Induction of δ -Aminolaevulinate Synthetase under Environmental-Stress Conditions

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1. Exposure of rats to environmental-stress conditions of hypobaria, hypoxia and cold did not alter the activity of hepatic δ -aminolaevulinate synthetase. 2. Induction of the enzyme by diethoxycarbonyldihydrocollidine was inhibited when the rats were exposed to hypobaria before or during the treatment with the drug but not after the initial phase when the process of induction was initiated. Neither increased concentration of the drug nor the time of induction had any effect on the inhibition under hypobaria. 3. A period of 12–24 h of pre-exposure to hypobaria gave the maximum inhibition, and on longer exposure the inhibitory effect was decreased. 4. The inhibition was not a permanent effect and could be substantially reversed in 12 h of withdrawal to ambient pressure. 5. Inhibition of induction was found under hypobaria and hypoxia, but not on exposure to cold. This suggests a specific effect of lack of O_2 rather than a general effect of stress. 6. It appears possible that alteration of concentration of endogenous adenine nucleotides may control the process of diethoxycarbonyldihydrocollidine-mediated induction of δ -aminolaevulinate synthetase, since treatment with ATP, cyclic AMP or theophylline produced inhibition similar to that under hypobaria and hypoxia.

Drug potency has been known for a long time to be altered under environmental-stress conditions. Lehman & Hanzlik (1932) observed a decreased LD_{50} for digitalis in cats at high altitude. A similar increased toxicity was reported for strychnine (Moore & Ward, 1935) and reserpine (Baumel *et al.*, 1967). On the other hand morphine sulphate and hexobarbital have been found to be less effective under the same conditions (Nedzel, 1955; Robinson *et al.*, 1968). However, the mechanisms of such an alteration in drug potency under stress conditions are not understood. In the present study an attempt has been made towards understanding this phenomenon by using the induction of hepatic δ -aminolaevulinate synthetase by a porphyrinogenic drug as the model system. The reasons for choosing this system are: (1) a variety of substances have been shown to induce δ -aminolaevulinate synthetase, including some drugs and steroids [see Tschudy & Bonkowsky (1972) for a review] and the site of action at the molecular level for some of the compounds has been well worked out; (2) δ -aminolaevulinate synthetase has been shown to be the rate-limiting enzyme in the biosynthesis of porphyrins from a variety of sources, such as micro-organisms (Burnham & Lascelles, 1963), plants (Ramaswamy & Nair, 1973), erythroid cells (Lever & Granick, 1965) and liver (Granick & Urata, 1963), and is therefore expected to be under regulation. In fact, it has been shown to be inhibited by haem, the end product of the biosynthetic pathway (Granick, 1966). In some of the photosynthetic micro-

organisms, low O_2 partial pressure in the environment has also been shown to play an important role in regulating this enzyme (Lascelles, 1959; Cohen-Bazire & Kunisawa, 1960). The increased haemoglobin concentration under high-altitude conditions is a well-documented phenomenon (see Folk, 1966). But not much is known about the regulation of δ -aminolaevulinate synthetase under hypobaric hypoxia in a non-erythropoietic tissue like liver. The results reported in the present paper indicate that the induction of hepatic δ -aminolaevulinate synthetase by 3,5-diethoxycarbonyl-1,4-dihydrocollidine is inhibited by hypobaria and hypoxia, but not in the cold.

Materials and Methods

Animals and exposure

Albino rats of both sexes (body wt. 100–120 g) from this Institute colony were used in all the experiments. The animals were starved for 24 h before diethoxycarbonyldihydrocollidine treatment. They were exposed to an atmospheric pressure of 0.05 MPa (corresponding to an altitude of about 6100 m) in a decompression chamber for the required period, as described by Inamdar *et al.* (1972).

Preparation of diethoxycarbonyldihydrocollidine and dosage

Diethoxycarbonyldihydrocollidine was prepared by the method of De Matteis & Prior (1962) starting

with ethyl acetoacetate, acetaldehyde and $(\text{NH}_4)_2\text{CO}_3$.

Diethoxycarbonyldihydrocollidine dissolved in peanut oil (50mg/ml) was administered subcutaneously at a dose of 500mg/kg body wt. and the animals were killed 6h after the treatment. The dosage and the period of treatment were the same in all the experiments, unless otherwise stated.

Enzyme assay

δ -Aminolaevulinate synthetase activity in the liver was assayed by the method of Marver *et al.* (1966a). The livers were homogenized in 3 vol. (w/v) of 0.05M-Tris-HCl buffer, pH 7.4, containing 0.9% NaCl and 0.5mM-EDTA. The incubation mixture contained 1 ml of the homogenate, 200 μ mol of glycine, 20 μ mol of EDTA and 0.1 M-Tris-HCl buffer, pH 7.4, to give a final volume of 2.0ml. The incubation was carried out at 37°C for 1h with continuous shaking. The reaction was stopped by the addition of 1 ml of 12.5% (w/v) trichloroacetic acid. δ -Aminolaevulinate was determined directly in the supernatant by using acetylacetone as the condensing agent and modified Ehrlich's reagent for colour development (Mauzerall & Granick, 1956).

Protein determination

Protein was determined by the biuret method (Gornall *et al.*, 1949).

Chemicals

ATP, theophylline, dibutyl cyclic AMP (6-N,2'-O-dibutyladenosine 3':5'-cyclic monophosphate) and insulin (crystalline) are from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Results

Effect of hypobaria on δ -aminolaevulinate synthetase induction

Exposure of rats to hypobaria for 6-72h did not alter the activity of hepatic δ -aminolaevulinate synthetase in normal animals. The results presented in Table 1 show the effect of exposure for 30h. When the last 6h of exposure was accompanied by treatment with diethoxycarbonyldihydrocollidine, the enzyme induction was decreased considerably compared with the corresponding diethoxycarbonyldihydrocollidine-treated normal animals. A similar inhibition was observed in animals treated with diethoxycarbonyldihydrocollidine at ambient pressure after a pre-exposure to hypobaria for 24h and also on treatment with twice the amount of diethoxycarbonyldihydrocollidine. The inhibition of induction may not be due to a change in the rate of enzyme induction or a lag thereof, since the enzyme activity in the hypobaria-exposed (24h) animals was always low compared with that in normal animals at all the time-intervals of diethoxycarbonyldihydrocollidine treatment studied (Fig. 1). The extent of inhibition was the same in both male and female animals. There seems to be some species specificity in this inhibitory effect, because no inhibition of induction was observed with mice. Wada *et al.* (1969), working with mice exposed to hypoxia, also observed no change either in basal or in diethoxycarbonyldihydrocollidine-induced activity in the liver, though the enzyme activity in spleen was increased severalfold.

Minimum period of pre-exposure

It is clear from the previous experiments that exposure for 6h during or for 24h before the diethoxycarbonyldihydrocollidine treatment was sufficient to inhibit the enzyme induction. Whether

Table 1. *Effect of hypobaric stress on δ -aminolaevulinate synthetase activity in the livers of normal and diethoxycarbonyldihydrocollidine-treated rats*

Male animals were exposed to about 0.5 atmospheric pressure (hypobaric) for 30h, the last 6h being accompanied with diethoxycarbonyldihydrocollidine treatment (50mg/100g body wt.). The values are means $\pm$ s.d. for six animals. *P* values were calculated by Student's *t* test. N.S., Not significant.

Treatment	Enzyme activity (nmol/h per mg of protein)		<i>P</i> values hypobaric versus normobaric
	Normobaric	Hypobaric	
None	0.29 $\pm$ 0.03	0.22 $\pm$ 0.04	N.S.
Diethoxycarbonyldihydrocollidine	1.06 $\pm$ 0.36	0.49 $\pm$ 0.19	<0.05
<i>P</i> values diethoxycarbonyldihydrocollidine versus normal	<0.002	<0.02	

exposure to short intervals before diethoxycarbonyldihydrocollidine treatment will also inhibit the induction was next studied. The animals were exposed to hypobaria for different time-intervals and then treated with diethoxycarbonyldihydrocollidine for 6h at ambient pressure. Pre-exposure of 6-9h was ineffective, and a minimum of 12h was necessary for obtaining a maximum of 60% inhibition (Fig. 2). Prolonged pre-exposure of 72h was still inhibitory, though the inhibition was only 40%. It was shown in

this laboratory that succinate dehydrogenase, an enzyme activated under hypobaria and hypoxia, reverted to normal activity on prolonged exposure to the stress (Susheela & Ramasarma, 1973). That a similar reversal may occur with the inhibition of δ -aminolaevulinate synthetase induction after several days of pre-exposure is indicated by the present results.

Fig. 1. Effect of different time-intervals of diethoxycarbonyldihydrocollidine treatment on the decreased induction of δ -aminolaevulinate synthetase under hypobaric stress

The animals were exposed to about 0.5 atmospheric pressure(hypobaric) for 24h, given diethoxycarbonyldihydrocollidine (50mg/100g body wt.) and then killed at specified time-intervals. The values are means, with $\pm$ S.D. indicated by bars ($n = 6$). Normobaric ($\bullet$); hypobaric ($\circ$).

Fig. 2. Effect of different periods of pre-exposure to hypobaric stress on the induction of liver δ -aminolaevulinate synthetase by diethoxycarbonyldihydrocollidine

The animals were exposed to about 0.5 atmospheric pressure (hypobaric) for the specified periods. Groups of three animals were then treated with diethoxycarbonyldihydrocollidine (50mg/100g body wt.) for 6h at ambient pressure. Mean values of enzyme activity (nmol/h per mg) and as a percentage of the control value without the exposure to stress are given.

Table 2. Effect of hypobaric stress during and after diethoxycarbonyldihydrocollidine treatment on δ -aminolaevulinate synthetase activity of rat liver

The animals were given diethoxycarbonyldihydrocollidine (50mg/100g body wt.) and exposed to about 0.5 atmospheric pressure (hypobaric) for the stated periods of time where indicated by +. The values are means $\pm$ S.D. N.S., Not significant.

Period of diethoxycarbonyldihydrocollidine treatment	Hypobaric stress			No. of animals	Enzyme activity (nmol/h per mg)	P value hypobaric versus normobaric
	0-3h	3-6h	6-9h			
0-6h	-	-		3	1.13 $\pm$ 0.28	-
	+	+		7	0.56 $\pm$ 0.15	<0.002
	-	+		5	1.00 $\pm$ 0.36	N.S.
	+	-		4	0.43 $\pm$ 0.18	<0.01
0-9h	-	-	-	3	1.44 $\pm$ 0.10	-
	-	-	+	5	1.43 $\pm$ 0.26	N.S.

Exposure to hypobaria during and after enzyme induction

To determine at what stage of induction exposure to stress would bring about effective inhibition, animals were treated with diethoxycarbonyldihydrocollidine and exposed to hypobaria at different time-intervals of the inducer administration. The results in Table 2 show that the induction was inhibited when the exposure to hypobaria for either 3 or 6h was immediately after diethoxycarbonyldihydrocollidine treatment. However, if the exposure was started at 3 or 6h after diethoxycarbonyldihydrocollidine treatment, no inhibition was observed. It appears therefore that the initial periods of drug treatment were sensitive to the stress effect.

Reversibility of the inhibition

To see whether the inhibition of induction is reversed on removal of the stress, animals were exposed to hypobaria for 24h, withdrawn to normal pressure for different time-intervals and treated with diethoxycarbonyldihydrocollidine for 6h at ambient pressure. The results in Fig. 3 indicate that about 80% of the control activity was restored within 12h of withdrawal. A similar reversal was observed with tryptophan pyrrolase and tyrosine aminotransferase (Inamdar *et al.*, 1972). The stress effects appear to be reversible and the normal enzyme concentrations were restored in about 1 day after withdrawal.

Inhibition by other factors

The inhibition of δ -aminolaevulinate synthetase induction by diethoxycarbonyldihydrocollidine under hypobaria may be due to either low pressure or a decrease in O_2 or a general stress effect. The effect of hypoxia and cold on the enzyme induction by diethoxycarbonyldihydrocollidine was therefore

studied. Exposure of rats to low environmental temperature of 0–5°C for 12h before diethoxycarbonyldihydrocollidine treatment did not alter δ -aminolaevulinate synthetase activity. However, animals exposed to hypoxia immediately after diethoxycarbonyldihydrocollidine injection showed an inhibition of enzyme activity (Table 3). Thus the inhibition seems to be due to the lack of O_2 but not an unspecific stress response.

Fig. 3. Effect of withdrawal from hypobaric stress on the induction of liver δ -aminolaevulinate synthetase by diethoxycarbonyldihydrocollidine

The animals were exposed to about 0.5 atmospheric pressure (hypobaric) for 24h, and groups of animals (three to five) were withdrawn to ambient pressure for the specified periods before diethoxycarbonyldihydrocollidine (50mg/100g body wt.) treatment of 6h. Mean values of enzyme activity (nmol/h per mg) and as a percentage of the control value without the exposure to stress are given.

Table 3. Effect of hypoxic and cold stress on diethoxycarbonyldihydrocollidine-induced δ -aminolaevulinate synthetase activity

For cold stress, the animals were exposed in a refrigerated chamber (0–5°C) for 12h and then treated with diethoxycarbonyldihydrocollidine for 6h at room temperature. For hypoxic stress, the animals were exposed for 6h to an atmosphere of 10% O_2 in N_2 during the period of diethoxycarbonyldihydrocollidine treatment. The number of animals in each group is given in parentheses. Only the decrease on hypoxic stress in diethoxycarbonyldihydrocollidine-treated animals is significant (* $P < 0.05$). The values are means $\pm$ s.d.

Treatment	Enzyme activity (nmol/h per mg)		
	Normal	Cold stress	Hypoxic stress
None	0.18 $\pm$ 0.04 (3)	0.12 $\pm$ 0.04 (3)	—
Diethoxycarbonyldihydrocollidine	1.05 $\pm$ 0.30 (3)	1.07 $\pm$ 0.23 (3)	0.40 $\pm$ 0.18* (6)

Table 4. *Effect of some compounds on diethoxycarbonyldihydrocollidine-induced δ -aminolaevulinate synthetase in the livers of normal rats*

ATP, theophylline and dibutyl cyclic AMP ($6\mu\text{mol}/\text{animal}$) were given intraperitoneally in two equal doses, one before and another 3 h after diethoxycarbonyldihydrocollidine treatment. Insulin ($1\text{ i.u.}/\text{animal}$) was given subcutaneously in one single dose before diethoxycarbonyldihydrocollidine treatment. The values are means $\pm$ S.D.

Treatment	No. of animals	Enzyme activity (nmol/h per mg)	P values experimental versus no treatment
None	6	1.06 ± 0.16	—
ATP	7	0.16 ± 0.02	<0.001
Theophylline	3	0.33 ± 0.13	<0.02
Dibutyl cyclic AMP	5	0.28 ± 0.06	<0.001
Insulin	4	0.25 ± 0.10	<0.002

Marver *et al.* (1966b) observed an inhibition of allylisopropylacetamide-induced δ -aminolaevulinate synthetase activity by glucose. Subsequently, Gajdos & Gajdos-Torok (1970) reported increased ATP activity after glucose treatment and suggested ATP as the possible inhibitor in the glucose effect on enzyme induction. In the present experiments, not only ATP but also 3':5'-cyclic AMP could also inhibit the induction (Table 4). Theophylline inhibition further supported the role of 3':5'-cyclic AMP. Insulin has been shown to decrease tissue 3':5'-cyclic AMP concentrations (Jefferson *et al.*, 1968) and was therefore expected to antagonize the effect of cyclic AMP. However, it was inhibitory to the induction of δ -aminolaevulinate synthetase.

Discussion

The above results indicate that the inhibitory effect on induction may not directly involve enzyme synthesis in view of the lack of effect of hypobaria on the basal as well as the induced enzyme activities. It is therefore conjectured that the interference of the stress is with the action of the drug. Since exposure in the first half of the period of diethoxycarbonyldihydrocollidine administration was inhibitory and not in the second half, inhibition of the entry of diethoxycarbonyldihydrocollidine to the target tissue appears to be a possibility, especially in view of the findings of Racz & Marks (1972a,b) that the extent of the induction of δ -aminolaevulinate synthetase is proportional to the amount of the compound present in the liver. Altered body-fluid compartmentalization has been reported under several stress conditions (Reissmann, 1951; Fryers, 1952), which may affect the body distribution of the compound. The metabolism of any drug in the liver is important in determining its potency under a given environmental condition. For example, Merritt & Medina (1968) found an increased hexobarbital metabolism under simulated altitude conditions with a concomitant

decrease in the hexobarbital sleeping time. Solomon & Figge (1960) have shown that some of the derivatives of diethoxycarbonyldihydrocollidine are not porphyrinogenic to experimental animals. Whether diethoxycarbonyldihydrocollidine is more rapidly metabolized to its inactive derivatives under hypobaria should be determined. Microsomal drug-metabolizing enzymes, however, do not seem to have a role in this process, since under conditions where they increased, such as phenobarbital treatment (De Matteis, 1972) and cold exposure (Furner & Stitzel, 1968), the induction of δ -aminolaevulinate synthetase by diethoxycarbonyldihydrocollidine was unaffected. Inhibition by exogenous ATP and cyclic AMP similar to that seen with hypobaria and hypoxia suggests that alteration of the nucleotide pools might explain stress-mediated effects, and is worthy of further investigation.

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