SOME FACTORS AFFECTING ENZYME RELEASE FROM CEREBRAL LYSOSOMES: INHIBITORY EFFECTS OF LEAD

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Abstract—The mode of the inhibitory effect of lead ion on the release of enzymes from cerebral lysosomes isolated from young Wistar rats was examined. The incubation of cerebral lysosomes in a low pH medium or with adenosine triphosphate (1 mM) at neutral pH resulted in the decrease of the release of acid phosphatase (EC 3.1.3.2) and β -N-acetylglucosaminidase (EC 3.2.1.30) activities. Multivalent cations such as Mn^{2+} , Co^{2+} and La^{3+} inhibited the enzyme release, while Ca^{2+} facilitated the release. On the other hand, lead ion suppressed the Ca^{2+} -induced enzyme release, but this suppressive effect of lead ion was eliminated by the treatment of lysosomes with phospholipase C and phospholipase A_2 . These results suggest that lead ion may alter the ionic permeability of cerebral lysosomal membrane by reacting with membraneous phospholipids, and thus may prevent the release of lysosomal enzymes in vitro.

The pathogenesis of neurological disorders associated with lead poisoning seems to be one of the important problems which should be clarified urgently. The neurotoxic effects of lead, however, has not been well understood, although various neurochemical or morphological studies have been employed on this disease (1–4). In a previous report (5), we have indicated that the accumulation of lead in the brain is larger in young animals than in adult animals, and the release of cerebral lysosomal enzymes in vitro is inhibited by lead acetate. The present study has been carried out to determine the mechanism of action of the inhibitory effect of lead ion on the release of cerebral lysosomal enzymes. Some factors affecting the enzyme release were also examined.

Materials and Methods

Male young rats (Wistar strain) weighing 50–60 g were used in all experiments. After

the decapitation, the brains were quickly removed and homogenized in cold 0.44 M sucrose with a Teflon glass homogenizer. Lysosomes from the brain homogenate were prepared as described previously (5). Isolated lysosomes were resuspended in 0.44 M sucrose-0.177 M KCl solution. The release of lysosomal enzymes was determined as follows: the lysosomal suspension (1.5 ml) was incubated with each test compound and 10 mM Tris-HCl buffer (pH 7.1) in a final volume of 2.0 ml at 37°C for 15 min, except otherwise described. When total enzyme activity in the lysosomes was determined, 0.1% Triton X-100 was added in the incubation medium. After cooling in ice-water, each reaction mixture was centrifuged at 13,000×g for 10 min, and 0.3 and 0.5 ml of the resultant supernatant were used to determine the activities of acid phosphatase (EC 3.1.3.2) and β -N-acetylglucosaminidase (EC 3.2.1.30), respectively.

Acid phosphatase and β -N-acetylglucosaminidase activities were measured spectrophotometrically according to the methods of Barrett and Heath (6) as indicated in our previous report (5). The magnitude of the enzyme release was shown as the percentage of the control or the percentage of total enzyme activity.

Significance of the results was analyzed by the Student's paired *t*-test, and the differences having $P < 0.05$ were considered to be statistically significant.

Materials: P-Nitrophenyl-N-acetyl-D-glucosaminide, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), phospholipase A₂ (porcine pancreas), phospholipase C (*C. welchii*), glutathione, dithiothreitol and N-ethylmaleimide were purchased from the Sigma Chemical Co. (Saint Louis, Mo, U.S.A.). Other chemicals used were guaranteed grade and obtained from Nakarai Chemicals, Ltd. (Kyoto, Japan).

Results

Extralysosomal pH and the release of enzymes: It is probable that the pH gradient across the lysosomal membrane is maintained in living cells since most of the lysosomal

enzymes have acidic pH optimums. Therefore, lysosomal particles were incubated with 5 mM Tris maleate buffer having various pH values

Fig. 1. Relationship between extralysosomal pH and the release of lysosomal enzymes. Cerebral lysosomes were incubated in the medium buffered with 5 mM Tris maleate described in the text. Enzyme activity released was indicated as the percentage of total activity which was determined after solubilizing with 0.1% Triton X-100. Open and closed circles represent β -N-acetylglucosaminidase and acid phosphatase activities, respectively. Each point is the mean of two separate experiments.

Table 1. Effect of adenine nucleotides on release of lysosomal enzymes

		Enzyme activity (% of control) $\pm$ S.E.		
		Released (E ₁)	Total (E ₂) [#]	(E ₁ /E ₂) $\times$ 100
Control		100	100	100
ATP (1 mM)	AP	61 $\pm$ 3	107 $\pm$ 2	57 $\pm$ 2***
	AG	65 $\pm$ 3	99 $\pm$ 2	66 $\pm$ 2***
ADP (1 mM)	AP	82 $\pm$ 9	104 $\pm$ 6	78 $\pm$ 6*
	AG	86 $\pm$ 4	100 $\pm$ 2	86 $\pm$ 4*
AMP (1 mM)	AP	97 $\pm$ 11	95 $\pm$ 7	103 $\pm$ 12
	AG	100 $\pm$ 1	99 $\pm$ 1	102 $\pm$ 2

Cerebral lysosomes were incubated with each test compound in the presence (E₂) or absence (E₁) of 0.1% Triton X-100. After centrifugation, enzyme activity in the resultant supernatant was measured. Each value indicates the mean $\pm$ S.E. obtained from four separate experiments. [#] Determined in the presence of 0.1% Triton X-100. *** $P < 0.01$ and * $P < 0.05$, compared with each control value. Abbreviations used: AP, Acid phosphatase; AG, β -N-Acetylglucosaminidase.

Table 2. Effect of metallic cations on release of lysosomal enzymes

Cations	Enzyme activity released (% of control) $\pm$ S.E.	
	AP	AG
Control	100	100
Ba ²⁺ (1 mM)	81 $\pm$ 8	83 $\pm$ 6
Mg ²⁺ (1 mM)	83 $\pm$ 8	84 $\pm$ 3**
Mn ²⁺ (1 mM)	55 $\pm$ 6***	73 $\pm$ 1***
Co ²⁺ (1 mM)	50 $\pm$ 10**	60 $\pm$ 4***
La ³⁺ (1 mM)	50 $\pm$ 15*	40 $\pm$ 11*

Enzyme activity appearing in the supernatant was determined as described in the text. The mean $\pm$ S.E. obtained from four experiments is shown. *** $P < 0.01$, ** $P < 0.02$, * $P < 0.05$, compared with each control value. Abbreviations used: AP, Acid phosphatase; AG, β -N-Acetylglucosaminidase.

adjusted by NaOH solution. The release of acid phosphatase and β -N-acetylglucosaminidase decreased in parallel with the decrease of extralysosomal pH, and it showed a minimum at pH 5.4 (Fig. 1).

Effect of adenine nucleotides on the release of enzymes: The addition of ATP inhibited the lysosomal release of both enzymes without directly affecting the activity of each enzyme. ADP was less effective than ATP on the release of the enzymes, and AMP was found to be ineffective for preventing the release of both enzymes (Table 1).

Effect of metallic cations on the release of enzymes: Table 2 shows the effect of metallic cations, which were added as chloride salts, on the release of lysosomal enzymes. Ba²⁺ poorly inhibited the release of both enzymes, and Mg²⁺ slightly inhibited the release. On the other hand, Mn²⁺, Co²⁺ and La³⁺ drastically inhibited the release of both enzymes from cerebral lysosomes. These cations, however, did not interfere with the assay of both enzyme activities (data not shown).

Interaction between Pb²⁺ and Ca²⁺ on the release of lysosomal enzymes: Calcium is well known to play important roles in various cell functions, for example, in the release of neurotransmitters (7). In addition, calcium-lead interaction in neuronal tissues has been demonstrated (2, 8–10). The addition of

Fig. 2. Effect of lead acetate on calcium-induced release of lysosomal enzymes. Cerebral lysosomes were incubated with various concentrations of CaCl₂ alone (open circle) or with CaCl₂ and lead acetate (10⁻⁴ M) (closed circle). The extent of the enzyme release was expressed as percentage of the control value which was determined in the absence of Ca²⁺ and Pb²⁺. Each point represents the mean of two separate experiments or the mean $\pm$ S.E. obtained from four separate experiments. ^a $P < 0.01$, ^b $P < 0.05$, compared with each control value.

10⁻⁴–10⁻³ M calcium significantly stimulated the release of both acid phosphatase and β -N-acetylglucosaminidase activities. The calcium-induced enzyme releases, however, disappeared completely in the presence of 0.1 mM lead acetate (Fig. 2).

Effect of sulfhydryl compounds on the release of lysosomal enzymes: Since Van Caneghem (11) has shown that sulfhydryl

compounds stabilize the hepatic lysosomes of the rat, we have also examined the effect of sulfhydryl compounds on the release of enzymes from cerebral lysosomes (Table 3). The total activity of acid phosphatase was increased 2 or 3-fold by the addition of reduced glutathione or dithiothreitol, whereas β -N-acetylglucosaminidase activity remained unchanged. When the ratio of apparent activity released (E_1) to total activity (E_2) was calculated, it became clear that the amount of enzymes released was diminished by the addition of reduced glutathione, N-ethylmaleimide or dithiothreitol. Oxidized glutathione slightly inhibited the release of acid phosphatase. On the other hand, reduced glutathione alone significantly inhibited the release of β -N-acetylglucosaminidase. Dithiothreitol also had a tendency to inhibit the

enzyme release. These results strongly suggest that the reduction of SH groups in the lysosomal membrane may suppress the release of lysosomal enzymes.

Pb^{2+} (10^{-4} M), as reported previously, prevented the lysosomal releases of acid phosphatase and β -N-acetylglucosaminidase without having an effect on the enzyme activities. The addition of an equimolar concentration of a thiol compound with Pb^{2+} hardly modified the preventative effect of Pb^{2+} .

Interaction between Pb and phospholipase on the release of lysosomal enzymes: The addition of phospholipase C or phospholipase A_2 increased the release of both lysosomal enzymes. Acid phosphatase was more sensitive to the addition of phospholipases than β -N-acetylglucosaminidase. Furthermore, the

Table 3. Effect of sulfhydryl compounds on release of lysosomal enzymes

			Enzyme activity (% of control) $\pm$ S.E.		
			Released (E_1)	Total (E_2) [#]	(E_1/E_2) $\times$ 100
Control			100	100	100
GSH	(1 mM)	AP	117 $\pm$ 4	282 $\pm$ 10	57 $\pm$ 3***
		AG	85 $\pm$ 4	102 $\pm$ 2	83 $\pm$ 4*
GSSG	(1 mM)	AP	108 $\pm$ 14	155 $\pm$ 7	70 $\pm$ 10
		AG	99 $\pm$ 2	99 $\pm$ 2	101 $\pm$ 4
NEM	(1 mM)	AP	93 $\pm$ 5	125 $\pm$ 9	76 $\pm$ 7*
		AG	109 $\pm$ 2	101 $\pm$ 2	108 $\pm$ 4
DTT	(1 mM)	AP	91 $\pm$ 13	204 $\pm$ 14	44 $\pm$ 4***
		AG	82 $\pm$ 5	91 $\pm$ 3	91 $\pm$ 7
Pb^{2+}	(0.1 mM)	AP	68 $\pm$ 5	98 $\pm$ 4	69 $\pm$ 6*
		AG	88 $\pm$ 2	100 $\pm$ 1	88 $\pm$ 1***
{ Pb^{2+} + GSH	(0.1 mM)	AP	62 $\pm$ 5	104 $\pm$ 7	60 $\pm$ 8*
		AG	84 $\pm$ 4	101 $\pm$ 2	83 $\pm$ 3**
{ Pb^{2+} + GSSG	(0.1 mM)	AP	63 $\pm$ 5	98 $\pm$ 5	65 $\pm$ 8*
		AG	87 $\pm$ 3	101 $\pm$ 2	86 $\pm$ 2***
{ Pb^{2+} + DTT	(0.1 mM)	AP	84 $\pm$ 3	127 $\pm$ 9	67 $\pm$ 4**
		AG	89 $\pm$ 2	98 $\pm$ 1	92 $\pm$ 3

Enzyme activity was measured as described in the text and footnote of Table 1. The mean $\pm$ S.E. from three or four separate experiments are shown. [#] Determined in the presence of 0.1% Triton X-100. *** $P < 0.01$, ** $P < 0.02$, * $P < 0.05$, compared with each control value. Abbreviations used: GSH, Reduced glutathione; GSSG, Oxidized glutathione; NEM, N-Ethylmaleimide; DTT, Dithiothreitol; AP, Acid phosphatase; AG, β -N-Acetylglucosaminidase.

Fig. 3. Interaction between lead and phospholipases on the release of lysosomal enzymes. Each value indicates the mean $\pm$ S.E. obtained from four separate experiments. ^a $P < 0.01$, ^b $P < 0.02$, ^c $P < 0.05$, compared with each control value which was determined in the absence of lead ion and phospholipases. Abbreviations used: PLC, phospholipase C; PLA₂, phospholipase A₂.

addition of both phospholipases eliminated the Pb-induced inhibition (Fig. 3).

Discussion

Intralysosomal pH is thought to be more acidic than extralysosomal pH in living cells (12, 13). Two mechanisms which maintain such a low pH in the inside of lysosomes have been postulated: one of these hypotheses is that a low intralysosomal pH is the consequence of a Donnan equilibrium resulting from the non-diffusible anions inside of the lysosomes and the selective permeability of the membrane to cations (14), and the other hypothesis is that an ATP-dependent proton pump which is linked to ATPase activity is present in the lysosomal membrane (15–17). On the other hand, it has been reported that the internal pH of secondary lysosomes isolated from the liver is strongly influenced by the pH of the surrounding medium: the intralysosomal pH increases as the external pH is raised, especially when a high concentration of potassium ion is added into the medium (14, 18).

If the above findings are applicable to brain lysosomes, the decrease in the release

of enzymes with the fall of extralysosomal pH found in the present study may be attributed to the changes in the intralysosomal pH. This explanation may be further supported by the fact that the addition of ATP attenuates the release of enzymes, since ATP is known as an agent which lowers the intralysosomal pH in vitro probably due to the stimulation of the ATP-dependent proton pump (17). Although the exact reason why a low intralysosomal pH prevents the enzyme release is unknown at present, it may be possible that the dissociation of enzymes from the binding sites may be facilitated under these conditions. In fact, it has been reported that acidification of lysosomes induces the dissociation of bound enzymes from receptors (19, 20). In addition, Gonzalez-Noriega et al. (21) showed that chloroquine, which increases intralysosomal pH, enhanced the secretion of lysosomal enzymes and inhibited enzyme pinocytosis in human fibroblast.

Henning (14) indicated that metallic cation-proton exchange across the lysosomal membrane decreased with the decrease of atomic weight and with increasing charge of the cation. Furthermore, he showed that

the difference in the rate of metallic cation-proton exchange was due to the difference in the size of hydrated cation. In the present study, we have also found that metal ions, except for Ca^{2+} , inhibited the release of lysosomal enzymes. Considering these facts, we are attempting to postulate that the inhibitory effect of lead ion on the release of lysosomal enzymes may correlate with the dissociation of lysosomal enzymes from binding sites within lysosomes which are endowed with denatured potassium-proton exchange by Pb^{2+} . Since we incubated the lysosomal particles in the medium containing a high concentration of potassium at pH 7.1, the hindrance of potassium-proton exchange in the lysosomes treated with Pb^{2+} might result in a lower pH within the lysosomes than that in the control.

One of the important findings obtained in this study is that calcium ion alone stimulates the release of lysosomal enzymes, but Pb ion eliminates the calcium-induced release. Considering the fact that Ca^{2+} did not alter the intralysosomal pH (14), the facilitation of lysosomal enzyme release by calcium ion may be attributed to different mechanisms from the pH shift within lysosomes. One possible interpretation may be that an interaction between endogenous Ca^{2+} and added Pb^{2+} is present and contributes to the inhibitory effect of lead, but it remains to be tested in further studies.

In the present study, it has been also found that thiol compounds are able to stabilize cerebral lysosomal membranes as Van Caneghem (11) indicated in liver lysosomes. It is uncertain, however, whether or not Pb ion prevents the release of lysosomal enzymes by reacting with SH groups in lysosomal membranes since oxidized glutathione and alkylating agents such as N-ethylmaleimide inhibited the release of acid phosphatase alone, probably reacting with a sulfhydryl moiety in the enzyme molecule (22, 23).

Furthermore, the addition of equimolar concentration of SH compounds with Pb^{2+} did not modify the Pb-induced alteration. We have concluded, therefore, that the interaction of Pb^{2+} with SH moieties in the lysosomal membrane may not be an essential factor involved in the inhibitory effect of Pb ion.

The inhibitory effect of Pb on lysosomal enzyme release disappeared when exogenous phospholipases were added. This result suggests that the action of Pb ion may be due to the interaction of Pb^{2+} with membrane components such as phospholipids, which are known to be involved in the maintenance of permeability of the lysosomal membrane (24). In mammalian brains, phospholipids are known to be broken down by various kinds of phospholipases which have different pH optimums, different sensitivity to Ca^{2+} , and different subcellular distribution (25–29). Exact molecular mechanisms underlying the interaction between Pb^{2+} and lysosomal membranous phospholipids, however, remain to be examined.

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