An increase in Na^+/K^+ -ATPase activity of erythrocyte membranes in workers employed in a lead refining factory

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ABSTRACT To clarify the relationship between erythrocyte Na^+/K^+ -ATPase activity and haematological findings, several clinical laboratory examinations were performed on 31 male workers employed in a scrap lead refining factory and, as controls, 50 male workers employed in railway construction. The results were: (1) Values for erythrocyte Na^+/K^+ -ATPase activity, blood and urine lead, urine δ -aminolaevulinic acid, and urine coproporphyrin of lead workers were significantly higher than those of the controls ($p < 0.01$). (2) A strongly positive relationship between blood lead and erythrocyte Na^+/K^+ -ATPase activity was observed in lead workers ($r = 0.473$, $p < 0.01$). (3) A strongly negative relationship between Na^+/K^+ -ATPase activity and intracellular sodium was observed in both groups (lead workers; $r = -0.601$, $p < 0.01$; controls; $r = 0.595$, $p < 0.01$).

Sodium and potassium dependent adenosine triphosphatase (Na^+/K^+ -ATPase) exists in erythrocyte membranes and regulates the active transport of cations. Lead is an inhibitor of Na^+/K^+ -ATPase, and this inhibition is non-competitive and reversible.¹⁻³ A reduction of Na^+/K^+ -ATPase activity has been reported in erythrocyte membranes of workers exposed to lead.⁴⁻⁶ We⁷ observed an increase in erythrocyte Na^+/K^+ -ATPase activity in workers employed in a lead refining factory, the opposite to the results of Hasan *et al*⁴ and Secchi and Alessio⁵ and Secchi *et al*.⁶

The activity of Na^+/K^+ -ATPase in recent years has been found to be dependent on the presence of phospholipids.⁸⁻¹² The enzyme was inactive or impaired after lipid extraction, treatment with phospholipid-splitting enzymes, or solubilisation with detergent, and reactivated after the addition of phospholipids. On the other hand, the enzyme activity was inhibited by the addition of lysophosphatidylcholine (LPC).¹³ Our recent work¹⁴ showed increased phosphatidylcholine (PC) and decreased lysophosphatidylcholine in the erythrocyte membranes of workers exposed to lead. Therefore, we

assume that the increased enzyme activity relates to the change in the phospholipid composition of erythrocyte membranes. On the other hand, we cannot ignore the influence of the tendency towards anaemia of workers exposed to lead on the enzyme activity. In this study we try to clarify the relationships between Na^+/K^+ -ATPase activity and various haematological findings in workers occupationally exposed to lead and in controls.

Materials and methods

Thirty-one men exposed occupationally to lead in a scrap lead refining factory were studied. Their mean age was 45.7 years (range 23-73). Fifty men employed in railway construction served as controls; their mean age was 43.5 years (range 19-65). All the procedures described below for both groups were carried out at almost the same time to exclude any differences that might have been due to seasonal fluctuations. Blood samples were taken from a cubital vein into heparinised tubes for the following measurements.

Purified ATPase and erythrocyte membrane fragments—Erythrocyte ATPase was purified according to the method of Ohta *et al*¹⁵ with modi-

Received 12 October 1981
Accepted 27 November 1981

fications. All further procedures were carried out at 4°C. The blood was centrifuged for 10 minutes at 3000 rpm. After removal of the serum and the buffy coat, the erythrocytes were washed three times with 0.15 M NaCl and lysed by forceful injection of deionised water, then shaken vigorously. The haemolysates were left standing for 15 minutes and centrifuged for 20 minutes at 13 000 rpm. The erythrocyte membranes were washed with 2.5 mM Tris-HCl buffer (pH 7.5) three times, 0.1 M Tris-glycylglycine buffer (pH 8.2) once to remove haemoglobin completely from the membranes and to accelerate fragmentation, and twice 2.5 mM Tris-HCl buffer (pH 7.5).

ATPase activity—A membrane fragment suspension (0.1 ml) was incubated in 1 ml of a combination of substrates A and B for one hour at 37°C. Both substrates contained 140 mM NaCl, 14 mM KCl, 5 mM MgCl_2 , 2.5 mM disodium ethylenediaminetetra-acetate (EDTA), 50 mM tris(hydroxymethyl)aminomethane, and 3 mM adenosine 5'-triphosphate (ATP) (Sigma, USA), with substrate B also containing 0.2 mM g-strophanthin (Merck, FDG) (pH 7.5). Substrates without membrane fragments were incubated separately as blanks (substrate C). After incubation, 0.1 ml of membrane fragments was added to substrate C and 1 ml of 10% of trichloroacetic acid (TCA) was added to substrates A, B, and C to stop the enzyme reaction. Next, the mixtures were centrifuged for 10 minutes at 3000 rpm at 4°C. Inorganic phosphorus (Pi) concentrations in the supernatant were measured by the method of Fiske and Subbarow.¹⁶ Activities were calculated using the following equations: Na^+/K^+ -ATPase activity = (pi of substrate A — Pi of substrate B)/protein concentration of fragment suspension. Protein was measured by the method of Lowry *et al.*¹⁷

Erythrocyte count—An automatic blood cell counter (Coulter Counter Model ZF, Coulter Electronics, USA) was used.

Haematocrit—The capillary tube method was used. The mean corpuscular volume was calculated by dividing the haematocrit value by the erythrocyte count.

Blood and urine lead concentrations—Ten millilitres of blood and 100 ml of urine were dried at 80°C on a hot plate and ashed at 500°C in a muffle furnace, then subjected to analysis by the dithionite-spectrophotography method.¹⁸

Urine δ -aminolaevulinic acid (δ -ALA)—The method of Tomokuni and Ogata¹⁹ was used.

Urine coproporphyrin—The method of Rimington modified by Sano²⁰ was used.

Statistical significance was tested by Student's *t* test.

Results

The haematological data from the two groups are shown in table 1. The values for Na^+/K^+ -ATPase of the lead workers was higher than those of the controls, while the values for erythrocyte count and haematocrit were lower. We found significant differences in Na^+/K^+ -ATPase activity ($p < 0.01$). Table 2 shows the laboratory data on lead exposure levels for the two groups. The values for blood and urine lead, urine δ -ALA, and urine coproporphyrin were significantly higher in the lead workers than in the controls ($p < 0.01$). Significant correlations were observed in the lead workers between blood lead concentration and Na^+/K^+ -ATPase activity ($r = 0.473$, $p < 0.01$) (fig 1), and in both groups between Na^+/K^+ -ATPase and intracellular sodium (lead workers; $r = -0.601$, $p < 0.01$; controls; $r = -0.595$, $p < 0.01$) (figs 2 and 3).

Discussion

The addition of lead to erythrocyte membrane fragments strikingly inhibits Na^+/K^+ -ATPase activity in in-vitro experiments.¹⁻³ Our result in vivo is just opposite to the in-vitro experiments and those of Hasen *et al.*⁴ and Secchi and Alessio⁵ and Secchi *et*

Table 1 Haematological data for lead workers and controls

	Lead workers (n = 31)	Controls (n = 50)
RBC ($10^6/\text{mm}^3$)	465 $\pm$ 40	479 $\pm$ 52
Haematocrit (%)	43.5 $\pm$ 3.3	44.7 $\pm$ 4.4
MCV (μ^3)	93.8 $\pm$ 7.8	93.7 $\pm$ 5.7
Na^+/K^+ -ATPase in RBC (Pi nM/mg protein/hr)	357 $\pm$ 85** (n = 31)	308 $\pm$ 44 (n = 24)
Na^+ in RBC (mEq/L)	28.0 $\pm$ 4.1	29.6 $\pm$ 3.2
K^+ in RBC (mEq/L)	97.1 $\pm$ 6.5	96.6 $\pm$ 4.9

RBC = Red blood cell; MCV = mean corpuscular volume; Na^+ = sodium; K^+ = potassium.

**Significant difference at $p < 0.01$.

Table 2 Laboratory data on lead exposure levels for lead workers and controls

	Lead workers (n = 31)		Controls (n = 50)	
	GM	DF	GM	DF
Blood lead ($\mu\text{g}/\text{dl}$)	36.0	1.39**	4.29	1.51
Urine lead ($\mu\text{g}/\text{l}$)	100.1	2.34**	11.6	1.50
Urine δ -ALA ($\mu\text{g}/\text{l}$)	6710	4.53**	2350	1.31
Urine coproporphyrin ($\mu\text{g}/\text{l}$)	51.8	4.01**	10.0	1.68

GM = Geometric mean; DF = dispersion factor;

ALA = aminolaevulinic acid.

**Significant difference at $p < 0.01$.

Fig 1 Correlation between erythrocyte Na^+/K^+ -ATPase activity and blood-lead concentration in lead-exposed workers.

Fig 2 Correlation between erythrocyte Na^+/K^+ -ATPase activity and intracellular sodium in lead-exposed workers.

Fig 3 Correlation between erythrocyte Na^+/K^+ -ATPase activity and intracellular sodium in controls.

al⁶ in vivo. Our method of preparing membrane fragments was almost the same, but the Na^+/K^+ -ATPase was determined differently, being measured with EDTA. In our previous study of lead workers⁷ we observed a significant increase in erythrocyte Na^+/K^+ -ATPase activity measured with EDTA but no significant change in the activity measured without EDTA. This might indicate that the preparation of the erythrocyte membranes removed lead ion from the membranes incompletely, but that treatment with EDTA did so completely. Therefore, by using EDTA in this study we tried to investigate a change in Na^+/K^+ -ATPase affected by the erythrocyte membrane structure in workers exposed to lead for long periods and not by the presence of lead. We also observed fragmentation of the erythrocyte membrane by microscope, although there was no difference between the two groups.

Why did the Na^+/K^+ -ATPase activity of erythrocyte increase in workers exposed to lead? Recently, many studies⁸⁻¹² have reported the relationship between Na^+/K^+ -ATPase activity and phospholipids. Tanaka and Abood²¹ observed that removal of phospholipids resulted in a loss of Na^+/K^+ -ATPase activity, which paralleled the loss of phospholipid and that the addition of various phospholipids restored the activity. The addition of phosphatidylserine (PS) re-activated the enzyme activity.^{22,23} On the other hand, Karli *et al*²⁴ observed in vitro that the addition of lysophosphatidylcholine (LPC) inhibited Na^+/K^+ -ATPase activity, and, in vivo, that the injection of heparin in rabbits caused a pronounced inhibition of the enzyme activity of heart sarcolemma. This might be attributed to an increased FFA and LPC concentration in the plasma due to the activation of lipoprotein lipase and phospholipase A₁. We observed increased PC and decreased LPC in erythrocyte membranes of lead workers in our previous study.¹⁴ From these results, we assume that the decrease in membrane LPC eliminated an inhibitory effect on Na^+/K^+ -ATPase activity and contributes to the increase in the enzyme activity. On the other hand, we observed an increased reticulocyte in workers exposed to lead compared with the controls, but there was no significant difference between the two groups. Reticulocyte has an increased activity of various enzymes and a decreased intracellular sodium compared with erythrocyte. We therefore assume that increases in reticulocyte and young erythrocyte in peripheral blood of workers with anaemic tendency who are exposed to lead may relate to the increased Na^+/K^+ -ATPase activity.

Recently, Barltrop and Smith^{25,26} and Ong and Lee²⁷ reported that most of the lead in blood is bound to haemoglobin not to the erythrocyte

membrane where Na^+/K^+ -ATPase exists; it may thus have a less inhibitory effect on enzyme activity than expected.

In this study we observed a strong negative correlation between Na^+/K^+ -ATPase and intracellular sodium in both groups (figs 2 and 3). Na^+/K^+ -ATPase in erythrocyte membranes controls the active transport of sodium and potassium. An increase in Na^+/K^+ -ATPase activity, therefore, increases intracellular potassium and decreases intracellular sodium. Although we observed that intracellular sodium was lower in workers exposed to lead than in controls, intracellular potassium was almost the same in both groups. The concentration of intracellular cations is also affected by passive transport, thus the increase in Na^+/K^+ -ATPase activity does not necessarily lead to an increase in intracellular potassium and a decrease in intracellular sodium because of the possibility of a change in passive transport caused by membrane alterations. On the other hand, Na^+/K^+ -ATPase transports cations actively by hydrolysing adenosine 5'-triphosphate (ATP). When one mole of ATP is hydrolysed by Na^+/K^+ -ATPase, two ion of potassium flow in and three ions of sodium flow out.²⁸ The effect of Na^+/K^+ -ATPase activity thus affects intracellular sodium 1.5 times more than intracellular potassium. Since the intracellular sodium concentration is about one-fifth that of intracellular potassium, the change in intracellular sodium appears to be five times that of intracellular potassium. In total, Na^+/K^+ -ATPase activity alters intracellular sodium by about 7.5 times that of intracellular potassium. This is in approximate agreement with our results showing a negative relationship between Na^+/K^+ -ATPase activity and intracellular sodium, and no relationships between the enzyme activity and intracellular potassium in both groups. We can also estimate the increase in Na^+/K^+ -ATPase in workers exposed to lead from the negative relationships between Na^+/K^+ -ATPase activity and intracellular sodium in both groups, and the decrease in intracellular sodium in the lead exposed group.

This study supports the view that Na^+/K^+ -ATPase activity in erythrocytes is increased in workers exposed to lead; the difference between these results and those of Hasan *et al*⁴ and Secchi and Alessio⁵ and Secchi *et al*⁶ is probably caused by a difference in experimental method. The measurement of Na^+/K^+ -ATPase with EDTA is recommended as a method that approximates physiological conditions more closely.

We thank Assistant Professor Shuzo Ōtani (Department of Biochemistry, Osaka City

University Medical School) for his kind advice on Na^+/K^+ -ATPase measurement and Miss Yumiko Hiroishi for her help.

This work was supported in part with a Grant-in-Aid for Encouragement of Young Scientists (1981) from the Ministry of Education, Science, and Culture of Japan.

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