BENZONAL: AN INDUCER OF PHENOBARBITAL TYPE OF THE MONOOXYGENASE SYSTEM

T. P. Novozheeva, A. S. Saratikov, A. Yu. Grishanova, N. I. Gutkina, R. R. Akhmedzhanov, O. G. Khatsenko, and N. E. Kozlovskaya

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Because of its marked inducing effect on the monooxygenase system (MOS) of the liver, the original Soviet anticonvulsant benzonal is being used for a different purpose, as an enzyme-inducer [12]. By the strength and duration of its effect, benzonal is not inferior to phenobarbital and is superior to Zixoryn (Flumecinol) [6, 10, 11]. However, the type of enzymic induction of MOS produced by benzonal has not yet been established, although it is known to bind, like phenobarbital, with cytochrome P-450 according to type I [7].

In the investigation described below modern methods of xenobiochemistry, including immunochemical determination of individual cytochrome P-450 isozymes and of their catalytic activity, the type and possible mechanism of induction of MOS by benzonal were determined.

EXPERIMENTAL METHOD

Experiments were carried out on 30 noninbred male albino rats weighing 140-160 g. The inducers were given in the form of a suspension in 1% starch mucilage internally in three equimolar doses: benzonal 70 mg/kg, phenobarbital 50 mg/kg. Animals of the control group received an equal volume of mucilage. The animals were decapitated 48 h after the last injection of the inducers and the microsomal fraction of the liver was isolated [1]. The cytochrome P-450 and b_5 concentrations were determined as in [17], activity of NADPH-cytochrome P-450-reductase as in [3], and protein by Lowry's method. The velocity of N-demethylation of amidopyrine was determined by measuring the formation of formaldehyde, and the rate of *p*-hydroxylation of aniline by measuring *p*-nitrophenol formation [3]; the velocity of O-de-ethylation of 7-ethoxyresorufin and of O-dealkylation of pentoxyresorufin was determined fluorometrically, using resorufin as the standard [13]. Metabolism of androstenedione was studied as described previously, corticosterone being used as the internal standard; the metabolites were analyzed by high-pressure chromatography [21]. Molecular isoforms of cytochrome P-450 from liver microsomes of rats induced with phenobarbital ($P\text{-}450_b$) and with 3-methylcholanthrene ($P\text{-}450_c$) were obtained by the method described previously [15]. Antibodies against the forms of cytochrome P-450 were obtained by immunizing adult noninbred rabbits with preparations of the cytochromes, as described previously [2]. Immunoglobulins were isolated from the immune sera by fractionation with ammonium sulfate and were used for immunochemical analysis of the microsomes. Ouchterlony's double immunodiffusion test was carried out as described previously [19]. Concentrations of isoforms of cytochrome P-450 ($P\text{-}450_b$ and $P\text{-}450_c$) were determined by immunoelectrophoresis [18]. Proteins of the microsomal fraction were separated by SDS-PAG electrophoresis [16]. The results were subjected to statistical analysis by Student's *t*-test.

*Deceased.

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TABLE 1. Effect of Benzonal and Phenobarbital on Rat Liver Microsomal MOS ($M \pm m$)

Parameters	Control	Benzonal	Phenobarbital
Microsomal protein, mg per organ	56,2±2,50	72,3±4,07*	75,6±7,35*
Cytochrome P-450, nmoles/mg protein	0,53±0,07	1,35±0,07*	1,21±0,08*
Immunochemically determined $P\text{-}450_{b+e}$, nmoles/mg protein	0,02±0,002	0,60±0,03*	0,56±0,07*
Cytochrome b_5, mmoles/mg protein	0,48±0,01	0,55±0,03	0,48±0,04
NADPH-cytochrome C-reductase, nmoles/min/mg protein	153±13,0	342±40,0*	321±25,0*
Mono-oxygenase activity, nmoles/min/mg protein			
Amidopyrine N-demethylase	1,44±0,15	5,29±0,33*	4,78±0,37*
Aniline p-hydroxylase	0,54±0,01	1,11±0,22*	1,12±0,07*
7-Ethoxyresorufin O-de-ethylase	0,07±0,002	0,08±0,02	0,06±0,01
7-Pentoxyresorufin O-dealkylase	0,07±0,01	0,83±0,05*	1,20±0,03*
Androstenedione hydroxylase			
7α OH	0,17±0,01	0,17±0,06	0,01±0,01
16α OH	1,96±0,13	2,69±0,67	0,87±0,28
6β OH	1,07±0,30	1,96±0,43	1,36±0,36
16β OH	0,82±0,20	X3,33±0,71*	3,11±0,32*

EXPERIMENTAL RESULTS

The general characteristics of MOS of the liver microsomes after three doses of benzonal and phenobarbital were given internally to the rats are shown in Table 1. Like phenobarbital, benzonal raised the microsomal protein level, the cytochrome P-450 concentration, and activity of NADPH-dependent cytochrome P-450-reductase, but did not change the cytochrome b_5 concentration.

Qualitative estimation of induced forms of cytochrome P-450 in the liver microsomes after administration of benzonal and phenobarbital to the animals was undertaken by Ouchterlony's double immunodiffusion test, using antibodies against $P\text{-}450_b$ and $P\text{-}450_c$. Microsomes isolated from the liver of rats receiving the inducers formed a precipitation line with anti-$P\text{-}450_b$ but did not interact with anti-$P\text{-}450_c$. Consequently, in microsomes induced by benzonal, a form of cytochrome P-450 immunologically identical with the phenobarbital-induced form ($P\text{-}450_b$) was formed, but the methylcholanthrene-induced form ($P\text{-}450_c$) was absent.

The content of the form of cytochrome P-450 immunologically identical with the phenobarbital-induced form was determined by "rocket" electrophoresis using anti-$P\text{-}450_b$. It was taken into account that form $P\text{-}450_b$ is immunologically identical with form $P\text{-}450_e$, which also is induced by inducers of phenobarbital type [5, 20]. These forms have similar molecular mass, a high degree of homology in their primary structure, and they differ only in substrate specificity and catalytic activity [20]. The total amount of cytochromes $P\text{-}450_{b+e}$ was therefore determined. As Table 1 shows, the absolute and relative content of these forms of the hemoprotein was similar in experiments with benzonal and with phenobarbital.

The molecular mass of forms of the hemoprotein induced by benzonal and phenobarbital was determined by SDS-PAG electrophoresis. The increase in the intensity of the protein bands corresponds to a molecular mass of 52 kilodaltons, and indicates induction of forms of cytochrome P-450 characteristic of phenobarbital, i.e., forms $P\text{-}450_{b+e}$ [5, 20].

Functional activity of cytochrome P-450-dependent microsomal MOS was assessed relative to the following xenobiotic substrates: amidopyrine, aniline, ethoxyresorufin, pentoxyresorufin, andostenedione, and hexobarbital. Both inducers significantly increased activity of amidopyrine N-demethylase and aniline *p*-hydroxylase (Table 1). However, the metabolism of these substrates is known not to be specific for any one form of hemoprotein, but reflects only the possibility that, in principle, the metabolism of a wide class of substrates can be accelerated by the inducer, a feature which is characteristic of inducers of phenobarbital type [4, 5]. To assess substrate specificity of forms of cytochrome P-450 induced by benzonal, the substrates chosen had a metabolism strictly linked with definite forms of this hemoprotein. For instance, 7-ethoxyresorufin is metabolized only by ethoxyresorufin O-d-ethylase and it is the substrate f form $P\text{-}450_c$, induced by 3-methylcholanthrene and other polycyclic aromatic hydrocarbons [13]. In our experiments, under the influence of benzonal and phenobarbital catalytic activity of this form was not found, confirming results obtained by its immunochemical determination. Meanwhile, the velocity of O-dealkylation of pentoxyresorufin, a substrate characteristic of form $P\text{-}450_b$, increased. With the aid of antrostenedione metabolism, activity of four forms of cytochrome P-450 can be assessed [15, 21]. Benzonal accelerates hydroxylation of androstenedione in the 16β position, which is linked with the $P\text{-}450_b$ form [15]. The velocity of metabolism of this hormone in positions 7α, 16α, and 6β did not differ from the control. The results are evidence that benzonal, in its effect on MOS, is an inducer of phenobarbital type, and that its effect is not weaker than that of phenobarbital.

The inducing effect of benzonal on the hepatic MOS has been demonstrated experimentally [6, 10, 11]. However, there is evidence that benzonal is found in the human and animal body only in the form of its principal metabolite, phenobarbital, which also determines its anticonvulsant effect [8, 9]. It is considered that benzonal, when administered internally, is metabolized by alkaline hydrolysis in the small intestine to phenobarbital and benzoic acid [8]. It is logical to suggest that the enzyme-inducing activity of benzonal also is due to the action of phenobarbital. This hypothesis is supported by our own data and, in particular, the similar effect of the two inducers on the level of cytochrome P-450 and its isoforms $P\text{-}450_{b+e}$, on the velocity of metabolism of model substrates (Table 1). Nevertheless, the inducing activity of benzonal molecules themselves, bound according to type I with cytochrome P-450, cannot be ruled out; in the case of induction by phenobarbital, moreover, affinity of benzonal for the hemoprotein increases considerably [7]. Since in these experiments benzonal was added to a suspension of microsomes immediately before the optical density was measured, its metabolism in microsomes must be excluded.

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