Protective Effect of Sclerin on a Lead-acetate Injury Affecting the Growth and Porphyrin Metabolism in Rat

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Received September 25, 1975

While a continuous ingestion of lead acetate added in drinking water suppressed the rat growth, depressing in some degree the level of hepatic δ -aminolevulinic acid (ALA) dehydratase, a very small amount of sclerin (SCL) added simultaneously in the water restored the growth and dehydratase level. Moreover, subcutaneous injection of SCL to the rat not only maintained the ALA dehydratase level, but prevented a marked depression of the level of mitochondrial ALA synthetase in liver caused by intraperitoneal injection of lead acetate. Injection of SCL alone increased tolerably (about 1.8 times) the mitochondrial ALA synthetase, but little the extramitochondrial synthetase. The treatment by SCL was attended by a initial decrease, then a gradual increase in the activity of microsomal drug metabolizing enzyme.

Sclerin (SCL), a physiologically active substance produced by a strain of *Sclerotinia libertiana*, influences widely the growth and enzyme formation in various kinds of organisms including plants, microorganisms and animals.^{1~3)} SCL has previously been reported to stimulate the formation and activity of peroxidase in the *Sclerotinia* fungus itself.⁵⁾ SCL also stimulates the chlorophyll formation in plants and chlorella cells, reducing the phytotoxicity of agricultural chemicals such as 2, 4-dichlorophenoxyacetate and phenylmercuric acetate.^{4, 6)} Peroxidase is a heme enzyme and since heme and chlorophyll are both metal complexes of porphyrin, the above effects of SCL suggest a common regulatory function for synthesis of porphyrins.

The porphyrin synthesis in liver has a profound relation with the activity of microsomal mixed-function oxidase system,^{7~12)} which involves cytochrome P-450, a heme protein, metabolizing steroids, drugs and 'foreign compounds.' Lately, we have found that SCL exhibits an activating effect *in vitro* on the microsomal aniline hydroxylase in rat liver tissues.⁴⁾

On the other hand, lead is known to inhibit *in vitro* the key enzymes in heme biosynthesis,¹³⁾ and air-borne lead, an urban environmental poison, has been found to cause some abnor-

malities in porphyrin metabolism.¹⁴⁾ Also, lead acetate is a powerful inhibitor of protein synthesis *in vitro*.¹⁵⁾ In the present paper, effect of SCL on a lead-acetate injury in the rats was investigated in reference to the growth and porphyrin metabolism.

MATERIALS AND METHODS

Animals. Male Wistar rats were fed on a diet of Nippon Clea Co. CE-2 and water *ad libitum*.

Treatment of animals. For injection tests, rats (100~130 g) were fasted for 24 hr. Stock solution containing 0.1% SCL was prepared by dissolving SCL in 0.1 N NaOH and neutralizing with a diluted HCl solution to pH 6.6~6.8 and was stored in a refrigerator. When SCL was employed, this stock solution was diluted by 0.9% NaCl and given subcutaneously as indicated in respective tables. Lead acetate was dissolved in distilled water and administered by intraperitoneal injection. Control rat was treated by 0.9% NaCl unless otherwise stated. Rats were maintained fasting throughout the injection experiments.

Assay of enzyme activity

δ -Aminolevulinic acid (ALA) dehydratase. Rats were killed by decapitation and livers were removed in a chilled dish. The liver was homogenized with 20 vol. of 0.25 M sucrose in Potter-Elvehjem homogenizer with a Teflon pestle, and the homogenate was centrifuged at 10,000 $\times g$ for 15 min. The supernatant was used as enzyme solution. Enzyme activity was estimated as described by Wilson *et al.*, using the Thunberg tubes.¹⁶⁾

The tube contained 0.5 ml of enzyme solution in 1.5 ml of a reaction mixture of the following final composition: 0.02 M reduced glutathione, 0.1 M sodium phosphate buffer (pH 6.8) and 0.01 M ALA. After evacuation and preincubation for 1 hr at 37°C, substrate was added from the cap of the tube. Incubation was then continued for additional 30 min and reaction was terminated by the addition of 1.5 ml of 10% trichloroacetic acid (TCA) containing 0.1 M HgCl₂. Porphobilinogen (PBG) formed was measured by the modified Ehrlich's method of Mauzerall and Granick.¹⁷⁾ The enzyme activity was expressed as μ moles of PBG formed/hr/g liver.

ALA synthetase. The rat liver removed as described above was homogenized with 6 vol. of 0.25 M sucrose containing 0.05 M Tris-HCl buffer (pH 7.5), 0.025 M KCl, 0.025 M MgCl₂ and 0.1 mM pyridoxal phosphate in Potter-Elvehjem homogenizer, and the homogenate was centrifuged at 800 $\times g$ for 10 min. Mitochondria were sedimented from the resulting supernatant solution by centrifugation at 10,000 $\times g$ for 15 min, and were washed and resuspended in the same medium. Enzyme activity was determined by the method of Hayashi *et al.*¹⁸⁾ Reaction mixture contained 150 μ moles Tris-HCl buffer (pH 7.5), 200 μ moles glycine, 20 μ moles succinate, 20 μ moles ATP, 0.3 μ moles pyridoxal phosphate, 0.3 μ moles CoA, 10 μ moles MgCl₂, 8 μ moles EDTA, succinyl-CoA synthetase, catalysing the formation of 16.2 μ moles of succinyl-CoA/hr and 1 ml of mitochondrial suspension, in the final volume of 2 ml. The reaction was carried out in a test tube at 37°C for 60 min with shaking, and stopped by the addition of 0.5 ml of 12.5% TCA. ALA formed was estimated as described by Narisawa and Kikuchi.¹⁹⁾ The enzyme activity was expressed as nmoles of ALA formed/hr/g liver. The activity in cytosol was assayed by employing the 10,000 $\times g$ supernatant fraction.

Aniline hydroxylase. Enzyme activity was measured

by the method of Imai *et al.*²⁰⁾ The liver perfused *in situ* with 0.9% NaCl was homogenized with 4 vol. of 1.15% KCl in Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 $\times g$ for 15 min. Microsomes were sedimented from the resulting supernatant solution by centrifugation at 105,000 $\times g$ for 60 min. The sediments of microsomes were washed once with 1.15% KCl and resuspended in the same KCl solution. Reaction mixture contained 5 mM aniline, 0.5 mM NADP, 5 mM glucose-6-phosphate, 2.5 mM MgCl₂, 1 unit of glucose-6-phosphate dehydrogenase, 8 mM nicotinamide, 100 mM Tris-acetate buffer (pH 8.0) and 1 ml of microsomal suspension, in the final volume of 2 ml. The reaction was carried out for 20 min at 37°C with shaking and stopped by the addition of 1 ml of 20% TCA. After centrifugation *p*-aminophenol formed was assayed in 1 ml of the supernatant after adding 0.5 ml of 10% Na₂CO₃ and 1 ml of 2% phenol in 0.2 N NaOH by measuring the E_{830} after 30 min. The enzyme activity was expressed as μ moles of *p*-aminophenol formed/min/g liver.

Chemicals. SCL was prepared from the extract of a bran culture of *Sclerotinia libertiana*.^{1,21)} Other chemicals used were obtained from the following sources: glucose-6-phosphate dehydrogenase (EC 1.1.1.49), from Boehringer Mannheim Biochemical Co.; succinyl-CoA synthetase (EC 6.2.1.4), ALA and PBG, from Sigma Chemical Co.; pyridoxal phosphate and reduced glutathione, from Fluka Co.; CoA, ATP, NADP and glucose-6-phosphate, from Kyowa Hakko Kogyo Co., Ltd.

RESULTS

Effect of SCL and lead acetate on the rat growth

As shown in Tables I and II, when lead acetate was administered in drinking water

TABLE I. EFFECT OF SCL AND LEAD ACETATE ON THE RAT GROWTH

Administration	Body weight (g) ^{c)}			Significance ^{d)} (P)
	Initial	Final	Gain	
Control	75.0 $\pm$ 4.7	281.0 $\pm$ 16.6	206.0 $\pm$ 16.9	
SCL ^{a)}	75.0 $\pm$ 2.6	288.5 $\pm$ 13.4	213.5 $\pm$ 9.8	N.S.
Lead acetate ^{b)}	72.8 $\pm$ 4.4	259.4 $\pm$ 28.7	186.6 $\pm$ 30.5	<0.2
SCL+lead acetate	79.2 $\pm$ 5.1	291.0 $\pm$ 16.4	211.8 $\pm$ 13.3	N.S.

^{a)} SCL was continuously administered in drinking water at 0.1 μ g/ml.

^{b)} Lead acetate was continuously administered in drinking water at 0.1% for the first 18 days, 0.2% for 4 days, 0.3% for 8 days, 0.4% for 4 days and then 0.5% for the last 2 days.

^{c)} Rats were fed on a diet of Nippon Clea Co. CE-2 (crude protein 24%) and drinking water *ad libitum* for 36 days. The values are means $\pm$ S.D. of 5 animals.

^{d)} Significance was statistically calculated referring to control. For *P* values greater than 0.2, the term N.S. (not significant) was employed.

TABLE II. EFFECT OF SCL AND LEAD ACETATE ON THE RAT GROWTH

Administration	Body weight (g) ^{c)}			Significance ^{d)} (P)
	Initial	Final	Gain	
Lead acetate ^{a)}	73.2±4.5	247.4±24.1	174.2±23.9	
Lead acetate + SCL ^{b)}	73.6±4.7	266.0±14.0	192.4±13.9	<0.02

^{a)} Lead acetate was intermittently (2 days within 1 week) administered in drinking water, with the gradual increase in concentration (0.2% for the first 1 week, 0.3% for the second 1 week, 0.4% for the third 1 week and 0.5% for the last 3 weeks).

^{b)} SCL was also intermittently (1 day within 5 days) administered in drinking water at 0.5 µg/ml.

^{c)} Rats were kept for 37 days as in Table I. The values are means±S.D. of 10 animals.

^{d)} Significance was statistically calculated referring to lead acetate alone.

daily or intermittently at an interval of days for 36~37 days, the young rats suffered an injurious effect on growth, reducing the gain of body weight. In contrast, a very small amount of SCL administered simultaneously in the drinking water protected somatic development of the rats and restored the body weight gain. SCL alone had little effect on the rat growth under such conditions. SCL was supposed to give a biochemical effect on the rats. As shown in Table III, though the ingestion of lead acetate

TABLE III. EFFECT OF SCL AND LEAD ACETATE ON THE LEVEL OF HEPATIC ALA DEHYDRATASE

Administration ^{a)}	ALA dehydratase activity ^{b)} µmoles of PBG formed/hr/g liver
Control	1.41±0.08
SCL	1.53±0.13
Lead acetate	1.14±0.10
SCL+lead acetate	1.30±0.04

^{a)} The same as in Table I.

^{b)} The activity was assayed at the 36th day. The values are means±S.D. of 5 animals.

for 36 days depressed to some extent the level of hepatic ALA dehydratase in the rats, combination of SCL and lead acetate just maintained the level. Ingestion of SCL alone had little effect on the level. It was indicated that some interactions between SCL and lead acetate occurred on the porphyrin metabolism in the rats during the growth.

Effect of SCL and lead acetate on level of the ALA dehydratase and synthetase in liver

As shown in Table IV, while intraperitoneal

TABLE IV. RESTORING EFFECT OF SCL ON THE LEVEL OF HEPATIC ALA DEHYDRATASE DEPRESSED BY LEAD ACETATE

Administration ^{a)}	ALA dehydratase activity ^{b)} µmoles of PBG formed/hr/g liver
Control	1.57±0.09 (100.0%) ^{c)}
SCL	1.62±0.06 (103.2)
Lead acetate	0.63±0.17 (40.1)
SCL+lead acetate	1.32±0.31 (84.2)

^{a)} SCL (10 µg/100 g body weight) was subcutaneously, and lead acetate (30 mg/100 g body weight) was intraperitoneally injected.

^{b)} The activity was assayed 24 hr after injection. The values are means±S.D. of 3 animals.

^{c)} Parenthesis shows relative activity.

injection of lead acetate (30 mg/100 g body weight) depressed the level of ALA dehydratase approximately to 40% of control after 24 hr, subcutaneous injection of SCL (10 µg/100 g) performed simultaneously prevented the depression, maintaining more than 80% of the control level of the dehydratase. SCL alone hardly affected the level. This may indicate that the interaction between SCL and lead acetate did not occur only on the level of ALA dehydratase.

As shown in Table V, the injection of SCL had an inducing effect on the ALA synthetase in the liver, and increased specially the enzyme activity in mitochondria at a small dosage, 1 µg per 100 g body weight. The effect of SCL attained to a peak at 10 µg and increased the activity in mitochondria about 1.8 times, but only slightly in the soluble part. The mitochondrial ALA synthetase is known to differ

from the soluble enzyme in molecular size, the former being smaller than the latter.²²⁾ Contrary to this, as shown in Table VI, the injection

TABLE V. EFFECT OF SCL ON THE LEVEL OF HEPATIC MITOCHONDRIAL ALA SYNTHETASE

SCL ^{a)} μg/100 g body weight	ALA synthetase activity ^{b)} nmoles of ALA formed/hr/g liver		
	Mitochondria	Cytosol	Total
0	52.7 ± 4.8 (100.0%) ^{c)}	12.1 ± 2.1	64.8 ± 5.9
1	91.7 ± 7.2 (174.0)	14.6 ± 3.0	106.3 ± 8.0
10	98.0 ± 12.7 (186.0)	15.6 ± 1.9	113.6 ± 13.1
50	81.2 ± 11.3 (154.1)	13.3 ± 3.4	94.5 ± 11.9

^{a)} SCL was administered by subcutaneous injection.

^{b)} The activity was assayed 24 hr after injection. The values are means ± S.D. of 3 animals.

^{c)} Parenthesis shows relative activity.

TABLE VI. RESTORING EFFECT OF SCL ON THE LEVEL OF HEPATIC MITOCHONDRIAL ALA SYNTHETASE DEPRESSED BY LEAD ACETATE

Admini- stration ^{a)}	ALA synthetase activity ^{b)} nmoles of ALA formed/hr/g liver		
	Mitochondria	Cytosol	Total
Control	53.8 ± 6.0 (100.0%) ^{c)}	13.3 ± 2.4	67.1 ± 6.2
SCL	96.1 ± 15.2 (178.6)	16.0 ± 1.8	112.1 ± 14.7
Lead acetate	15.7 ± 3.9 (29.2)	82.9 ± 17.3	98.6 ± 18.1
SCL + lead acetate	48.6 ± 8.1 (90.3)	78.6 ± 19.5	127.2 ± 20.2

^{a)} SCL (10 μg/100 g body weight) was subcutaneously, and lead acetate (30 mg/100 g body weight) was intraperitoneally injected.

^{b)} The activity was assayed 24 hr after injection. The values are means ± S.D. of 3 animals.

^{c)} Parenthesis shows relative activity.

of lead acetate (30 mg/100 g) markedly decreased the mitochondrial ALA synthetase with rather increasing the 'soluble form.' When SCL and lead acetate were simultaneously administered, the level of mitochondrial synthetase was maintained approximately normal, but that of the 'soluble form' con-

siderably increased. Accordingly, it was proved that the level of ALA dehydratase in liver had a close relation with the mitochondrial synthetase, but no relation with the soluble enzyme.

Effect of SCL on level of the microsomal aniline hydroxylase in liver

As presented in Fig. 1, the injection of SCL at 10 μg per 100 g body weight, at first, rapidly

FIG. 1. Time Course of Change in the Level of Microsomal Aniline Hydroxylase after SCL Injection.

The activity was assayed 2, 4, 8, 16 and 24 hr after subcutaneous injection, respectively, as described in MATERIALS AND METHODS and the relative activity was represented as % of control. Each point is mean with S.D. (bar) of 3 animals.

x—x, control; ○—○, 10 μg SCL/100 g body weight; ●—●, 50 μg SCL/100 g body weight.

lowered the level of microsomal aniline hydroxylase in the liver, but soon restored raising the level gradually within 24 hr. The effect of SCL resembled a pattern of the enzyme regulation by certain porphyrinogenic drug such as allylisopropylacetamide.^{10,23)} Though the injection of SCL at 50 μg also exhibited a similar effect initially, the rise in the level of aniline hydroxylase did not continue from 8 hr forth and equilibrated with control. Administration of an excessive amount of SCL may produce some harmful effect on the enzyme induction.

DISCUSSION

As shown in the above experiments, though the intraperitoneal injection of lead acetate

increases the 'soluble form' of ALA synthetase in liver, it markedly decreases the mitochondrial synthetase. ALA synthetase is believed to be synthesized originally in the microsomal system and subsequently transferred into mitochondria where it settles, accompanying the modification of the enzyme protein.^{22,24)} The soluble ALA synthetase may be a precursor of the mitochondrial ALA synthetase. Lead acetate appears to inhibit the transfer of soluble ALA synthetase into mitochondria, because mitochondria of liver Kupffer and parenchymal cells are known to be structurally altered by non-toxic doses of lead.^{14,25)} On the other hand, administration of SCL to the rat increases the mitochondrial ALA synthetase and prevents the depression by lead acetate of the level of the mitochondrial synthetase. This may be due to a protective effect of SCL on the structure and function of mitochondrial membrane.²⁶⁾ It has previously been shown that SCL stimulates the transfer of proteins synthesized in extramitochondrial system into mitochondria.²⁷⁾

The ALA synthesized in mitochondria is then transferred into cytosol and may induce the ALA dehydratase, an extramitochondrial enzyme. Administration of porphyrinogenic drugs or ALA to animals is known to increase the dehydratase.^{28,29)} The exit of ALA from the mitochondria is a possible site of metabolic regulation.¹³⁾ It seems reasonable that SCL restores the level of ALA dehydratase depressed by lead acetate through the induction of mitochondrial ALA synthetase. It was found that SCL hardly affects the level of ALA dehydratase maintained in normal, regardless of the elevation of mitochondrial synthetase. This may imply the regulation of porphyrin metabolism by the so-called 'succinate-glycine cycle' in mitochondria, regenerating succinate or succinyl-CoA from ALA.

Regarding the effect of SCL on microsomal aniline hydroxylase, initial decrease in the enzyme activity was noticed, which suggests the involvement of the degradation of cytochrome P-450 in the induction of ALA synthetase by SCL, as in the case with allylisopropyla-

cetamide, a potent porphyrinogenic drug.^{7~12)} On the other hand, potency of SCL on the ALA synthetase induction is not so high but rather resembles with phenobarbital, a weaker porphyrinogenic drug, which markedly increases cytochrome P-450 content.¹²⁾ These drugs are known to elicit an increase in liver weight, microsomal protein, RNA and phospholipid contents as well as the rate of protein synthesis, *in vivo* and *in vitro*.^{12,30~32)} SCL also has a stimulatory effect on protein synthesis *in vitro* in extramitochondrial system, as reported previously.^{2,4,27)} There may be a common basis involving the anabolic effects in the regulation of microsomal mixed-function oxidase system coupled with the ALA synthetase induction. Also, for further elucidating the induction mechanism of ALA synthetase by SCL, detailed studies are necessary on interaction of SCL with some steroid metabolite or heme, acting as a derepressor or corepressor respectively in the heme biosynthesis.^{33~36)}

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