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Phenobarbital-induced increase of NADH-cytochrome b_5 reductase activity in rat liver microsomes

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The enhanced rate of oxidative drug metabolism after chronic administration of phenobarbital is currently attributed to an increased activity of the microsomal mixed-function oxygenase [1, 2]. This system has been further resolved into three main components: cytochrome P-450, NADPH-cytochrome P-450 reductase and phospholipids [3]. The content of both cytochrone P-450 and its reductase has been shown to increase in liver microsomes after phenobarbital administration [4]. In addition it was shown that cytochrome b_5 participates in the mixed-function oxygenase system by transferring one electron from NADH to cytochrome P-450 [5–7]. Indeed cytochrome b_5, which is the only other cytochrome present in microsomes, also increases after repeated doses of phenobarbital [8, 9]. However we are not aware of studies on the effect of phenobarbital on the enzyme which reduces cytochrome b_5: NADH-cytochrome b_5 reductase (EC 1.6.2.2).

In this report evidence is provided that the activity of this reductase is increased in liver microsomes of phenobarbital-treated rats.

MATERIALS AND METHODS

Sprague–Dawley CD female rats (Charles River, France) were first maintained on a standard pellet chow (and in the same housing conditions) for 8 days prior to experimentation. Six rats were given increasing doses from 8 to 14 mg/100 g body wt. per day [10] of phenobarbital sodium salt (Merck, Darmstadt) intraperitoneally; phenobarbital was dissolved in 500 μl of 0.15 M NaCl and injected twice a day for 4 days. An identical volume of 0.15 M NaCl was injected at corresponding times to six control rats. Immediately after the final injection, the animals were starved and sacrificed 24 hr later. At that time, their body wt. was 180–210 g. Under light ether anesthesia, the liver was washed by injecting 20 ml of ice-cold 0.15 M NaCl into the portal vein; the liver was then quickly excised, weighed and homogenized in 4 volumes of ice-cold 0.25 M sucrose. The homogenate was centrifuged at 10,000 *g* for 15 min and the supernatant was subsequently centrifuged at 100,000 *g* for 60 min. The resulting pellet was resuspended in 0.25 M sucrose. It was controlled that the corresponding 100,000 *g* supernatant was not contaminated with microsomes since it was devoid of any measurable activity of NADPH-cytochrome *c* reductase and UDP-glucuronosyltransferase.

NADH-cytochrome b_5 reductase was assayed as follows. The assay mixture was that of Mihara and Sato [11] and contained 100 mM potassium phosphate buffer, pH 7.5 and 10 μM cytochrome b_5. Each determination was performed in duplicate, using either 5 and 10 μg of microsomal protein, or 25 and 50 μg of cytosolic protein. The final volume was 500 μl. The reaction was initiated by the addition of NADH 100 μM final, and followed at 556 nm. This wavelength corresponds to a peak of absorption of reduced cytochrome b_5 and was preferred to 423 nm because it lessens the interference due to any contaminating protein which would also absorb in the Soret region. The reaction was monitored on a Gilford 2400 spectrophotometer with a full-scale expansion of 0.1 OD and a chart speed of 2 inch/min. Under these conditions the reduction of cytochrome b_5 is extremely rapid, with pseudo-first order kinetics with respect to cytochrome b_5 concentration, as already shown by Ohnishi *et al.* [12]. Therefore the velocity of the reaction was defined as the apparent first order rate constant k_r (min^{-1}). There was a linear correlation between the calculated rate constant and the amount of extract up to a protein concentration of 300 μg in the cuvette. The cytochrome b_5 used in this assay was prepared from trypsinized rat liver microsomes according to the procedure of Omura and Takesue [13]. Cytochrome b_5 was quantitated in microsomal suspensions from the difference-spectrum between oxidized and NADH-reduced samples. An increment in extinction coefficient of 185 $cm^{-1}mM^{-1}$ between 424 nm and 409 nm was utilized [14]. Cytochrome P-450 was measured in microsomal suspensions according to Schoene *et al.* [15] using a Perkin–Elmer 356 double beam spectrophotometer. Ethylmorphine *N*-demethylase activity was assayed by measurement of the formation of formaldehyde [16] according to the colorimetric procedure of Nash. The reaction mixture contained: 3 mM ethylmorphine, 100 mM phosphate buffer, pH 7.4, 25 mM $MgCl_2$, 0.66 mM NADP, 3 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase and 2 mg/ml microsomal protein. The final volume of the reaction mixture was 360 μl. The reaction was carried out in a shaking bath for 10 min at 37°. Aniline hydroxylase activity was assayed according to the method of Mazel [17]. Final concentrations in the reaction mixture were: 1.25 mM aniline, 50 mM phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 0.16 mM NADP, 2.5 mM glucose-6-phosphate, 12.5 mM nicotinamide and 2 mg/ml microsomal protein. The reaction was carried out for 30 min at 37° with a final vol. of 400 μl.

Protein content in homogenates, in cytosolic and in microsomal suspensions was determined by the method of Lowry *et al.* [18]. Statistical analysis of the results was performed according to the nonparametric test [19] of Mann and Whitney.

RESULTS AND DISCUSSION

The microsomal and cytosolic cytochrome b_5 reductase response to phenobarbital was compared to that of other inducible systems: cytochrome P-450, cytochrome b_5, ethylmorphine demethylase and aniline hydroxylase. After 4 days of phenobarbital treatment, liver weight increased from 7.14 ± 0.61 (S.E.M.) to 9.37 ± 0.79 g (+ 31 per cent) ($P < 0.001$) and the liver:body weight ratio (g/100 g body wt. × 100) increased similarly from 3.67 ± 0.41 in the controls to 4.70 ± 0.25 in the phenobarbital-treated rats ($P < 0.001$). Liver protein content remained essentially unchanged, amounting to 110.5 ± 4.5 and 97.0 ± 11.8 mg/g of liver in the controls and the treated rats, respectively. The amount of cytochrome P-450 per g of liver, estimated in the homogenates, increased significantly from 42.1 ± 3.6 in the controls to 70.4 ± 10.4 nmoles/g of liver ($P < 0.001$).

In phenobarbital-treated rats, microsomal cytochrome b_5 reductase activity was significantly increased (+ 53 per cent, $P < 0.001$). This increase was of the same order of magnitude as that observed for cytochrome b_5 (Table 1).

Table 1. The effect of phenobarbital on the rat liver cytochrome b_5 system and on some mixed-function oxygenases involved in drug metabolism

	Controls	Phenobarbital	% Increase
Microsomal cytochrome b_5 (nmoles × mg protein^{-1})	0.296 ± 0.066	0.394 ± 0.20*	33
Microsomal NADH-cytochrome b_5 reductase (k_r† × mg protein^{-1})	11.44 ± 0.09	17.50 ± 2.01*	53
Cytosolic NADH-cytochrome b_5 reductase (k_r† × mg protein^{-1})	1.58 ± 0.31	1.64 ± 0.20‡	4
Microsomal cytochrome P-450 (nmoles × mg protein^{-1})	0.73 ± 0.05	1.51 ± 0.24*	107
Aniline hydroxylase (nmoles × min^{-1} × mg protein^{-1})	3.78 ± 0.92	6.38 ± 0.75*	69
Ethylmorphine demethylase (nmoles × min^{-1} × mg protein^{-1})	2.86 ± 0.41	6.90 ± 1.18*	141

All values are means ± 1 S.E.M. for six rats.
* $P < 0.001$.
† k_r is the apparent first order reaction constant.
‡ Not significant.

No rise in cytosolic cytochrome b_5 reductase activity could be detected after phenobarbital. This result must be interpreted with caution however, owing to the very low activity of this enzyme in hepatic cytosol (unpublished data).

The stimulation of the drug hydroxylation system by phenobarbital was more pronounced. In phenobarbital-treated rats, microsomal cytochrome P-450, aniline hydrolase and ethylmorphine demethylase increased by about 110, 70 and 140 per cent respectively; the results are detailed in Table 1.

Our results indicate that liver microsomal NADH-cytochrome b_5 reductase, as well as cytochrome b_5, is induced in rat liver following a 4-day treatment with phenobarbital. Under the same experimental conditions, the expected induction of cytochrome P-450, aniline hydroxylase and ethylmorphine demethylase was observed. In contrast, the soluble form of NADH-cytochrome b_5 reductase which was recently shown to exist in several tissues [20] was not significantly increased by phenobarbital. The microsomal and soluble forms of cytochrome b_5 reductase appear to be under the same genetic control, since they are both lacking in man in the inherited disease of congenital methemoglobinemia with severe encephalopathy [21]. The relationship between the two forms of cytochrome b_5 reductase is still unknown. It has been postulated that the cytosolic enzyme results from the post-translational modification (possibly proteolytic) of the microsomal enzyme [20]. The results of the present work neither suggest nor preclude the possibility that the induced microsomal cytochrome b_5 reductase might be a precursor of the cytosolic soluble enzyme. For instance, a long-term effect of phenobarbital remains possible and further study is clearly needed.

The induction of cytochrome b_5 reductase as well as that of cytochrome b_5 by phenobarbital leads us to postulate that such stimulation of the NADH-dependant portion of the electron transport chain may contribute to the enhancement of microsomal drug oxidation.

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