) to compare the abilities of the metals to induce zinc-thionein (Zn–Th) in the liver. Metallothionein (MT) level was determined for cadmium-replaced liver supernatant fractions by high performance liquid chromatography-atomic absorption spectrophotometry. All the metals induced Zn–Th to some degree; the strongest inducers were zinc and indium, and the weakest was iron. Selenium, previously reported to be unable to induce MT, also gave a fairly high level of Zn–Th at the lower dose of 30 μmol/kg (it was lethally toxic at the standard dose). Changes in the concentrations of some essential metals in the liver were also determined by the use of an inductively coupled plasma-atomic emission spectrometer in order to correlate the extents of MT induction with the changes of essential metal levels.

Keywords——metallothionein; zinc-thionein; high performance liquid chromatography; ICP; heavy metals; zinc; calcium; liver

Metallothionein (MT) is a low-molecular-weight protein (about 6000—7000 daltons) with a high content of cysteine residues (about 30%), and it has a strong affinity for group IIb and Ib metals.[1,2] MT was first isolated as a unique Cd and Zn-binding protein[3] and shown to be inducible by the injection of group IIb and Ib metal ions as a protein loaded with the injected metal.[4,5] On the other hand, recent studies have revealed that MT plays an important role in zinc homeostasis and that the protein can be induced as zinc-thionein (Zn–Th) by stress,[6] food restriction,[7] and the injection of many organic chemicals.[8–12] As for inorganic metals other than group IIb and Ib ones, it has been reported that some induced Zn–Th and others did not when administered at different molar doses.[13–15] Moreover, it has been reported that even cadmium and mercury, which belong to group IIb, can induce Zn–Th when injected as a suspension[16] and as methylmercury,[17] respectively. Therefore, the induction of Zn–Th seems to be a common response of the living body to a wide variety of injected metals.

We have been interested in comparing the abilities of many heavy metals to induce Zn–Th. The present study was designed to compared the amount of Zn–Th induced in the liver after administration of several heavy metals at an equimolar dose. Zn–Th was determined on a gel permeation column by high performance liquid chromatography–atomic absorption spectrophotometry (HPLC–AAS).[18] Changes in the levels of some essential metals in the liver were also determined by inductively coupled plasma-atomic emission spectrometry (ICP–AES)[19] to detect the disturbance of homeostasis after heavy metal loadings.

Materials and Methods

Solutions for Injection——Manganese acetate, ferrous sulfate, ferric chloride, nickel acetate, zinc acetate, cobaltous chloride, indium sulfate and lead acetate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Chromic chloride and sodium selenite were obtained from Kanto Chemical Co. (Tokyo, Japan) and Merck (Darmstadt, West Germany), respectively. The metal salts except for lead acetate were dissolved (24 mM) in saline freshly prepared from doubly distilled water (bubbled through with nitrogen gas beforehand). Lead acetate was dissolved in degassed doubly distilled water. The concentration of indium

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was calibrated with an atomic absorption spectrophotometer (Hitachi 170-50A), because the amount of combined water in the indium sulfate used was unknown.

Injection of Metals——Male ICR mice (JCL, Clea Japan, Tokyo; 5 weeks old; body weight ±S.D., 32.5±1.8 g) were injected intraperitoneally with the above-mentioned metal solutions at a dose of 150 μmol/kg (0.2 ml/mouse) (6 mice/group). In the treatments with selenium and indium, a 5-fold diluted solution was also injected (30 μmol/kg) due to the low LD_{50} value. Control mice were given saline solution. The animals were fed on a standard laboratory chow and distilled water *ad libitum* during the experimental period and killed 24 h (unless otherwise noted) after the injection by cardiac puncture under ether anesthesia. To follow the time-courses of hepatic calcium and zinc levels, other mice (JCL, Clea Japan; 6 weeks old; body weight ±S.D., 35.3±1.8 g) were injected with lead, 5-fold diluted indium and saline solutions, and killed 1 or 7 d after the injection.

Determination of Essential Metal Concentrations——The liver (about 0.3 g portion) from each mouse was digested with 1 ml of mixed acid (HNO_3: $HClO_4$=5: 1, v/v) and the solution was diluted to 10 ml with doubly distilled water. The concentrations of several essential metals were simultaneously determined by ICP-AES (Jarrell-Ash Model 975 Plasma Atomcomp).

Preparation of Liver Supernatant Fractions——A 0.3 g portion of liver from each mouse in a given group was pooled and homogenized in 3 volumes of 0.1 M Tris-HCl buffer solution (pH 7.4, 0.25 M glucose) with a Polytron homogenizer under ice-water cooling in an atmosphere of nitrogen. The homogenates were centrifuged at 170000×*g* for 60 min at 2°C. To detect the induced Zn–Th as Cd–Th, cadmium acetate (1000 ppm, 10 μl) was added to 300 μl of each supernatant and the excess cadmium was removed as Cd-containing denatured high-molecular-weight proteins by heat treatment (80°C, 5 min)[20] and centrifugation (10000×*g*, 1 min).

Determination of Induced MT——A 100 μl portion of the liver supernatant fraction was subjected to HPLC [Toyo Soda HLC 803A equipped with a gel permeation column (TSK GEL SW 3000 column, Toyo Soda, 7.5×600 mm, with a 7.5×75 mm precolumn)], and the column was eluted with 50 mM Tris–HCl buffer solution (pH 8.0 at 25°C) at a flow rate of 1 ml/min. The cadmium level of the eluate was continuously monitored by AAS (Hitachi 170-50A).[18]

Results and Discussion

No enzymatic activities of MT have been reported, so MT can be estimated only by determining bound metals or apo-protein. Examples of the former approach are the methods of Piotrowski *et al.*[21] and of Kotsonis and Klaassen,[22] and an example of the latter is radioimmunoassay.[23] Our HPLC–AAS method belongs to the former category in principle and has the advantage that MT can be estimated at the isometallothionein level. Although the induced Zn–Th could be monitored by measuring the zinc level in a heat treated sample,[16] the replacement of zinc by cadmium gave several times higher sensitivity.

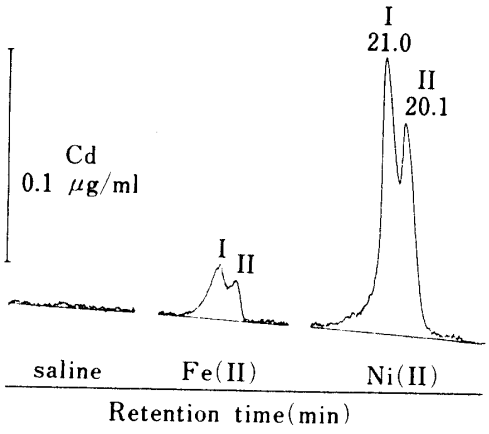

Fig. 1. Gel Permeation–Cd Atomic Absorption Chromatograms of Liver Supernatants after Injection of Ni (II), Fe(II) and Saline Solutions

Mice were killed 24 h after the injection (150 μmol/kg). Livers were combined in each group and homogenized in 3 volumes of 0.1 M Tris–HCl buffer solution, then the homogenates were centrifuged at 170000×*g* for 60 min. Cadmium acetate was added to the original supernatant and the excess cadmium was removed by heat treatment and centrifugation. A 100 μl portion of the cadmium-replaced supernatants was subjected to HPLC–AAS and the atomic absorbance of cadmium was continuously monitored. The detector level of AAS was set as indicated by the vertical bar. I and II indicate MT-I and -II, respectively.

Fig. 1 shows some representative gel permeation-cadmium atomic absorption chromatograms of the converted liver supernatants obtained from mice injected with a metal-containing solution and killed 24 h after the administration (in general, the maximum amount of induced Zn–Th is observed about 24 h later[24]). MTs were detected as two distinct cadmium peaks (the isometallothioneins, MT-I and -II, are indicated as I and II, respectively); MT-I showed a somewhat larger peak than MT-II in all cases examined. Thus, MT can be estimated easily at the isometallothionein level by the HPLC–AAS method.

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The amounts of total hepatic MT induced by the metal loadings are represented in Table I as normalized values with the value for zinc-loading (the induction of MT by zinc has already been confirmed[24]) taken as unity. The peak area on zinc loading corresponded to 1.12 μg Cd (namely, 0.65 μg Zn) per 100 μl of the cadmium-replaced and then heat-treated liver supernatant. Absolute values of cadmium content were estimated from the relative pesk area with respect to a standard Cd–Th solution obtained from cadmium-loaded rats. Selenium and indium are the most toxic of the metals used[25]; the mice injected with the standard dose of selenium were all dead within 24 h, and 15 animals out of 18 injected with indium were dead within 48 h, though no death was observed within 24 h. Therefore, the results obtained with one-fifth of the standard dose are also included in the table.

Table I. Zinc-thionein Levels induced by Various Metals[a]

Metal	MT	Metal	MT	Metal	MT
Saline	<0.01	Fe(III)	0.15	Se(IV)[b]	0.26
Cr(III)	0.36	Co(II)	0.15	In(III)	0.62
Mn(II)	0.25	Ni(II)	0.31	In(III)[b]	0.54
Fe(II)	0.06	Zn(II)	1.00	Pb(II)	0.20

a) The value obtained after zinc loading was taken as unity.
b) One-fifth of the standard dose was given (*i.e.* 30 μmol/kg).

The data in Table I suggest that all the metals induced MT when injected intraperitoneally at the dose of 150 μmol/kg. Even Fe(II) gave MT peaks one order of magnitude larger than the control (saline treatment). Although the metal solutions used for injection are somewhat hypertonic, this factor is ruled out as the cause of MT induction by the following observations (data not shown). (1) Injection of hypertonic (1.35%) NaCl solution induced only a negligible amount of MT (comparable to that induced by isotonic saline treatment). (2) Aqueous and saline solutions of nickel acetate induced similar levels of MT.

Another factor to be taken into consideration is the acidity of the solutions injected. In the case of trivalent cations (Cr(III), Fe(III) and In(III)), the pH values of the solutions were 2—4, and the injection of such an acidic solution may induce MT because it is stressful.[6] In fact, the intraperitoneal injection of 10^{-2} N HCl (0.2 ml) resulted in detectable induction of MT (0.05 relative to zinc-loading). Therefore, a considerable part of the difference in the amount of induced MT between Fe(II) and Fe(III) could be explained in terms of the difference in acidity. On the other hand, In(III) and Cr(III) ions appear to possess high ability to induce MT *per se*.

Indium showed the second highest ability to induce MT, and even one-fifth of the standard dose produced MT in a considerable amount. The high ability of indium to induce Zn–Th was comparable to that of zinc ion. It is interesting to note that the In(III) ion has a $4d^{10}$ electron configuration and tends to form polynuclear complexes[26] with sulfur-containing ligands,[27] which are also the case for Cd(II) ion. Therefore, the incoropration of indium into MT is not unexpected. However, it was previously reported that the indium ion, which is classified as a hard acid,[28] was not detected in the MT fraction on filtration.[13] Although indium can be monitored by HPLC–AAS, the sensitivity is low.

Selenium injected as sodium selenite also induced Zn–Th. Although selenium so far has been classified as one of the metals which are unable to induce MT,[14,15] the present study clearly shows that selenium can induce Zn–Th.

Zn is one of the metals which directly induce MT *per se* and are incorporated into the induced MT. On the other hand, the other metals tested here are considered to induce Zn–Th indirectly *via* an altered hormone level[29] or the disturbance of zinc homeostasis[9] by stress,[6] or disease[10] upon intraperitoneal injection of a large amount of heavy metal. Thus, it is possible that some part of the MT induction by zinc is also due to indirect mechanism(s).

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The concentrations of some essential metals in the liver after administration of various metal ions are given in Table II. No significant changes were observed in magnesium and copper concentrations, while the levels of three other metals were influenced in several cases. Significantly increased zinc concentrations were detected after metal treatments which produced a Zn–Th level larger than 0.20 (Table I). The concentrations correlated reasonably well with the MT levels.

TABLE II. Concentrations of Some Essential Metals in the Liver (μg/g wet weight)[a] after Loading with Various Metals

Injected metal	Mg	Ca	Fe	Cu	Zn
Saline	261±10	38.7±2.2	147±43	6.1±0.8	29.6±2.4
Cr(III)	254± 5	44.2±2.2**	136±35	6.9±0.8	34.7±3.9*
Mn(II)	251±12	42.9±4.4	160±28	5.9±0.9	35.2±3.2*
Fe(II)	259± 4	42.4±2.7	231±48*	6.7±1.0	29.3±2.1
Fe(III)	261± 5	51.0±5.8**	183±48	7.0±1.1	32.3±2.7
Co(II)	255± 9	36.5±2.0	128±31	5.5±0.7	31.9±3.2
Ni(II)	255± 8	38.6±2.1	200±29*	6.2±0.7	40.2±5.6**
Zn(II)	267± 8	46.0±7.7	173±36	6.7±1.0	54.6±8.8***
Se(IV)[b]	264± 9	38.9±3.2	130±24	6.8±0.8	35.7±4.4*
In(III)	259±10	67.9±13.5***	217±54*	6.5±0.7	46.8±6.9***
In(III)[b]	265±12	59.2±8.8***	177±32	6.5±1.0	46.1±3.6***
Pb(II)	269±11	119±42**	144±35	6.7±0.7	33.1±2.2

a) Values are means ±S.D. ($n = 6$). Significant differences from saline treatment are represented as follows: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.
b) Dose was reduced to one-fifth.

We have reported that the time-course of calcium concentration in the liver correlated well with that of Zn–Th level (transitory increase with a maximum at 1 or 2 dauys after the injection), when MT was induced by certain organic chemicals [12]and metal.[16] Therefore, the time-courses of calcium concentration in the liver were followed for the metal loadings which produced the largest (lead) and the second largest (indium) changes of calcium level at 24 h post-injection. The calcium concentrations in the liver at 1 and 7 days after the injection of lead and indium are shown in Fig. 2A. The increase of calcium level was transitory on indium

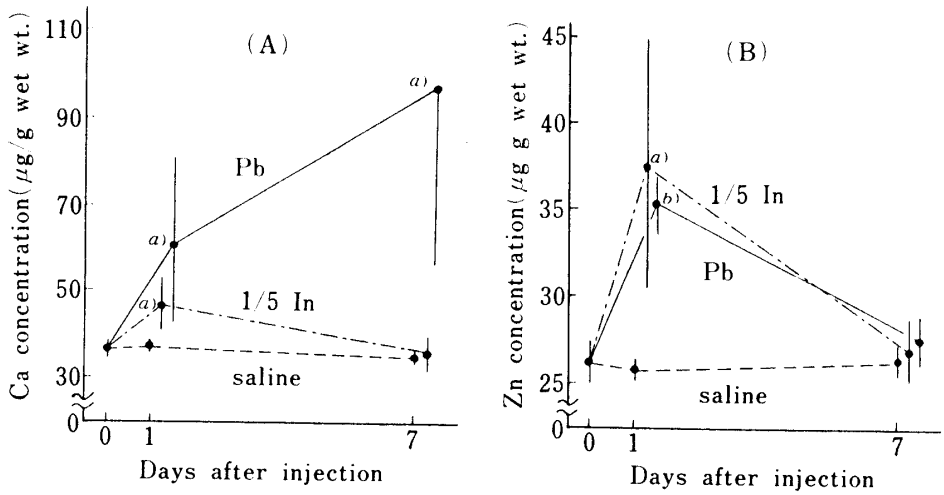

Fig. 2. Changes of Calcium (A) and Zinc (B) Concentrations in Mouse Liver after Injection of Pb(II), 1/5 In(III) and Saline Solutions

Control mice (at day 0) were killed without any treatment. Significant differences from saline treatment are represented as follows ; *a*) $p<0.05$, b) $p<0.001$. Pb, —— ; 1/5 In, —·—· ; saline, ---.

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loading, but prolonged on lead administration. On the other hand, the zinc level (namely Zn–Th level) had fallen to the control (saline treatment) value at 7 days after both injections (Fig. 2B).[24] Consequently, on indium loading, there appeared to be a correlation between calcium and Zn–Th levels with the induction of a large quantity of MT. The induction of the large quantity of MT may be one of the responses of the liver to a pathological condition which accompanies the transitory increase of calcium level. However, an increase of calcium level is not always necessary for the induction of MT (Table II).

Lead tends to form insoluble salts with various anions. Hence, intraperitoneally injected lead is precipitated to some extent in the peritoneal cavity,[30] and a white deposit adhering to the surface of the liver was observed both at day 1 and day 7. A liver sample that was prepared by gathering only parts of the liver with this precipitate showed extraordinarily high lead and calcium concentrations (about 400 and 1600 μg/g wet weight, respectively). Therefore, a considerable part of the unusally high calcium level observed in the liver at days 1 and 7 in the present study seems to be due to the deposit on the liver. Yamaguchi *et al.* reported similar long-lasting deposition of lead in the liver after injecting lead acetate into rats,[31] and explained the observation in terms of the mobilization of calcium from bone[32] and the deposition of the metal in the nuclear fraction with lead.[32] Although our dose was one-fifth of that of Yamaguchi *et al.*, a considerable part of the high calcium level observed in the present study at days 1 and 7 may be produced by the same mechanism as that described by Yamaguchi *et al.*[31–33] On the other hand, no deposit was observed and the increase of calcium level was temporary in the case of indium loading. Therefore, the persistent high calcium level observed after lead loading was different in origin from that after indium administration.

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