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BEHAVIOR OF HEPATIC MICROSOMAL CYTOCHROMES AFTER TREATMENT OF MICE WITH DRUGS KNOWN TO DISTURB PORPHYRIN METABOLISM IN LIVER

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Abstract—Induction of the liver microsomal cytochromes, P-450 and b_5, and mitochondrial delta-aminolaevulinic acid (ALA) synthetase were attempted in mice by treatment with various porphyria-inducing chemicals and several other drugs known to precipitate acute attacks in human hepatic porphyria.

On administration of porphyria-inducing chemicals except 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC), the level of microsomal cytochromes showed a gradual increase after a transient decrease probably due to disturbed heme metabolism, and activity of ALA synthetase showed a marked increase concomitantly with the decrease of the level of cytochromes. In DDC poisoning, the level of cytochromes showed a persistent decrease, while the increase of ALA synthetase and porphyrin accumulation were most prominent. Treatment with drugs which precipitate acute attacks in human hepatic porphyria resulted in a marked increase of the level of cytochromes without showing a decrease phase, whereas the activity of ALA synthetase remained at a level 1·5–2 times higher than normal without an increase of porphyrin. Incorporation of ^{14}C-glycine into microsomal heme *in vivo* and activity of microsomal *N*-demethylase parallelled the level of cytochrome P-450. An impairment in synthesis of sufficient heme for hepatic hemoproteins in need of them and a positive feedback mechanism for induction of ALA synthetase were suggested as a possible cause of experimental porphyria.

A WIDE range of drugs and chemicals such as barbiturates[1] and chlorinated hydrocarbon-insecticides[2] increase the activity of hepatic microsomal drug-metabolizing enzyme system. Two microsomal cytochromes, P-450 and b_5, participating in the drug metabolism increase remarkably on administration of these drugs to animals.[3, 4] On the other hand, allylisopropylacetamide (AIA), an analogue of barbiturates, and hexachlorobenzene (C_6Cl_6), one of the insecticides, are known to induce an increased activity of mitochondrial delta-aminolaevulinic acid (ALA) synthetase,[5] causing an overproduction of porphyrins to produce hepatic porphyria in the recipient animals.[6] As ALA is the initial precursor of heme moiety of microsomal cytochromes and is believed to be synthetized only in mitochondria,[7] a close relationship might exist between the activity of mitochondrial ALA synthetase and the level of microsomal cytochromes.

The present investigation was undertaken to study the effect of the porphyria-inducing chemicals and the other drugs known to precipitate acute attacks in human hepatic porphyria on the level of microsomal cytochromes and on the activity of

mitochondrial ALA synthetase. The former chemicals studied include 3,5-dicarbethoxy-1,4-dihyrocollidine (DDC), AIA, griseofulvin and C_6Cl_6, and the latter drugs include phenobarbital, tolbutamide, progesterone and estrogen.

METHODS

Preparation of animals

Male mice of D-D strain, weighing 18–22 g, were obtained from the Research Institute of Infectious Disease in Tokyo. AIA (100 mg/kg), DDC (500 mg/kg), phenobarbital (80 mg/kg), progesterone (50 mg/kg) and estradiol (0·1 mg/kg) were suspended in saline and injected every 12 hr. Griseofulvin, C_6Cl_6, and tolbutamide were each mixed in a powder diet (2·5% w/w) and fed to mice ad libitum after 24 hr starvation. Animals were sacrificed every 6 hr by decapitation.

Preparation of tissue sample

Liver mitochondria were prepared in cold isotonic sucrose according to the method of Hogeboom-Schneider.[8] Washed mitochondria were suspended in the isotonic sucrose so that 1 ml of the suspension contained the mitochondria obtained from 1 g of liver. Microsomal fraction (henceforth termed 'microsomes' for convenience) was separated from the 9000 *g*-supernatant fraction by centrifugation for 60 min at 104,000 *g* in a Hitachi 55p type ultracentrifuge. In order to remove the bulk of sucrose and hemoglobin originated from red cells, the microsomes were resuspended in 0·15 M KCl and recentrifuged. The pellet was suspended in 0·1 M phosphate buffer, pH 7·4, so that the microsomes obtained from 1 g liver were contained in 10 ml of suspension.

Assay

ALA synthesis in liver mitochondria was determined by incubating 0·3 ml of mitochondria suspension at 37° for 60 min with glycine, 50 μmole; phosphate buffer (pH 7·4), 100 μmole; α-ketoglutarate, 3 μmole; pyridoxal phosphate, 0·2 μmole; $MgCl_2$, 4μmole; EDTA 2 μmole and Coenzyme-A 0·25 μmole; in a final volume of 0·5 ml. The reaction was terminated by adding 1 ml of 5% trichloracetic acid. The ALA in the aliquot of deproteinized supernatant was determined by the method of Granick and Urata.[9]

N-demethylase activity against 3-methyl-4-monomethyl aminoazobenzene as substrate was measured by the method of Terayama,[10] using the 9000 *g* supernatant as enzyme solution.

Cytochrome P-450 was determined by measuring the CO-difference spectrum of dithionite-reduced microsome suspension. The increment of molar extinction between 450 and 490 mμ was assumed to be 91 cm^{-1} mM^{-1}.[11]

Cytochrome b_5 was quantitated from the difference spectrum between NADH-reduced and air-saturated microsomes based on an extinction coefficient between 424 and 409 mμ of 185 cm^{-1} mM^{-1}.[12]

Determination of porphyrin concentration

Liver porphyrins were extracted with ethylacetate-glacial acetic acid (4:1), separated by solvent extraction, and measured spectrophotometrically, essentially as described by Dresel and Falk.[13]

Incorporation of ^{14}*C-glycine into heme* in vivo

2-^{14}C-glycine (200 μc/kg) was injected i.p. 60 min prior to sacrifice. Mice were killed at 24 and 48 hr after poisoning, and mitochondrial and microsomal fractions, obtained from 1 g liver by the method as described above, were washed twice with 1·15% KCl and suspended in 2 ml of 0·1 M phosphate buffer. After 1 ml of red blood cell hemolysate was added as a carrier, hemin was crystallized by the method of Chu and Chu.[14] The crystallized hemin was dissolved in a small volume of 10% ammonia. Radioactivity of the hemin was counted with a windowless gas flow counter (ALOKA, model FC-IE). Heme concentration was measured colorimetrically after adding potassium cyanide.

RESULTS

One gram of normal liver synthetizing about 20 mμmole (20·0 $\pm$ S.E. 1·2) ALA/hr contained approximately 24 mμmole (24·2 $\pm$ S.E. 2·2) cytochrome P-450 and 10 mμ-mole (10·3 $\pm$ S.E. 0·92) cytochrome b_5.

As shown in Fig. 1, the level of cytochrome P-450 in AIA or griseofulvin poisoned mice was slightly increased ($P < 0·05$) at 6 hr followed by an obvious decrease

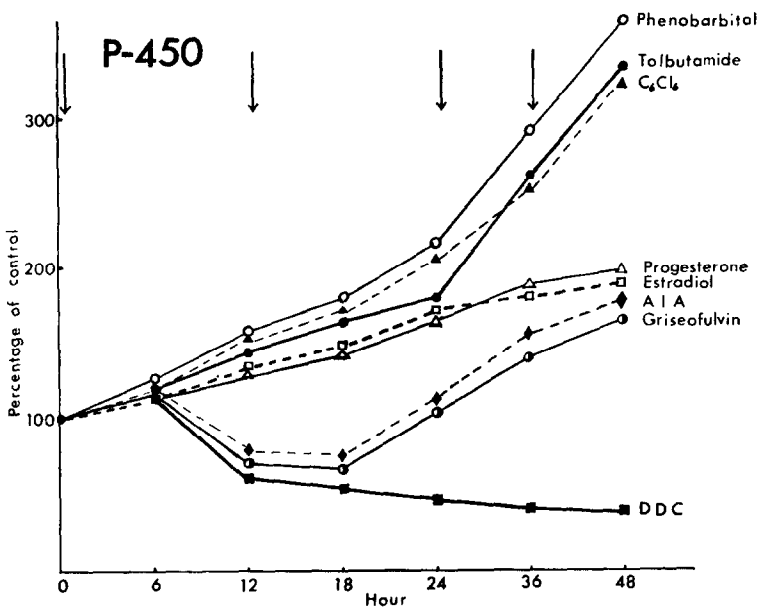

FIG. 1. Effects of various drugs on the microsomal content of cytochrome P-450. The averages of the amount of cytochrome P-450 per one gram liver of the treated group (6 mice) are plotted in relation to the corresponding values for the control group (6 mice.) The arrows indicate the injections.

($P < 0·01$) at 12–18 hr after treatment. However, it returned to the normal level about 24 hr and was elevated to 1·5 to 2-fold the normal level ($P < 0·01$) 48 hr later. The level of cytochrome b_5 (Fig. 3) resembled the pattern of cytochrome P-450, although the phase was shifted about 12 hr.

The activity of *N*-demethylase (Fig. 4) was parallel to the content of cytochrome P-450. Activity of ALA synthetase (Fig. 2) began to increase markedly at the time

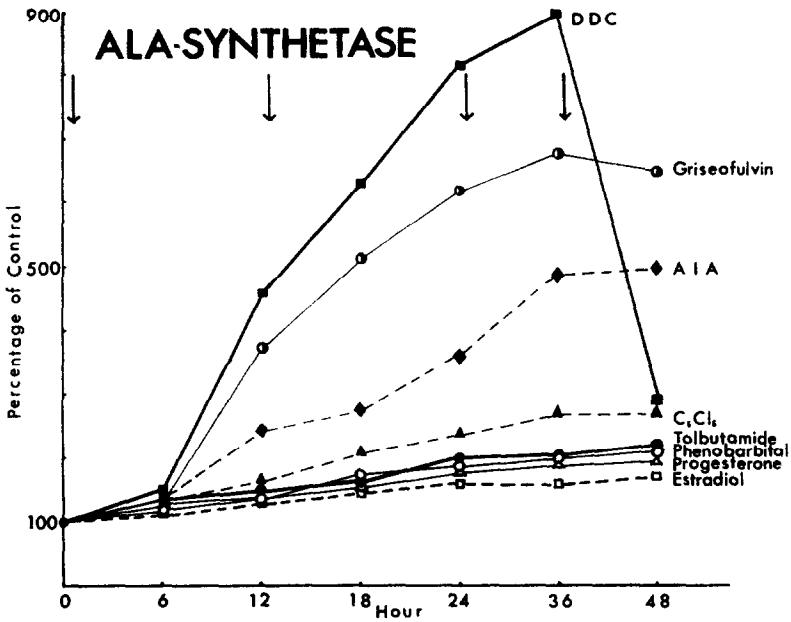

FIG. 2. Enhanced rate of the mitochondrial ALA synthesis after treatment of mice with various drugs. Each value plotted represents an average of the activities per one gram liver of 6 experimental animals as compared with those of 6 control mice. The arrows indicate the injections.

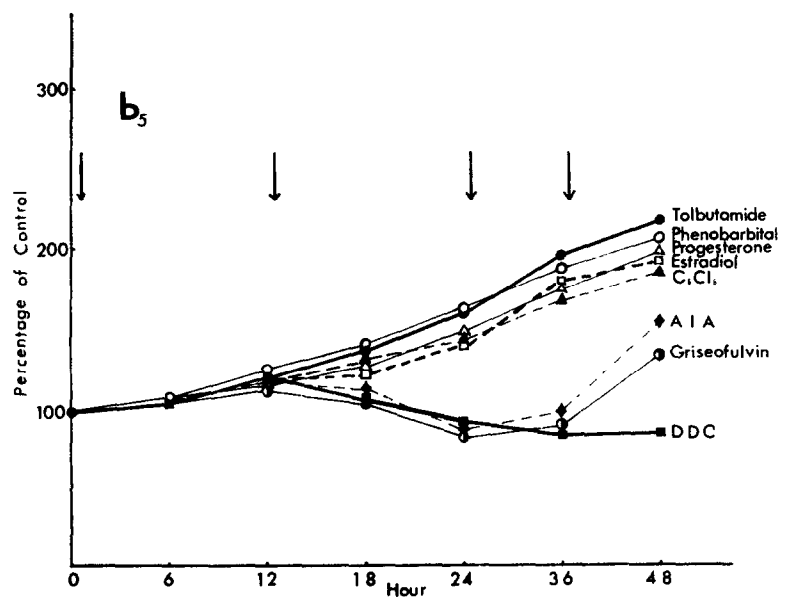

FIG. 3. Effects of various drugs on the microsomal content of cytochrome b_5. Each value plotted is an average of the amounts of cytochrome b_5/1 g liver of the treated group (6 mice) as compared with those of 6 control mice. The arrows indicate the injections.

when the level of the cytochrome P-450 was decreased, and continued to increase to about 7 times the normal level at 48 hr after treatment. When DDC was administered, the level of cytochrome P-450 and b_5 (Fig. 1,3) showed the same pattern as AIA or griseofulvin treated mice till 18 hr later. However, it continued to decrease without showing a phase of increase. In contrast, activity of ALA synthetase (Fig. 2) was

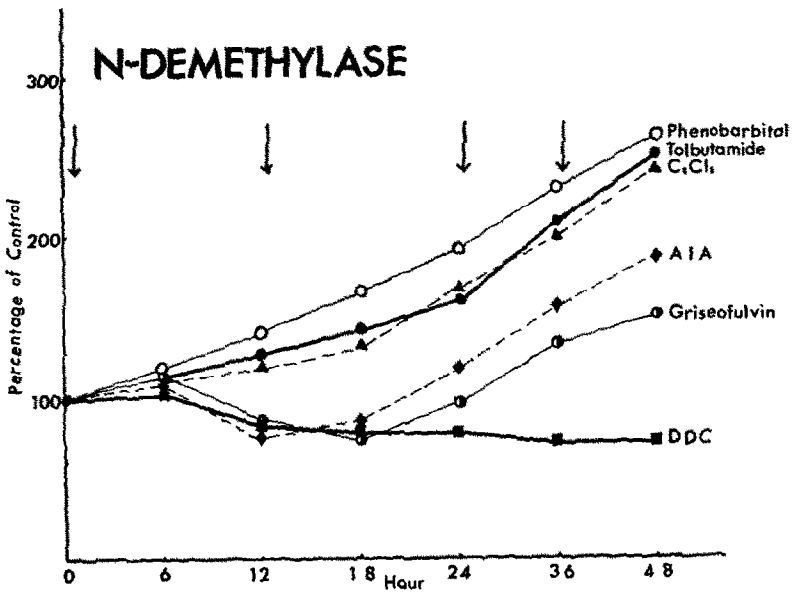

FIG. 4. Changes of aminoazo dye *N*-demethylase activities after treatment of mice with various drugs. Each value plotted represents an average of the activities per one gram liver of 6 experimental animals as compared with those of 6 control mice. The arrows indicate the injections.

increased most prominently and reached the maximal level at 36 hr after treatment, then it decreased in the subsequent 12 hr, reaching a level of twice or 3 times higher than normal.

Treatment of mice with phenobarbital, tolbutamide and female sex hormones resulted in a gradual increase of the level of cytochromes (Fig. 1,3) without showing a phase of decrease. Moreover increase of both cytochromes were higher than that of mice treated with AIA or griseofulvin, while activity of ALA synthetase (Fig. 2) remained at the level 1·5 to 2 times higher than normal. Accumulation of porphyrin (Table 1) was most prominent in the liver of mice treated with DDC. Moderate increase was observed in the liver of AIA or griseofulvin poisoned mice, whereas almost no increase was seen when mice were treated with phenobarbital, tolbutamide or female sex hormones.

Table 2 shows the inhibitory effect of DDC on the incorporation of ^{14}C-glycine into both microsomal and mitochondrial heme. Less incorporation of ^{14}C-glycine into microsomal heme than into mitochondrial heme was observed in mice treated with AIA or griseofulvin as compared with phenobarbital or C_6Cl_6. As shown in Table 3, the tendency to increase of cytochrome P-450 in mice treated with phenobarbital seems to be blocked with DDC administration.

TABLE 1. PORPHYRIN CONCENTRATION IN THE LIVERS OF MICE TREATED WITH DDC, AIA, GRISEOFULVIN (GF), C_6Cl_6, PHENOBARBITAL (PB), TOLBUTAMIDE (TB), PROGESTERONE (PG) AND ESTRADIOL (ED)

		Control	DDC	AIA	GF	C_6Cl_6	PB	TB	PG	ED
Coproporphyrin	Mean	1·1	165	58·5	105	4·5	1·2	1·4	1·2	1·1
	Range	0·5–2·0	96–210	40–73	80–135	2·5–5·5	0·5–2·1	0·5–3·0	0·5–2·4	0·6–2·5
Protoporphyrin	Mean	28·5	9800	1110	3250	78·5	30·5	32·5	34·5	29·5
	Range	18·3–34·5	8,100–11,200	950–1,350	2,500–4,100	60–105	20–35	30·2–37·5	23–37·5	19–38·5

Porphyrin values were determined at 48 hr after the first treatment and are expressed as μg per 100 g wet liver. Each figure was obtained from 6 mice.

DISCUSSION

Calculating from our data, the maximal ability of 1 g liver for ALA synthesis in normal mice appears to be about 480 mμmole/day (20 mμmole ALA × 24). As the half life of cytochrome P-450 is less than 12 hr,[12] ALA needed for maintaining the

TABLE 2. INCORPORATION OF 2-^{14}C-GLYCINE INTO HEPATIC MICROSOMAL AND MITOCHONDRIAL HEME *IN VIVO*

Duration of treatment		Control (6)	DDC (6)	AIA (6)	Griseofulvin (6)	C_6Cl_6 (6)	Phenobarbital (6)
				cpm/mg hemin/g liver			
24 hr	Microsome	36·0 ±0·7	12·9 ±1·2	59·8 ±1·8	39·0* ±2·2	64·0 ±3·0	69·0 ±2·0
	Mitochondria	20·5 ±1·0	6·3 ±0·5	62·5 ±2·0	70·0 ±4·8	60·1 ±2·9	61·0 ±1·8
48 hr	Microsome		18·9 ±2·5	66·2 ±2·0	48·0 ±2·2	130·0 ±3·1	141·0 ±4·2
	Mitochondria		12·5 ±1·5	58·0 ±2·2	111·3 ±5·9	55·3 ±1·2	61·0 ±3·8

Figures express mean ± S. E.
2-^{14}C-glycine was injected i.p. 60 min prior to sacrifice.
Data in parentheses indicate the number of mice.
Significance of difference between the means of control vs. those of the treated (*t* test) = $P < 0.01$ except*.

TABLE 3. EFFECT OF DDC ADMINISTRATION ON THE HEPATIC LEVELS OF CYTOCHROME P-450, ALA SYNTHETASE AND PROTOPORPHYRIN CONCENTRATION IN THE PHENOBARBITAL (PB) TREATED MICE

			P-450	ALA synthetase	Protoporphyrin
Control		(6)	100 ±8·1	100 ±9·0	1 ±0·2
PB	24 hr*	(6)	215 ±10·5	162 ±8·2	1·02 ±0·1
PB	24 hr*	(6)	112	456	30·2
DDC	12 hr*		±6·8	±20·8	±3·1
DDC	12 hr*	(6)	60·8 ±4·2	510 ±25·8	39·4 ±3·7
DDC	24 hr*	(6)	48·6 ±3·8	735 ±30·0	110 ±10·5
DDC	24 hr*	(6)	56·5	826	148
PB	12 hr*		±6·8	±38·5	±14·5
PB	12 hr*	(6)	158 ±12·0	138 ± 6·5	1·0 ±0·15

Figures express mean ± S.E.
* Injection time before sacrifice.
Values are expressed in relative units with the controls taken as 100 or 1.
Figures in parentheses indicate the number of mice.

normal level of cytochrome P-450 is at least 193·6 mμmole/g liver/day (24·2 mμmole × 8), which value is approximately 2/5 the normal maximum liver production. In phenobarbital treated mice at 48 hr after treatment, for example, 4-fold elevated level of cytochrome P-450 requires the synthesis of about 1060 mμmole ALA (193·6 × 3 + 480)/day for maintenance of that level. As considerable amount of ALA is used

for the increased content of cytochrome b_5, rate of ALA synthesis must be at least twice the normal level, provided that none of the other hepatic hemoprotein is increased as Schmid reported.[12, 17] That the assayed activity of ALA synthetase is about twice the control is not only comparable, but also means that ALA formed in mitochondria is smoothly utilized for the synthesis of microsomal cytochromes. On the contrary, AIA and griseofulvin, though they apparently showed the stimulatory effect in the initial stage, decreased the level of cyochromes until a considerable amount of ALA was supplied. The decreased level persists in case of DDC, resulting in the most prominent accumulation of protoporphyrin in liver in contrast to almost no accumulation in cases of phenobarbital, tolbutamide and female sex hormones. In view of isotopical studies, DDC seems to block heme formation as already reported by Onisawa *et al.*,[15] thought it must be taken into consideration that a change in a factor such as absorption rate or pool size could render results for glycine incorporation *in vivo* quite misleading as a measure of rate of synthesis of heme. It is well known that in microorganisms heme is involved in the regulation of the level of ALA synthetase[16] and that catalase, the hemoprotein, is decreased in liver of animals rendered prophyric by almost all kinds of porphyria-inducing chemicals.[17] As shown in this report, the level of cytochrome b_5 also showed a transient or permanent decrease, although a delay of appearance probably due to its longer turnover rate was observed. These facts appear to support the interpretation that in liver of AIA or griseofulvin poisoned mice a relative insufficiency of available hemes for synthesis of microsomal cytochromes is still occurring in spite of increased heme formation, although the attacking point of AIA or griseofulvin might be different from that of DDC. C_6Cl_6 poisoned mice showed similar changes in the level of microsomal cytochromes and the incorporation of ^{14}C-glycine into heme as phenobarbital poisoned mice, differing in the slightly higher porphyrin concentration and ALA synthetase activity. These findings are probably due to the weaker inhibitory effect of C_6Cl_6, and are in accordance with the observation of the slower evolution of the abnormality in C_6Cl_6-induced porphyria.[6] These results suggest that porphyria-inducing chemicals might block, partially or almost completely, the pathway anywhere from heme formation to heme utilization for synthesis of microsomal cytochromes, and a positive feedback seems to act to supply more heme precursor through a mechanism of induction of ALA synthetase, the rate-limiting enzyme in porphyrin synthesis.[7] It is also suggested that the amount of accumulated porphyrin would result from the difference between magnitude of ALA formation and that of protoporphyrin utilization, though the redox-environment in the liver might play an important role in determining both the pattern of excretion and extent of accumulation of porphyrins in certain types of porphyria as suggested by Rimington.[18]

In human subjects the elevated activity of ALA synthetase was reported in patients with acute intermittent porphyria.[19, 20] Furthermore, barbiturates,[21] female sex hormones,[22, 23] and sulfonylurea antidiabetic drugs[24, 25] have been reported to precipitate or aggravate human hepatic porphyrias, though these drugs did not cause increased porphyrin excretion in normal human beings.[26–28] It would be tempting to speculate from these facts and the results obtained from experimental porphyria that the metabolic defect of human hepatic porphyria might be a disturbed heme synthesis in the liver, and that the aggravation due to drug administration might well be due to the presence of an insufficiency of hepatic microsomal cytochromes or other

hemoproteins in the manifest stage due to a deficiency of available hemes compensated by increased ALA supply in a latent stage. Endogenous or exogenous toxic agents which escaped the detoxication by their livers are probably causing signs and symptoms of acute attacks in hepatic porphyria as suggested by many pathologists from autopsy findings[29] and by a successful treatment of a patient with extracorporal hemodialysis.[30]

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