Biochemical Pharmacology, Vol. 25, pp. 753–756. Pergamon Press, 1976. Printed in Great Britain.

INHIBITION OF INTRALYSOSOMAL PROTEOLYSIS IN MOUSE LIVER AND KIDNEY PHAGOLYSOSOMES BY ZINC

JOHN L. MEGO

Department of Biology and The Interdisciplinary Biochemistry Program, University of Alabama, University, Ala. 35486, U.S.A.

(*Received* 18 *June* 1975; *accepted* 8 *August* 1975)

Abstract—Previous studies have shown that cadmium ions cause disruption of albumin-filled phagolysosomes *in vitro*, and that cadmium and mercuric salts inhibit intralysosomal proteolysis and phagolysosome formation *in vivo* in mice after intraperitoneal injections [*Biochem. Pharmac.* **22**, 373 (1973) and **24**, 1227 (1975)]. Cupric, stannous, lead, mercuric and zinc salts also inhibited proteolysis when added to phagolysosome suspensions in 1 mM concentrations, and all of these substances caused phagolysosome disruption except zinc. Reduced rates of intralysosomal proteolysis were noted in liver and kidney phagolysosomes from mice injected intraperitoneally with zinc at less than LD_{50} doses, but the metal had no inhibitory effect of phagolysosome formation in the liver as measured by the uptake of labeled protein into osmotically releasable form. The inhibitory effects of zinc on intralysosomal proteolysis were evident in liver phagolysosomes up to about 6 hr after injections, but effects on kidney phagolysosomes were more prolonged. The results suggest that zinc may be taken up into phagolysosomes where it inhibits cathepsins B and C.

In previous studies, we have shown that intraperitoneal injections of cadmium acetate [1] or mercuric chloride [2] into mice inhibit phagolysosome formation and intralysosomal proteolysis of intravenously injected ^{125}I-labeled bovine serum albumin in kidneys or livers. Cadmium chloride was also found to inhibit intralysosomal proteolysis when added to phagolysosome suspensions. The inhibitory effects of cadmium *in vitro* were caused by phagolysosome disruption, as shown by the release of labeled protein into the medium. The fungal toxin rubratoxin also inhibited phagolysosome formation *in vivo* and inhibited intralysosomal proteolysis after intraperitoneal injections into mice, but this toxin had no effect on phagolysosome suspensions *in vitro* [3]. Similarly to rubratoxin, cadmium and mercuric ion, zinc acetate also inhibited intralysosomal proteolysis when injected intraperitoneally into mice, but unlike these substances, zinc had little effect on phagolysosome formation. Results of these studies are reported in this communication.

MATERIALS AND METHODS

Methods used in these studies have been reported elsewhere [2, 3]. Briefly, mice were injected intraperitoneally with the substance to be tested dissolved in physiological saline. After the interval of time shown in the tables, the animals were injected intravenously with 0·1 ml (1 mg) formaldehyde-treated ^{125}I-labeled bovine serum albumin. Thirty min later, the mice were anesthetized with ether and the livers and kidneys were removed and homogenized in cold 0·25 M sucrose. The homogenates were centrifuged to sediment the 500–30,000 *g* particulate fraction and these pellets, containing albumin-filled phagolysosomes, were resuspended and incubated for 60 min at 35° in 0·25 M sucrose, 50 mM mercaptoethanol and 25 mM Tris-acetate buffer, pH 5. Samples were removed at intervals and added to counting tubes containing 2 ml of 10% trichloroacetic acid. The radioactivity in these was counted; they were then centrifuged to sediment precipitated protein and the supernatants were decanted and counted to determine per cent of the total radioactivity converted to trichloroacetic acid-soluble form due to the action of proteases.

Intact phagolysosomes present in particulate suspensions were estimated by osmotic shock. This was accomplished by diluting samples of the suspensions 1:10 in cold 0·25 M sucrose buffered with 0·02 M Tris-acetate, pH 7·3, and in buffer alone. The dilutions were allowed to set on ice for 10 min and then these were centrifuged to sediment all particulate material. The supernatants were decanted and counted. Radioactivity in samples diluted in sucrose buffer was subtracted from the corresponding sample diluted in buffer and the results were calculated as per cent of the total radioactivity. This method was also used to determine phagolysosome formation in kidneys and liver in mice. If the particulate suspensions centrifuged from homogenates of these tissues contained the same relative quantities of osmotically active particles as untreated controls, phagolysosomes formation was assumed to be unaffected. The technique is a reasonably valid estimate of phagolysosome formation because particle-bound, non-osmotically active labeled protein remains relatively constant in tissue homogenates but osmotically active protein varies with the extent of the phagolysosome formation [4].

All counts were performed with a Packard Gamma Well Counter with automatic sample changer.

RESULTS

A number of heavy metal salts were screened for inhibitory effects on intralysosomal proteolysis in

Table 1. Effects of some metal salts on intralysosomal proteolysis and phagolysosome stability*

Salt	Proteolysis (% in 40 min) Kidney	Liver	Osmotically releasable radioactivity (% of total) Kidney 0 time	Kidney 20 min	Kidney 40 min	Liver 0 time	Liver 20 min	Liver 40 min
$CuSO_4$	4·8	18·1	10·6	0	0·5	66·4	19·5	16·9
$Zn(C_2H_3O_2)_2$	10·4	16·4	72·7	53·3	33·6	66·9	46·9	36·4
$ZnSO_4$	7·1		62·5	41·1	30·7	69·3	52·3	36·1
$Pb(NO_3)_2$	18·2	17·1	66·1	13·1	3·2	60·3	39·0	25·0
$SnCl_2$	9·9	17·9	71·3	12·2	4·3	78·2	52·2	37·0
$HgCl_2$	1·4	13·3	57·6	0·4	2·3	60·3	41·9	31·6
Controls	30·6 (1·6)	25·3 (1·5)	75·2 (5·6)	43·6 (12·7)	30·7 (5·3)	73·0 (6·4)	41·8 (10·8)	40·7 (5·2)

* Mice were injected intravenously with 1 mg (0·1 ml, about $4·4 \times 10^6$ cpm) ^{125}I-labeled albumin. The 500–30,000 *g* subcellular particulate fractions (unwashed) were incubated at 35° in the presence and absence of 1 mM concentrations of the salts in the incubation mixture described in Materials and Methods. Controls (no metals) are shown with standard deviations in parentheses. Samples were removed at 0, 10, 20, 40 and 60 min and assayed for trichloroacetic acid-soluble radioactivity and osmotically releasable (in dilute pH 7·3 buffer) radioactivity.

mouse kidney and liver phagolysosome suspensions. These included nickel chloride, sodium tungstate, zinc acetate, zinc sulfate, stannous chloride, cupric sulfate, lead acetate, lead nitrate, cobaltous sulfate, cobaltous chloride, mercurous chloride, mercuric chloride, mercuric acetate, ferrous sulfate, ammonium molybdate, sodium molybdate, strontium chloride, barium hydroxide, calcium chloride, manganous chloride, and beryllium nitrate. Only cupric sulfate, zinc acetate, zinc sulfate, lead nitrate, stannous chloride and mercuric chloride inhibited intralysosomal proteolysis in 1 mM concentrations (Table 1). With the exception of zinc salts, all these substances caused the disruption of kidney phagolysosomes but had little or no apparent effects on the integrity of liver phagolysosomes. Furthermore, mercuric acetate, like cadmium acetate [1] had no effect. Further studies showed that mercuric chloride in the presence of acetate buffer and mercaptoethanol also had no effect. If mercaptoethanol was omitted from the suspensions, mercuric acetate caused phagolysosome disruption similar to mercuric chloride. Similar results to these were obtained in studies with cadmium salts [1].

Since both mercuric and cadmium salt injections caused decreases in osmotically releasable radioactivity in mouse livers or kidneys after intravenous injections of ^{125}I-labeled serum albumin as well as inhibitions of intralysosomal proteolysis, it was of interest to determine whether zinc would produce similar effects. These studies should provide information concerning the action of metal ions on membrane systems, since effects on phagolysosomes *in vitro* might reflect their action *in vivo*.

The LD_{50} for zinc acetate in the mice used in this study, determined according to Weil [5], was 12·8 mg Zn/kg. Table 2 shows the effects of various concentrations of zinc acetate on intralysosomal proteolysis and on phagolysosome formation (osmotically active particles containing radioactive protein) in mouse liver and kidneys 2 hr after intraperitoneal injections.

Table 2. Effect of zinc acetate dose 1 hr after intraperitoneal injections on uptake of ^{125}I-labeled albumin in osmotically active mouse kidney and liver phagolysosomes and on proteolysis in these particles*

Zn dose (mg/kg)	Intralysosomal proteolysis (% in 40 min) Kidney	Liver	Osmotically releasable radioactivity (% of total) Kidney	Liver
6·0	24·7	25·3	77·8	72·0
7·0	20·3	24·6	66·0	63·0
8·0	20·0	16·7	64·0	72·6
13·0	16·5	9·1	58·2	64·5
16·0	9·5	8·0	45·3	66·8
20·0	9·9	6·2	27·6	46·9
None	36·5 ± 4·3	26·8 ± 4·7	75·8 ± 5·0	74·0 ± 4·3

* Osmotically releasable radioactivity refers to the per cent of the particle-bound (500–30,000 *g*) radioactivity released into the medium after suspension in a × 10 vol. of 0·01 M Tris-acetate buffer, pH 7·3. Proteolysis refers to the per cent of the particle-bound radioactivity converted to a trichloroacetic acid-soluble form during 40 min of incubation at 35°. Controls (same as Table 3) are shown with standard deviations of the means.

Table 3. Effect of time after injection of zinc acetate (8 mg/kg) on uptake of ^{125}I-labeled albumin in osmotically active 30,000 *g* mouse kidney and liver phagolysosomes and on proteolysis in these particles*

Time (hr)	No. of expts.	Intralysosomal proteolysis (% in 40 min)		Osmotically releasable radioactivity (%)	
		Kidney	Liver	Kidney	Liver
0	3	31·0	22·8	73	81
1	5	19·1 ± 1·3	13·8 ± 0·8	62 ± 2·2	72 ± 0·7
2	5	20·0 ± 1·8	16·7 ± 2·8	64 ± 1·2	73 ± 4·3
4	5	25·5 ± 1·3	19·5 ± 2·8	64 ± 3·4	77 ± 0·9
6	4	24·3 ± 0·8	22·4 ± 0·4	61 ± 1·8	71 ± 1·7
12	4	24·7 ± 0·6			
24	4	31·1 ± 2·1			
Control	13	36·5 ± 1·2	26·8 ± 1·3	76 ± 1·3	74 ± 1·1

* Time refers to the hours after intraperitoneal injections of zinc acetate that labeled albumin injections were performed. All figures, except 0 time, are shown with standard errors of the means.

These injections produced no apparent effect on protein uptake in the kidneys or livers except at doses higher than the LD_{50}. There were progressively increasing inhibitory effects on both intralysosomal proteolysis and on phagolysosome formation. At 8 mg/kg, a dose less than the LD_{50}, there appeared to be little effect on phagolysosome formation in the liver, but intralysosomal proteolysis was inhibited about 40 per cent. This dose was analyzed in further detail, and the results are shown in Table 3. At 8 mg/kg, intralysosomal proteolysis was inhibited in both liver and kidney phagolysosomes with a maximum effect at 1 hr after injection and slow recovery up to about 24 hr after injection. The kidney appeared to require a longer period for recovery than the liver. In spite of the inhibitory effects of zinc injections on proteolysis, there was no apparent effect of phagolysosome formation in the liver. Some inhibition (about 20 per cent) did occur in the kidneys, however, perhaps due to concentration in these organs during excretion of the metal.

DISCUSSION

Misaka and Tappel [6] have shown that zinc is a potent inhibitor of cathepsins B and C, and to a lesser extent cathepsin D. In the present study, 8 mg/kg of Zn^{2+} inhibited intralysosomal proteolysis, but had no effects on uptake of formaldehyde-treated ^{125}I-labeled serum albumin in the liver or kidneys or on phagolysosome formation at the same dose. This suggests that the metal might have accumulated in lysosomes where it exerted inhibitory effects on cathepsin activities. Tappel *et al.* [7] found relatively high concentrations of zinc as well as other metals in lysosomes of animals which suggests that zinc may become sequestered in these organelles.

The apparently greater sensitivity of mouse kidney phagolysosomes to heavy metal salts *in vitro* (Table 1) is difficult to explain. We have noted that kidney phagolysosomes generally appear to be more sensitive to a number of substances. For example, inhibitory effects of alkaline buffers and responses to ATP are greater in mouse kidney phagolysosomes [8]. Although all the metal salts shown in Table 1 inhibited intralysosomal proteolysis in liver phagolysosomes, there were no apparent effects on phagolysosome integrity. The reason for this is also not clear, and no explanation can be offered at the present time other than the possibility that the substances may have exerted their effects directly on cathepsin activity with the phagolysosomes.

In previous studies on interactions of various hepato- and nephro-toxins with mouse liver and kidney phagolysosomes formation and function, we have noted that some substances affect only liver, some affect only kidney and some affect both kidney and liver phagolysosomes. For example, mercuric chloride inhibited phagolysosome formation and function only in the kidneys [2], and the major effects of Cd^{2+} and rubratoxin were in the liver [1, 3]. The potent hepatotoxin carbon tetrachloride affected neither liver nor kidney phagolysosomes at lethal doses [9]. The reason for these differential effects is not clear at the present time. However, many substances, including heavy metal salts, may be taken up principally by either the kidney or the liver or both. For example, Cd^{2+} is first taken up in the liver and later it is excreted by the kidney where it becomes temporarily concentrated [10]. These changes in distribution correlate with effects on phagolysosomes [1], i.e. effects were noted in the liver at about 2 hr after injection of Cd^{2+} but kidney phagolysosomes were not affected until about 18 hr later. Furthermore, in all studies carried out thus far, those substances which inhibited intralysosomal proteolysis after intraperitoneal or intravenous injection also inhibited phagolysosome formation. All these substances except rubratoxin [3] caused phagolysosome disruption when added to suspensions of mouse kidney phagolysosomes. Zinc represents a notable exception with respect to phagolysosome formation in that it caused inhibition of intralysosomal proteolysis in mouse liver phagolysosomes after intraperitoneal injection but had no effect on the relative quantity of osmotically releasable radioactivity (phagolysosome formation) at the same dose.

Acknowledgements—Mrs. K. Chang performed the technical work in this study. The work was supported by Public

Health Service Grant ES 00591 from the National Institute of Environmental Health Science.

REFERENCES

1. J. L. Mego and Judith A. Cain, *Biochem. Pharmac.* **24,** 1227 (1975).
2. J. L. Mego and Judith Barnes, *Biochem. Pharmac.* **22,** 373 (1973).
3. J. L. Mego and A. W. Hayes, *Biochem. Pharmac.* **22,** 3275 (1973).
4. J. L. Mego and J. D. McQueen, *J. Cell. Physiol.* **70,** 115 (1967).
5. C. S. Weil, *Biometrics* **8,** 249 (1952).
6. E. Misaka and A. L. Tappel, *Comp. Biochem. Physiol.* **38B,** 651 (1971).
7. A. L. Tappel, S. Shibko, M. Stein and J. P. Susz, *J. Fd Sci.* **30,** 498 (1965).
8. J. L. Mego, R. M. Farb and Judith Barnes, *Biochem. J.* **128,** 763 (1972).
9. J. L. Mego and Judith A. Cain, *Biochim. biophys. Acta* **297,** 343 (1973).
10. L. Friberg, M. Piscator and G. Nordberg, *Cadmium in the Environment*, pp. 27–68. C.R.C. Press, Cleveland, Ohio (1971).