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Studies on Ergothioneine. X.[1] Effects of Ergothioneine on the Hepatic Drug Metabolizing Enzyme System and on Experimental Hepatic Injury in Rats

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When ergothioneine was administered to rats for 7 d, the pentobarbital sleeping time was reduced significantly. This action was specific to ergothioneine and was not shown by various ergothioneine-related compounds (cysteine, glutathione, carnitine and histidine). This action of ergothioneine did not appear to be caused by induction of the drug-metabolizing enzyme system. The level of ergothioneine in the liver decreased markedly when phenobarbital or ethionine was administered to rats. Ergothioneine inhibited hepatic injury and significantly decreased the level of lipid peroxide induced by ethionine administration.

Keywords——ergothioneine; drug metabolizing enzyme; ethionine induced hepatic injury; lipid peroxidation; ergothioneine deficient; ergothioneine excessive

Ergothioneine (Erg; 2-mercaptohistidine trimethylbetaine) is distributed in various organs of higher animals,[2] but its physiological role has not yet been established. Previously, we have shown that Erg is derived from food, and we discussed its possible actions in red blood cells of rats.[1] The metabolic turnover rate of Erg is low[2] and, indeed, Erg accumulates in the liver of rats during the aging process[3] reaching the highest concentration (approximately 10^{-4} M) of any organ in that species. In spite of these studies, its physiological role and its relationship with the liver function have not been elucidated. In contrast to Erg, there are many reports on physiological roles of glutathione (GSH), especially on its significance in the liver.

The present investigation was carried out to elucidate the role of Erg in the liver. We have already found that Erg administration significantly reduces the pentobarbital sleeping time in rat. Therefore, we attempted to obtain further data on the effects of Erg administration on the hepatic drug-metabolizing enzyme system and also on hepatic injury induced experimentally with ethionine.

Materials and Methods

Chemicals——L-Erg($2H_2O$) was purchased from Sigma Chemicals Co., and DL-ethionine, DL-carnitine·HCl, L-Cys·HCl·H_2O, GSH, L-His and others, from Wako Pure Chemical Industries, Ltd.

Animals——Wistar rats (Japan Clea Co., Ltd.) were maintained at 21±1°C, humidity 50—60%, light period 12 h/d. They were fed standard laboratory chow (CE-2; manufactured by Japan Clea Co., Ltd.), and water was given *ad libitum*.

[I] **Experiment on Drug-metabolizing Enzyme System**——(1) Male and female rats weighing 60 and 200 g were used in the sleeping test. Erg or a related compound was administered intraperitoneally (*i.p.*) for 7 d. In the phenobarbital-treated group, the rats were pretreated by *i.p.* injection with phenobarbital-Na (30 mg/kg body weight) twice a day for 4 d, overlapping the administration of Erg for the last 4 d. Pentobarbital sleeping time was examined in the rats 24 h after the final phenobarbital injection (PB induction group) or/and final Erg administration, at about noon at a room temperature of 21°C. The righting reflex of the rats was examined to judge the induction of sleeping and wakening. Thus, the duration of sleep was determined.

(2) The drug-metabolizing enzyme system was prepared in the following manner. Rats were sacrificed under ether anesthesia. The liver was freed of blood by perfusion with saline and homogenized in 1.15%

KCl–10 mM potassium phosphate buffer (pH 7.4). A liver homogenate (25%) was centrifuged at 10000 *g* for 30 min. The resulting supernatant was used as the enzyme solution. Pentobarbital hydroxylation was determined by the method of Brodie.[4] Reaction mixture (5 ml) containing 5 mM pentobarbital, 50 mM $MgCl_2$, 2 ml of enzyme solution, an reduced nicotinamide adenine dinucleotide phosphote (NADPH) generating system (0.65 μmol NADP, 10 μmol glucose-6-phosphate (G-6-P)) and 50 μmol nicotinamide was incubated for 30 min at 37°C. The residual pentobarbital extracted with *n*-heptane was determined by the use of a dual-wavelength (240 and 265 nm) spectrophotometer (Hitachi model 156). Pentobarbital hydroxylation activity is presented as nmole of residual pentobarbital, so a smaller value represents higher activity. Hepatic microsomes, prepared from liver homogenate in 1.15% KCl–10 mM phosphate buffer (pH 7.4) were suspended in 50 mM phosphate buffer (pH 7.4, ethylenediaminetetraacetic acid (EDTA) 1 mM) to a protein concentration of *ca.* 2 mg/ml. Cytochrome P-450 in the microsomes was determined by the method of Omura and Sato.[5]

[II] **Erg-deficient and Erg-excessive Rats**——Three-week-old male rats were fed Erg-free diet[3] for 20 weeks, and we confirmed that hepatic Erg was not detectable by high performance liquid chromatography.[2] The Erg-supplemented diet consisted of Erg mixed homogenously at a ratio of 5 mg per 100 g of the Erg-free diet.

[III] **Experiment on Hepatic Injury induced by Ethionine**——Administration of ethionine was performed by the method of Olivecrona.[6] Eight-week-old female rats were divided into 4 groups. Erg group: Erg (8 mg/100 g body weight) was administered subcutaneously (*s.c.*) for 7 d and saline was injected on the 7th day after 15 h starvation instead of ethionine. Ethionine group: Saline was administered for 7 d instead of Erg and ethionine was injected *i.p.* on the 7th day after 15 h starvation. Ethionine plus Erg group: Erg was administered for 7 d and ethionine was injected on the 7th day after 15 h starvation. Control group: Saline was administered *s.c* for 7 d and *i.p.* on the 7th day. All rats in each group were sacrificed under ether anesthesia on the 8th day after continuous starvation for 39 h. The lipid peroxidation in the liver was estimated by the method of Wills.[7] The liver homogenate was incubated at 37°C for 60 min and subjected to 2-thiobarbituric acid (TBA) reaction after deproteinization with TCA. The result obtained was expressed as the value of optial density (OD) at 532 nm (TBA value) or as nmole of malonylaldehyde (MDA). GSH was estimated by the enzymic method of Bergmeyer.[8] Erg was determined by high performance liquid chromatography (HPLC) as described previously,[2] serum aspartate aminotransferase (GOT) and alanine aminotransferase (GPT) by the method of Reitman–Frankel,[9] and protein by the biuret method.[10]

Results

Effect of Erg on the Pentobarbital Sleeping Time in Rats

The results in Fig. 1 suggest that in both non-treated and phenobarbital-treated rats, regardless of sex or age, Erg, administration caused a reduction in the pentobarbital sleeping time. It seemed that Erg itself might cause the induction of the drug-metabolizing enzyme system and, at the same time, enhance the inducing effect of phenobarbital. As can be seen in Fig. 2, the action to reduce the pentobarbital sleeping time seems to be characteristic of Erg, since it was not exhibited by cysteine, GSH (–SH radical), carnitine (betaine), or histidine (imidazole), although these substances are structurally related to Erg.

Effect of Erg on the Drug-metabolizing Enzyme System in Rats

The shortening of pentobarbital sleeping time *in vivo* suggests that Erg may induce or stimulate the drug-metabolizing enzyme system of the liver. However, no clear increase of drug-metabolizing enzyme activities (P-450, pentobarbital hydroxylation and aminopyrine demethylation) was apparent in Erg-treated rats (data not shown). In order to obtain further information on the effect of Erg on this system, Erg-deficient and Erg-excessive rats were used, and enzyme solutions were prepared from the liver of each rat. Unexpectedly, no significant difference in P-450 or pentobarbital hydroxylation activity was observed between the two groups. Thus, Erg failed to have the anticipated inducing effect on the drug-metabolizing enzyme system. On the other hand, Erg greatly reduced the level of lipid peroxide.

Preventive Effect of Erg on Hepatic Injury induced with Ethionine

Even though Erg has no direct effect on the drug-metabolizing enzyme system, it is possible that Erg inhibits experimental hepatic injury or stimulates the recovery from such injury

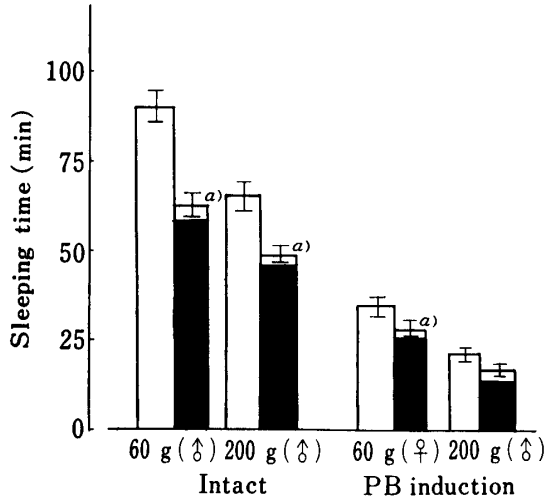

Fig. 1. The Pentobarbital Sleeping Time

Phenobarbital (PB) induction groups were pretreated with PB (30 mg/kg body weight, *i.p.*) twice a day for 4 d. Pentobarbital (35 mg/kg body weight, *i.p.*) was injected into rats at 24 h after the final PB treatment in the PB induction group and the final ergothioneine administration in the intact group. Open columns; Control (saline 0.1 ml/100 g body weight, *i.p.*). Closed columns; Erg administration (Erg 1.6 mg (6.03 μmol)/100 g body weight, *i.p.* daily, for 7 d). *a*) $p<0.01$ significant difference from control value. Each value represents the mean ± s.d. of 4 or 5 rats.

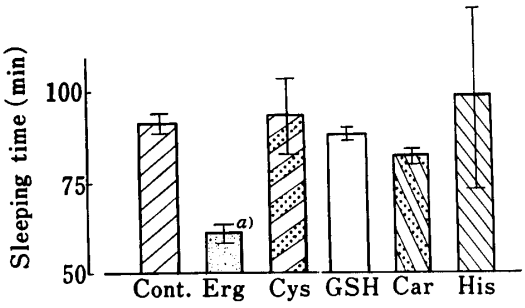

Fig. 2. Effect of Ergothioneine-related Compounds on the Pentobarbital Sleeping Time

Each compound was administered (6.03 μmol/100 g body weight) for 7 d by *i.p.* injection into male rats (200 g). Pentobarbital was injected *i.p.* (35 mg/kg body weight) at 24 h after the final administration of these compounds.

a) $p<0.01$ significant difference from control value. Each value represents the mean ± s.d. of 5 rats.

TABLE I. P-450, GSH, Pentobarbital Hydroxylation and Lipid Peroxidation Levels in Ergothioneine-deficient and-excessive Rats[a)]

	Erg-deficient	Erg-excessive
Erg (nmol/g liver)	1>	1400±152
P-450 (nmol/mg protein)	0.321±0.032	0.315±0.018
Pentobarbital hydroxylation (nmol/mg protein)	4.14±0.77	4.87±1.18
GSH (μmol/g liver)	2.996±0.384	3.056±0.244
Lipid peroxidation (MDA nmol/g liver)	14.63±1.40	10.31±0.62[b)]

The results are expressed as means s.d. of 4 rats.

a) Ergothioneine-deficient rats were fed ergothioneine-free diet for 20 weeks. Ergothione-excessive rats were fed ergothioneine-supplemented diet (Erg 5 mg/100 g of Erg-free diet) for 20 weeks.

b) Significant difference ($p<0.01$) from the value for the ergothioneine-deficient roup.

through some effects on the hepatic function.

As shown in Table II, the liver weight increased, the protein content decreased markedly and the liver turned reddish in color with a milky white tint on ethionine administration. Hsu *et al.* reported that ethionine administration increased the GSH content in rat liver at 24 h, and simultaneous administration of methionine with ethionine tended to reduce this effect of ethionine.[11)] Serum GOT and GPT (indicators of the hepatic function) increased remarkably after ethionine administration, whereas they were significantly lower than in the ethionine group after simultaneous administration with Erg and were the same in the group given Erg alone as in the control group. These results suggest that Erg does not induce hepatic injury but rather counteracts the effect of ethionine. Moreover, ethionine administration greatly reduced the level of Erg in the liver. The results suggest that Erg may alleviate hepatic injury induced by ethionine. It is well known that ethionine induces hepatic injury

by causing inhibition of protein synthesis and damage to cell membranes.[12] These phenomena are presumably caused by an increase in the level of lipid peroxide. Erg preadministration seemed to reduce the lipid peroxide-increasing effect of ethionine. In order to further clarify the effect of Erg, low doses of ethionine were examined. Table III demonstrates again that administration of Erg distinctly inhibited the dose-dependent increase in lipid peroxide caused by ethionine.

TABLE II. Effect of Ethionine and/or Ergothioneine Administration on Rat Liver

	Liver weight (g)	Protein (mg/g)	GSH (μmol/g)	Ergothioneine Liver (μ/g)	Ergothioneine Blood (nmol) (μ/ml)	MDA (nmol/g liver)	GOT (GPT) (Karmen unit)
Control	4.62±0.22	115.2±4.3	6.02±0.54	75.8±7.0	9.5±1.6	249±27	80.20±6.56 (18.55±2.01)
Ethionine	5.77±0.41[c]	80.6±3.5[c]	7.06±0.50[a]	30.9±4.9[b]	10.6±1.4	388±35[c]	172.44±14.67[c] (94.39±12.60)[c]
Ethionine + Ergothioneine	5.68±0.23	91.0±6.0[b]	4.75±0.51[c]	188.0±23.3	72.5±1.4	312±41	135.71±15.28[d] (49.50±4.53)[e]
Ergothioneine	4.89±0.13	112.0±3.3	5.81±0.55	672.0±21.1	76.7±8.9	271±46	80.49±4.13 (19.55±0.90)

The results are expressed as means ± s.d. of 5 rats.
Rats were sacrificed at 24 h after ethionine (100 mg/100 g body weight) and/or ergothioneine (8 mg/100 g body weight) administration as described in the text.
Significance of differences : *a*) $p<0.05$, *b*) $p<0.005$ and *c*) $p<0.001$ with respect to control value.
d) $p<0.005$ and *e*) $p<0.001$ with respect to value for ethionine-treated group.

TABLE III. Effect of Ergothioneine Administration on Lipid Peroxidation in the Liver of Rats treated with Ethionine[a]

Treatment	Liver weight(g)	TBA value
Control	4.67±0.47	0.155±0.006
Ergothioneine[b]	4.56±0.15	0.159±0.006
Ethionine (20 mg)	5.44±0.41[c]	0.228±0.040[c]
Ethionine (20 mg) +Ergothioneine	5.11±0.38	0.160±0.023[e]
Ethionine (60 mg)	6.46±0.49[d]	0.501±0.035
Ethionine (60 mg) +Ergothioneine	6.12±0.44[d]	0.243±0.027[f]

The results are expressed as means ± s.d. of 5 rats.
a) Ethionine (20 or 60 mg/100 g body weight) was injected intraperitoneally at 24 h before sacrifice.
b) Ergothioneine (8 mg/100 g body weight) was administered subcutaneously for 7 d.
Significance of differences; *c*) $p<0.05$ and *d*) $p<0.001$ with respect to control value.
e) $p<0.01$ and *f*) $p<0.001$ with respect to corresponding ethionine value.

Changes in Erg Content on Phenobarbital Treatment

Since administration of ethionine greatly decreased the level of Erg in the liver (Table II), we examined the change of the level of Erg caused by phenobarbital administration, which induces lipid peroxidation.[13] As shown in Table IV, treatment of phenobarbital reduced the Erg level by 30% in the liver and by 70% in the blood.

TABLE IV. Effect of Phenobarbital Administration on Ergothioneine Content in Rat Liver and Blood

Group	Liver (nmol/g)	Blood (nmol/ml)
Intact	127.0±13.8	16.3±6.1
Phenobarbital-treated	93.3±21.9	6.1±0.2

The results are expressed as means ± s.d. of 5 rats.
Phenobarbital was administered intraperitoneally to male rats (100 mg/kg body weight) for 3 d and then the ergothioneine contents in the liver and blood were determined by HPLC 24 h after the last injection.

Discussion

The present investigation was performed to clarify a possible relationship between the liver function and Erg, since Erg accumulates in the liver during aging. First, we carried out a sleeping test to examine the effect of Erg on the drug-metabolizing enzyme system. As a result of Erg administration, the pentobarbital sleeping time was greatly decreased (Fig. 1). However, Erg had no inducing effect on the enzymes. To account for this discrepancy, two hypotheses can be considered: (1) Erg accelerated the excretion of pentobarbital from the body and (2) since the Erg level decreased in the liver (Table IV), when pentobarbital was administered, Erg might react with it directly to reduce the content of the drug.

Moreover, it is well known that lipid peroxide formation is elevated after administration of phenobarbital.[13] This finding is of great interest in considering the significance of Erg in the liver. Indeed, Chiba *et al.* investigated the mitigating effect of 2-mercaptopropionylglycine on hepatic injury induced by ethionine in detail.[14] They reported that this compound is an ethyl-acceptor in the transfer reaction of the ethyl functional group. This compound and GSH do not induce the drug-metabolizing enzyme system, but increase the rate of excretion of pentobarbital from the blood, which is similar to the Erg effect. Furthermore, Sato *et al.*[15] pointed out that non-GSH compounds containing SH-radical were decreased by the induction of the drug-metabolizing enzyme system. This decrease can be regarded as a decrease of the Erg content, in view of the results in the present paper.

GSH is contained in the liver in high concentration.[16] It conjugates to some drugs for detoxification[17] and its level is greatly decreased by administration of tetrachlorides,[18] acetaminophen,[19] diethylmaleate[20] and chloroform.[21] These drugs may cause hepatic necrosis or cell damage with an increase in lipid peroxidation. This hepatic injury is ameliorated by administration of GSH or cysteine.[14] Also, it is well known that phenobarbital stimulates the production of lipid peroxide and that the substrates of lipid peroxidation exist in the membrane.[13] Based on our present findings that Erg inhibited the lipid peroxidation and partly prevented the increases of GOT and GPT induced by ethionine, it can be assumed that Erg contributes positively to the maintenance of the hepatic function.

Furthermore, like GSH and SH-compounds, which have an inhibitory effect on lipid peroxidation,[22] Erg appears to protect the hepatic cell membrane by inhibiting lipid peroxidation induced by various drugs. The mechanism of inhibition of lipid peroxidation by Erg has been investigated in our laboratory and the details will be described in subsequent papers.

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