INDUCTION OF LIVER MICROSOMAL MONOOXYGENASES IN EXPERIMENTAL CHOLESTASIS

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The intensity of the inducing action of phenobarbital on microsomal monooxygenase depending on the number of binding sites for this inducer in the active center of cytochrome P-450 was investigated. Cholestasis accompanied by the accumulation of hydroxylated metabolites of cholesterol, possessing detergent properties and disintegrating the substrate area for phenobarbital in the P-450 molecule, was chosen as the model. The ability of phenobarbital to induce under conditions excluding the stage of primary binding and metabolism of monooxygenase was demonstrated; this points to activation of the synthesis of the specific protein by the whole molecule of the inducer and not by its primary hydroxylation products in the microsomes.

KEY WORDS: microsomal monooxygenase; induction by phenobarbital; cholestasis.

Among substrates undergoing oxidative conversions catalyzed by cytochrome P-450 in the liver microsomes there are some with the ability to induce the formation of that enzyme de novo [5]. The understanding of the mechanism of this phenomenon, which is of clinical [13] as well as of theoretical importance, largely depends on the discovery of the nature of intracellular reception of the inducer and subsequent activation of the genome [9]. For phenobarbital, the most thoroughly studied member of this group of inducers [5], this receptor is not yet known. On the other hand, modern theories of induced enxyme formation attribute a key role to the initial stage of formation of the enzyme–substrate (inducer) complex; the role ascribed to the substrate is either that of stabilizing the enzyme or of stimulating synthesis of the specific protein by the products of its primary metabolism [1]. In this connection it was natural to assume that the active center of cytochrome P-450 itself acts as the receptor for phenobarbital.

To test this hypothesis the intensity of the inducing action of phenobarbital was studied in relation to the number of binding sites for this and other type I substrates [15] in the active center of cytochrome P-450. Experimental cholestasis was chosen as the model; in this condition hydroxy-derivatives of bile salts, with detergent properties and the ability to destroy the substrate area for barbiturates and pyrazolone derivatives [4] in the P-450 molecule, accumulate in the liver cells [8].

EXPERIMENTAL METHOD

Male Wistar rats weighing 180-200 g were used. The bile duct was ligated by the method of Greim et al. [8]. On the fourth day after the operation some animals received intraperitoneal injections of phenobarbital sodium (100 mg/kg) daily for 3 days. Rats undergoing a mock operation, who also received phenobarbital, acted as the control. Isolation of the liver microsomes and determination of the content of protein and of cytochromes P-450 and P-420, of activity of NADPH-cytochrome c reductase and NADPH-cytochrome-P-450 reductase, of maximal binding (the ΔA_{max} constant) of phenobarbital, aminopyrine, and aniline, the rate of N-demethylation of aminopyrine and of p-hydroxylation of aniline, and also activity of enzyme and ascorbate-dependent systems of peroxidation of microsomal lipids, were carried out as de-

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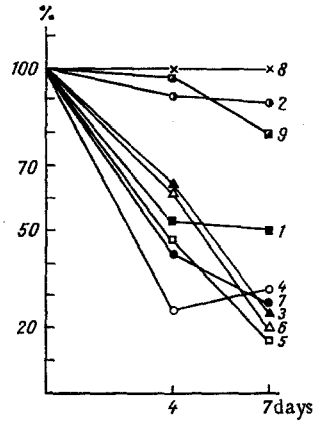

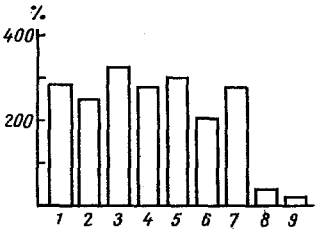

Fig. 1 Fig. 2

Fig. 1. Substrate-binding and metabolic activity of microsomal fraction of liver during development of cholestasis. 1) Content of cytochrome P-450 (100% = 0.95 ± 0.07 nmole/mg protein); 2) activity of NADPH-cytochrome c reductase (100% = 93 ± 6 nmoles cytochrome reduced in 1 min mg protein); 3) activity of NADPH-cytochrome P-450 reductase (100% = 2.0 ± 0.1 nmoles cytochrome reduced in 1 min/1 mg protein); 4) maximal binding of aniline (100% = 27.0 ± 1.2 O. D. units between 430 and 500 nm · 10^{-3}/mg protein); 5) maximal binding of aminopyrine (100% = 13.8 ± 0.9 O.D. units between 421 and 500 nm × 10^{-3}/mg protein); 6) rate of p-hydroxylation of aniline (100% = 0.56 ± 0.04 nmole p-aminophenol formed in 1 min/mg protein); 7) rate of N-demethylation of aminopyrine (100% = 3.76 ± 0.22 nmoles formaldehyde formed in 1 min/mg protein); 8) rate of enzymic preoxidation of lipids (POL) (100% = 164 ± 10 natoms oxygen utilized in 1 min/mg protein); 9) rate of ascorbate-dependent POL (100% = 180 ± 14 natoms oxygen utilized in 1 min/mg protein). Abscissa, time after ligation of bile duct (in days); ordinate, spectral and kinetic parameters of experimental microsomes (in % of control).

Fig. 2. Effect of phenobarbital on content and enzyme activity of components of microsomal monooxygenase from liver of control rats. 1) Content of cytochrome P-450; 2) activity of NADPH-cytochrome c reductase; 3) activity of NADPH-cytochrome P-450 reductase; 4) maximal binding of aminopyrine; 5) rate of N-demethylation of aminopyrine; 6) maximal binding of aniline; 7) rate of p-hydroxylation of aniline; 8) rate of enzymic POL; 9) rate of ascorbate-dependent POL. For values of control indices, see Fig. 1. Ordinate, inducing effect of phenobarbital (in % of control).

scribed previously [16, 17]. Activity of NADPH oxidase in the presence and absence of 1.6 mM phenobarbital was determined by Orrenius's method [11]. All spectral measurements were made on the Hitachi-356 differential spectrophotometer. Details of the experiments are given in the captions to the figures.

EXPERIMENTAL RESULTS AND DISCUSSION

As Fig. 1 shows, microsomal enzyme systems sensitive to all forms of injury to the phospholipid component of the membranes [6] have their activity considerably reduced during the development of cholestasis. The reason is evidently an excessive accumulation of secondary cholates, with a detergent action on the microsomal membrane [4], in the liver cells.

TABLE 1. Effect of Cholestasis on Maximal Binding of Phenobarbital and on NADPH-Oxidase Activity of Liver Microsomes (results of four experiments; M ± m)

Experimental conditions	ΔA_{max}*	Rate of oxidation of NADPH†	
		in absence of phenobarbital	1.6 mM phenobarbital
Control	5,9±0,29	11,8±0,73	14,3±0,8
Cholestasis (7 days)	0,1±0,01	5,1±0,4	5,1±0,4
Same + injection of phenobarbital, starting on 4th day	3,4±0,17	7,7±0,52	12,1±0,7
Control + injection of phenobarbital for 3 days	3,9±0,22	21,8±1,2	26,1±1,3

*Maximal binding in $\Delta A_{421-500} \cdot 10^3$ in O.D. units/min/mg protein.
†Rate of oxidation of NADPH in nmoles/min/mg protein.

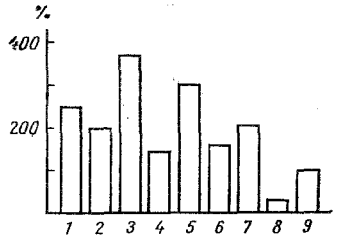

Fig. 3. Effect of induction of liver microsomal monooxygenases by phenobarbital during cholestasis. Abscissa, content and activity of monooxygenase components (legend, see Fig. 2); ordinate, intensity of inducing action of phenobarbital (in % of original activity on seventh day of cholestasis).

The choice of time for the administration of phenobarbital in these experiments during the development of cholestasis was determined by two factors. First, in the period between the 4th and 7th days after ligation of the bile duct, indices such as the cytochrome P-450 content, activity of NADPH–cytochrome c reductase, and binding of aniline, a substrate using the ligand of heme iron of P-450, came out onto a plateau (Fig. 1). Second, in this period the number of binding sites for type I substrates estimated from the value of ΔA_{max} [12], fell considerably for aminopyrine (Fig. 1) and was virtually indeterminable for phenobarbital (Table 1).

Furthermore, during the development of cholestasis the stimulating effect of phenobarbital on the rate of NADPH oxidation was not observed (Table 1); according to Martin [10], this is evidence of inhibition of the hydroxylation of this barbiturate by the enzyme system containing P-450. The ability of phenobarbital to induce microsomal monooxygenase can thus be estimated under conditions when the stage of primary binding and metabolism of the inducer by the enzyme is absent.

The results given in Figs. 2 and 3 indicate that triple injection of phenobarbital into both control and experimental animals is followed by a marked and practically equal increase in the content and enzymic activity of components of microsomal electron-transport chain. Meanwhile the rather smaller increase in maximal binding of aminopyrine can evidently be explained by competition between this substrate and hydroxylated metabolites of cholesterol, which are also type I substrates with high affinity for P-450 [8]. This conclusion is also supported by the greater increase in the rate of the NADPH–cytochrome P-450 reductase reaction in the liver microsomes of the experimental than of the control rats. On the other hand, the less marked increase in binding and metabolism of the type II substrate, aniline, observed in the experimental preparations cannot be completely understood; in this connection the possible detergent effect of deoxycholate on the phospholipid-dependent hydrophobic zone, incorporating the heme of cytochrome P-450, cannot be ruled out [3].

As Fig. 2 shows, microsomal membranes from the liver of induced rats have a low velocity of lipid peroxidation (POL) reactions. This applies equally to the rate of enzymic POL by the experimental microsomes (Fig. 3). As regards the activity of ascorbate-dependent POL, in this case the picture was similar to that with the control preparations, when treatment of microsomes in vitro with deoxycholate led to a

marked increase in the rate of POL, which was explained by the "loosening" of the membrane and the easier breakdown of the resulting hydroperoxides of the unsaturated fatty acids by iron ions [2].

The data given in this paper thus indicate marked induction of microsomal monooxygenases by phenobarbital, despite the absence of the stage of primary binding and metabolism in the active center of the enzyme so very important for a lipophilic inducer [7, 14]. The facts described above suggest that activation of the genome and of subsequent synthesis of specific protein in the mechanism of phenobarbital induction are carried out by the whole molecule of the inducer and not by the products of its primary hydroxylation in the microsomes.

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