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Studies on Ergothioneine. VII.[1] Some Effects of Ergothioneine on Glycolytic Metabolism in Red Blood Cells from Rats

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The lactate level in red blood cells from rats administered ergothioneine by *i.p.* injection for 7 d (1.6 mg/kg/d) then starved for 24 h was maintained at the normal level, whereas that of the control group (no ergothioneine) decreased to 60%. No effect of ergothioneine administration was observed on the lactate level in red blood cells when rats were fed *ad libitum*.

Addition of 10^{-3} M ergothioneine to either a suspension or a hemolysate of red blood cells from intact rats accelerated lactate formation. Moreover, the amounts of glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) in red blood cells decreased to 74% and 51% of the control level respectively. The amount of pyruvate in red blood cells did not change.

It is suggested that ergothioneine had the effect of accelerating the glycolytic metabolism in red blood cells, or effected an increase in the permeability of red blood cells to glucose.

Keywords——ergothioneine; ergothioneine administration; lactate in the red blood cells; effect on red blood cells; glycolysis in red blood cells

Ergothioneine (Erg: 2-thiolhistidine trimethyl betaine) is an SH-compound usually present in the living body, and a considerable amount of Erg is contained in the liver of mammals at a concentration of 10^{-4} M.[2] This concentration is almost equal to one-tenth that of glutathione (GSH), but the physiological role of Erg has not been clarified. We have been studying the significance of Erg from various points of view and have earlier reported some actions of Erg in the red blood cells (RBC). When Erg was added to an RBC suspension, the permeability of glucose into RBC was increased. The permeability, reduced in RBC treated with *p*-chloromercuribenzoic acid (PCMB), was restored to its normal level by Erg.[3] The previous paper reported that administered Erg was trapped and deposited in free form in RBC.[2] These results suggested that Erg has some role in RBC. On the basis of the findings mentioned above, and in view of the SH structure of Erg, the present study was performed mainly to examine the effect of Erg on the glycolytic system in RBC.

Materials and Methods

Chemicals——Erg (free form) was purchased from Nutritional Biochemical Corporation. The other chemicals (from Sigma Chemical Co.) were of analytical grade.

Animals——Wistar strain male rats (6 weeks of age) were fed on pelleted food CE-2 (Japan Clea Co.) and water *ad libitum*, and maintained at 23±1°C, 50—60% humidity, with light and dark conditions at 12 h intervals. Rats were administered Erg at a low dosage (0.8 mg/100 g body weight) or a high dosage (1.6 mg/100 g body weight) intraperitoneally (*i.p.*) for 7 d, and were killed 24 h after the last administration.

Red Blood Cells——Blood was collected from the aorta of rats with heparin treatment. RBC suspension was generally brought to the original volume of blood by suspending it in phosphate-buffered saline (containing 5 mM glucose) after washing it thoroughly to remove serum. For the experiments using a hemolysate, the RBC suspension was prepared to be of 30% hematocrit value. The other experimental conditions are described in the table or figures.

Methods of Measurement——RBC suspension was deproteinized and the resulting supernatants were used for measurement. Lactate was determined by the nicotinamide adenine dinucleotide (NAD) method using lactate dehydrogenase (LDH)[4] and it was confirmed that Erg did not affect the procedure for measuring

lactate. Glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P) and pyruvate kinase activity were determined by the method of Minakami *et al.*[5]

Results and Discussion

Effect of Erg Administration *in Vivo* on Lactate Level in RBC

Lactate was determined in RBC in both normally fed rats and fasted rats after daily Erg administration (*i.p.* injection) for 7 d. As shown in Fig. 1, no effect of Erg administration, in either the low dose or the high dose group, was observed on the lactate level of RBC from rats in normally fed groups. In the case of rats starved for 24 h after the final Erg administration, the lactate level was maintained at that of the normally fed groups, whereas the lactate level in the control group fell to 60% of this level. However, the effect of Erg was not dose-related at these dosages. These results suggest that an increase of Erg concentration in the body did not enhance the glycolysis in RBC, but maintained the normal level of lactate which would otherwise have decreased in the starved rats. Since the glucose level in RBC was decreased by starvation for 24 h, it is also suggested that Erg increased the membrane permeability to glucose and thus allowed the glycolysis in RBC to continue at a normal level. This is in accord with our previous results on the ability of Erg to increase the permeability of RBC *in vitro*.[3]

Effect of Erg on Glycolytic Metabolism of RBC *in Vitro*

Fig. 2 shows the effects of Erg on lactate formation *in vitro*. Erg was added to the hemolysate from intact rats. The control value without Erg addition was taken as 100 (under these conditions, lactate/ml of control hemolysate was 13.1 μmol). When 10^{-3} M Erg was added, the lactate formation was increased by 25%. These results indicate that Erg stimulates the glycolytic system in RBC.

Further, when an intact RBC suspension was used, as shown in Table I, the production of lactate was increased very significantly by the addition of 10^{-3} M Erg and significantly by

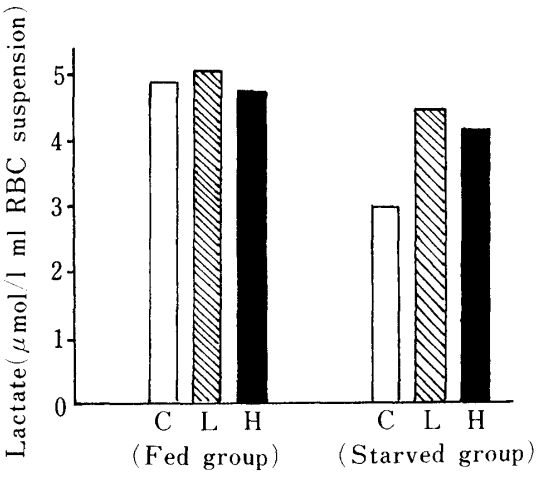

Fig. 1. Effect of Ergothioneine Administration on Lactate Level in Red Blood Cells

□ C: control, saline *i.p.* injection.
▨ L: 0.8 mg Erg/100 g body weight/day *i.p.* injection.
■ H: 1.6 mg Erg/100 g body weight/day *i.p.* injection for a week.

Fed group: Rats took food *ad libitum* until they were bled. Starved group: RBC were collected from rats starved for 24 h after the final administration of Erg.

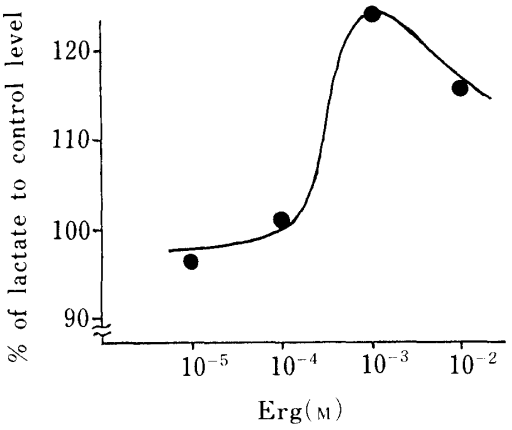

Fig. 2. Increase of Lactate Level produced in the Reaction Mixture with Hemolysate

The enzyme solution was an aliquot of supernatant from sonicated (15 s, 3 times) 30% RBC suspension. One ml of incubation mixture containing 120 mM NaCl, 5 mM KCl, 1 mM K_2HPO_4, 2 mM $MgCl_2$, 30 mM Tris buffer (pH 9.5), 5 mM glucose, 30 mM nicotinamide, 2 mM ATP, 2 mM NAD and 0.3 ml of enzyme solution was incubated for 1 h at 37°C. The supernatant from deproteinized incubation mixture was examined to determine lactate by the use of lactate dehydrogenase. The production of lactate is expressed as a percentage of the value obtained in the absence of Erg.

Table I. Effect of Ergothioneine on Lactate Formation in Red Blood Cells *in Vitro*

Ergothioneine		1 h incubation: Lactate (μmol/4 ml of RBC suspension)[a]	1 h incubation: Increase %	3 h incubation: Lactate (μmol/4 ml of RBC suspension)[a]	3 h incubation: Increase %
[Control]	Without	18.53±1.41	—	41.74±3.55	—
	10^{-5} M	20.52±1.11	10.7	42.83±4.64	2.7
	10^{-4} M	20.40±0.78[b]	10.1	42.51±2.17	1.8
	10^{-3} M	21.17±1.54[c]	14.2	43.78±3.29	4.9

Mean±s.d.

a) Washed RBC were used to make a 30% RBC suspension in medium containing 120 mM NaCl, 5 mM KCl, 1 mM K_2HPO_4, 2 mM $MgCl_2$, 30 mM Tris and 5 mM glucose (4 ml final volume). After incubation at 37°C with or without Erg, the RBC were separated from the medium and lactate levels in the RBC were determined after deproteinization.
Significant differences against control; $b<p=0.05$, $c<p=0.01$.

Table II. Effect of Ergothioneine on Glucose-6-phosphate and Fructose-6-phosphate Contents in Red Blood Cells

Glucose-6-phosphate (nmol/4 ml RBC suspension)	Control	120.20±14.67	—
	Ergothioneine	88.43± 9.62[a]	73.6%
Fructose-6-phosphate (nmol/4 ml RBC suspension)	Control	30.63± 1.09	—
	Ergothioneine	15.70± 0.70[b]	51.3%

Mean±s.d.

Significant differences against control; *a*)$<p=0.05$, *b*)$<p=0.01$ The washed RBC were incubated in the medium with or without 10^{-3} M Erg at 37°C for 1 h. The other conditions were the same as in Table I. G-6-P and F-6-P in the RBC suspension was determined by the use of G-6-P dehydrogenase and phosphoglucose isomerase, respectively.

10^{-4} M on incubation for 1 h. These results are similar to those obtained from the hemolysate. The result that Erg appeared to have little effect on lactate level of RBC after 3 h, was suggested decrease in glucose level in RBC.

We next investigated the intermediates of glycolysis. The results are shown in Table II. When 10^{-3} M Erg was added to an RBC suspension, G–6–P and F–6–P levels were reduced by about 26% and 50%, respectively. Though no measurement was performed on fructose-1,6-diphosphate, adenosinediphosphate (ADP) and adenosine triphosphate (ATP), these results suggested that Erg might stimulate phosphofructokinase activity. The activity of pyruvate kinase was unchanged (0.56—0.66 unit/ml of RBC suspension) (data not shown).

Although GSH was hardly taken up by the RBC, it was reported that a synthetic SH–compound was taken into RBC and stimulated the uptake of glucose.[6] Erg is a physiological SH–compound and might thus have a physiological role in the redox system of RBC. Further work is necessary to investigate the relationships between glycolysis and reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced nicotinamide adenine dinucleotide (NADH), ATP or methemoglobin reduction in order to study the redox system in RBC.

References and Notes

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