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THE EFFECT OF PHENOBARBITAL AND DEXAMETHASONE COADMINISTRATION ON THE ACTIVITY OF RAT LIVER P450 SYSTEM

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SUMMARY: Phenobarbital, the potent inducer of CYP2B and CYP3A, and dexamethasone, that induces CYP3A, are not able to elevate p-nitrophenol hydroxylase activity of CYP2E1. However, rats treated with phenobarbital and dexamethasone in combination showed threefold increase in p-nitrophenol hydroxylation and the activity correlates with an elevated amount of a 53.000 dalton protein. Biosynthesis of mRNA and P450 protein is required for the induction. 3-amino-1,2,4-triazole and anti CYP2E1 IgG inhibition studies show that CYP2E1 is not responsible for enhanced p-nitrophenol hydroxylation, but the residual activity indicates the participation of other isozyme(s). As a result of double induction,changes in the amount of CYP2E1 of microsomes were not detected by Western blot analysis compared to untreated rat liver microsomes.

The liver contains a family of microsomal hemoproteins called cytochromes P450 (P450) which are involved in the metabolic oxygenation of many lipophilic chemicals including drugs, pesticides or food additives. Many of the individual isozymes of P450 exhibiting distinct substrate specificities can be induced by these compounds through processes involving stimulated synthesis or stabilization of the mRNAs and cytochrome proteins (1). The induction of various P450s by a drug can alter metabolic properties of drugs that may result in changes in the pharmacological effects of the drug itself or of other ones. It is important to investigate responses of P450 system to combined exposure of products of human action having divers P450-inducing capacity.

Increased transcription of CYP2B1 and CYP2B2 genes is a major mechanism of P450 induction by phenobarbital (PB) (2). In addition, PB also induces CYP3A in primary cultures of rat hepatocytes (3). CYP3A, the most sensitive to glucocorticoid

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Abbreviations used: P450, cytochrome P450; PB, phenobarbital; DXM, dexamethasone.

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induction, is induced by dexamethasone (DXM), pregnenolone 16α-carbonitrile and spironolactone as well (4). Although increased transcription plays some role in the induction of CYP3A, a strong post-transcriptional influence believed to be message stabilization was found. It also appears that P4503A protein is stabilized by 'PB-like' compounds (3).

This study sought to define the response of monooxygenase system to combined action of PB and DXM and to compare the results to those obtained in liver microsomes prepared from rats treated with PB or DXM alone.

Methods

Chemicals: Dexamethasone and phenobarbital were obtained from Sigma (St.Louis, USA), isoniazid aqueous solution (Isonicid, 25 mg/ml) was from Chemical Works of Gedeon Richter, Ltd. (Budapest, Hungary); 3-amino-1,2,4,-triazole was purchased from Merck (Darmstadt, Germany); cycloheximide and actinomycin D were from Reanal (Budapest, Hungary).

Animals: Male Wistar rats (100 g) were purchased from LATI (Gödöllő, Hungary). They were treated with PB i.p. (80 mg/kg daily) or DXM p.o. (100 mg/kg daily) alone or in combination for 3 consecutive days. In additional experiments isoniazid (40 mg/kg i.p.) was administered for 3 consecutive days. Liver microsomes were prepared as described (5) and protein concentrations were determined by using the method of Lowry et al. (6), with bovine serum albumin as the standard.

Assay methods: Published methods were followed in the assays given below: aniline hydroxylation (7), p-nitrophenol hydroxylation (8), N-nitrosodimethylamine, ethylmorphine and aminopyrine N-demethylation detected by formaldehyde formation (9) and pentoxyresorufin O-dealkylation (10).

Inhibition studies: Preincubation mixtures usually contained: 0.1 M Tris-HCl buffer (pH 7.0), 1.5 mg/ml hepatic microsomal proteins, various concentrations of 3-amino-1,2,4-triazole or anti CYP2E1 IgG (kindly provided by M. Ingelman-Sundberg, Karolinska Institutet, Stockholm, Sweden) and NADPH generating system consisting of 0.5 mM NADP, 5 mM glucose 6-phosphate and 0.5 units/ml glucose 6-phosphate dehydrogenase. The mixtures were preincubated for 15 min. The reactions for the hydroxylation of p-nitrophenol were initiated with 0.2 mM p-nitrophenol and were terminated after 10 min with 0.5 ml 20% trichloroacetic acid. 4-nitrocatechol was determined spectrally at 522 nm in 2 ml supernatant after the addition of 0.1 ml 10 N NaOH.

Electrophoretic and immunoblot analysis: Microsomes were subjected to SDS-polyacrilamide gel electrophoresis with a discontinuous system according to Laemmli (11). The amount of CYP2E1 in microsomes was determined by Western blot analysis using polyclonal antiserum. Electrophoretic transfer of proteins to nitrocellulose and immunoblotting were performed as described (12).

Results and Discussion

Exposure of xenobiotics can alter activity of drug-metabolizing P450s through induction of different forms. Combined actions of drugs are quite common phenomena in therapy and can cause some more changes in responses of P450 system. Induction of P450 by xenobiotics is the result of multiple mechanisms: increased transcription of

Table I. Ethylmorphine and aminopyrine N-demethylase activities of rat liver microsomes from untreated, PB-, DXM- and PB+DXM-treated animals. Each value represents the group mean ±SD.

Treatment	Ethylmorphine N-demethylation*	Aminopyrine N-demethylation*
Control	3.74 ±0.8	3.36 ±0.6
PB	7.19 ±0.7	7.10 ±0.4
DXM	8.14 ±0.4	7.55 ±0.2
PB+DXM	8.68 ±1.2	8.16 ±0.8

* Values are expressed as nmol formaldehyde/mg protein/min.

P450 genes, message stabilization, enhanced transport of mRNA from the nucleus to the cytoplasm, elevated P450 protein synthesis or P450 enzyme stabilization.

PB and DXM used in our experiments are potent inducers of CYP3A. If the mechanism of CYP3A induction caused by PB or DXM is different, additive effect may be expected in ethylmorphine or aminopyrine N-demethylase activites of liver microsomes from rats coadministered with PB and DXM. The administration of PB or DXM resulted in a twofold increase in the ethylmorphine and aminopyrine N-demethylation, due to the induction of CYP3A. The effect of PB+DXM did not differ; double induction caused similar elevation of CYP3A activities as it could be seen in the groups of rats treated with PB or DXM alone (Table I).

However some alterations could be observed in P450 responses after coadministration of PB and DXM: p-nitrophenol hydroxylation increased about threefold comparing to the activity of microsomes from untreated rats. Microsomes from the animals treated with PB or DXM alone did not display similar elevation in p-nitrophenol hydroxylation (Fig. 1a) (13). An attempt to investigate the time dependency for the induction of this P450 enzyme activity by PB+DXM was carried out by determination of the rate of p-nitrophenol hydroxylation in liver microsomes prepared at different time intervals after treatment of rats (Fig. 1b). PB+DXM coadministration caused about threefold increase in p-nitrophenol hydroxylation after the first 24 hours and it did not change significantly by the end of the third day after the first treatment. P-nitrophenol hydroxylation assay is considered to be an indicator of participation of CYP2E1, an ethanol- or isoniazid-inducible P450 form (14). We tried to answer the question whether CYP2E1 was responsible for the unusual increase of p-nitrophenol hydroxylation activity in microsomes from PB+DXM-induced animals. Investigating aniline hydroxylation (15) and N-nitrosodimethylamine N-demethylation (16) considered to indicate participation of CYP2E1, it was found that treatments with PB and DXM alone

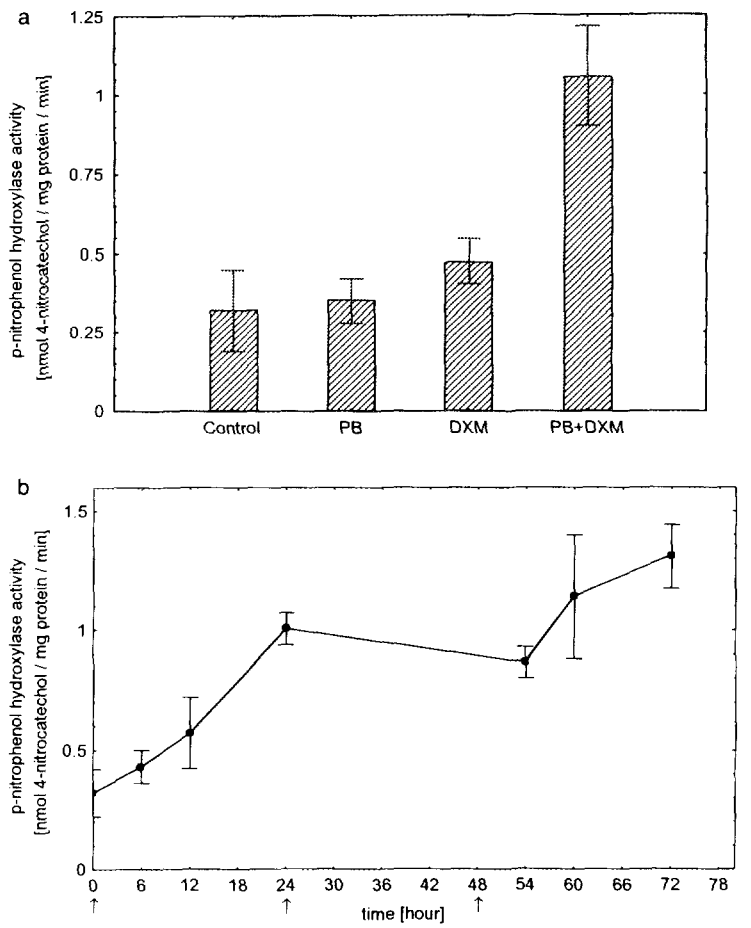

Fig. 1a. Effect of treatment with PB and DXM alone or in combination on p-nitrophenol hydroxylase activity in rat liver comparing to untreated group.

1b. Effect of time on the induction of P450 enzyme(s) responsible for hydroxylation of p-nitrophenol in liver microsomes after treatment of rats with PB+DXM. Animals were treated every 24 hours as arrows show and microsomes were prepared in different times after treatment.

or in combination increased neither aniline hydroxylase nor N-nitrosodimethylamine N-demethylase activities in rat liver microsomes; the same rate of enzyme activities could be detected as in case of untreated rat liver microsomes (data are not shown).

Nevertheless, the composition of microsomes determined by gel electrophoresis demonstrated an intensive protein band with a molecular mass of 53.000 daltons observed only in PB+DXM-induced rat liver microsomes, but not in microsomes prepared from animals administered PB or DXM alone (Fig. 2a) and the intensity of the protein band progressively increased in time after the beginning of the treatment (Fig. 2b). It also correlates with increasing p-nitrophenol hydroxylase activity that was

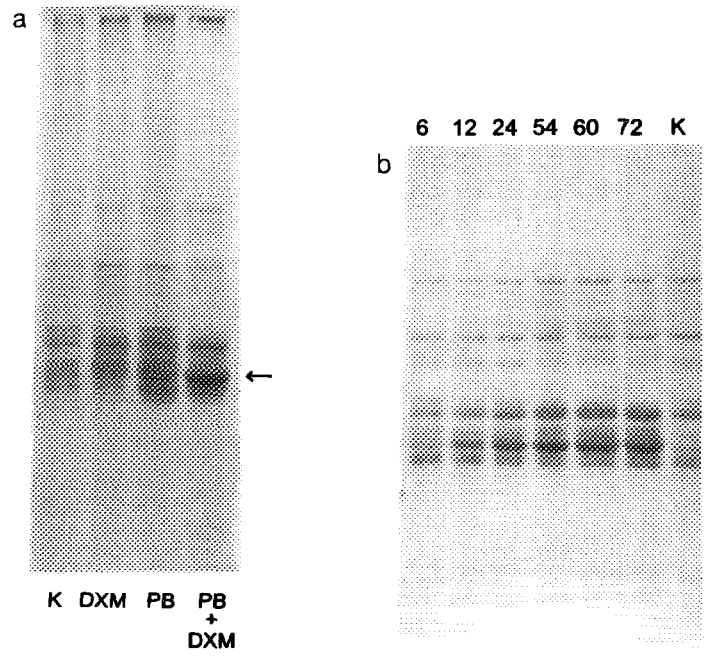

Fig. 2a. Electrophoretic patterns of microsomes from an untreated rat (K) and rats treated with PB and DXM alone or in combination. Microsomes from an untreated animal (lane 1) and microsomes from PB- (lane 2), DXM- (lane 3) and PB+DXM-treated rats were electrophoresed in 7.5% acrylamide gels.

2b. Electrophoretic analysis of PB+DXM-induced rat liver microsomes prepared in different times (as numbers indicate in hours) from the beginning of the treatment. Lane K shows the microsomes from an untreated animal.

(Fig. 1b). It may be suggested that the induction of this protein band is responsible for the enhanced activity.

Koop (17) reported that greater than 90% of p-nitrophenol hydroxylation activity of microsomes from ethanol treated rabbits can be inhibited specifically by the nonselective herbicide, 3-amino-1,2,4-triazole. In our experiments 95-100% of p-nitrophenol hydroxylase activity of microsomes from isoniazid-induced rats was inhibited by 3-amino-1,2,4-triazole and inhibitor concentration for half a maximal inhibition was 3.0 mM. The unexpected enhancement of p-nitrophenol hydroxylation as a result of double induction by PB+DXM could be inhibited by 3-amino-1,2,4-triazole, a specific inhibitor for CYP2E1 by about 60% (Fig. 3a) and inhibitor concentration for half a maximal inhibition was 3.0 mM.

Antibody to CYP2E1 was used to determine the role of the isozyme in the microsomal p-nitrophenol hydroxylation of PB+DXM-induced rats. Fig. 3b shows the results obtained when hepatic microsomes from isoniazid- and PB+DXM-treated rats were assayed in the presence of increasing amounts of anti CYP2E1 IgG. The antibody

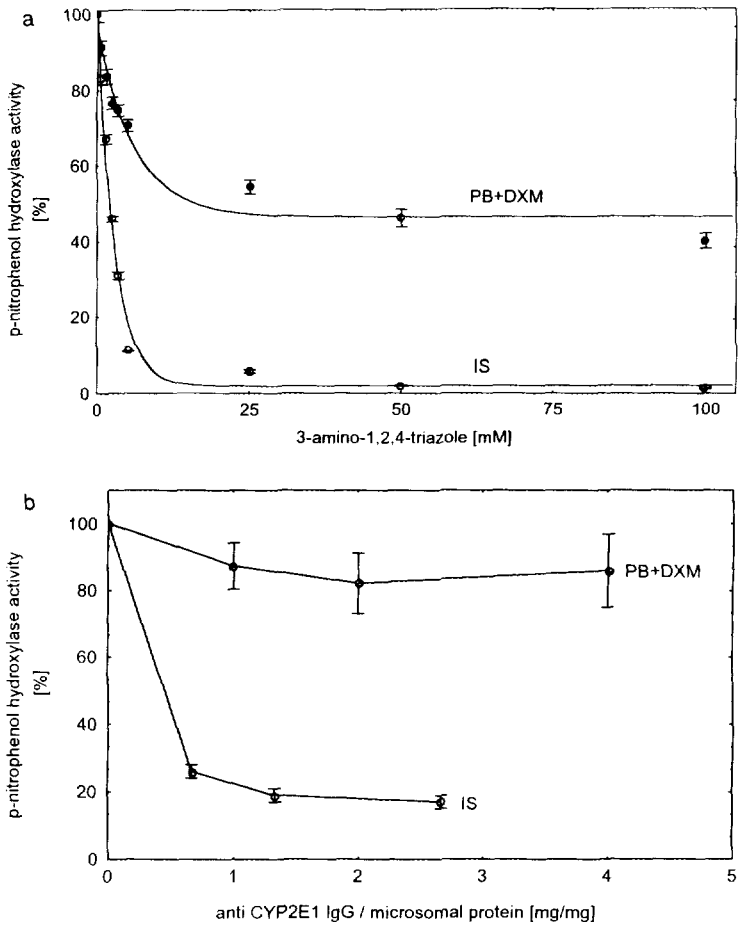

Fig. 3a. Effect of 3-amino-1,2,4-triazole on p-nitrophenol hydroxylation in microsomes from PB+DXM- and isoniazid (IS)-treated rats. The values represent the percentage of the activity of the mixture without inhibitor.

3b. Inhibition of microsomal p-nitrophenol hydroxylation by anti CYP2E1 IgG. Reaction mixtures contained 1.5 mg of microsomal protein from PB+DXM- and isoniazid (IS)-treated rats and increasing amounts of anti CYP2E1 IgG. Activities are expressed relative to p-nitrophenol hydroxylase activity of the mixture without specific antibody.

effectively inhibited p-nitrophenol hydroxylase activity of liver microsomes from isoniazid-induced rats. About 80% inhibition could be produced by 0.6 mg of anti CYP2E1 IgG per mg protein of microsomes. In contrast, p-nitrophenol hydroxylation of PB+DXM-treated rat liver microsomes could be decreased by only about 20% even using 4 mg anti CYP2E1 IgG / mg microsomal protein (Fig. 3b). The apparent disparity between the activities of microsomes from the two groups treated with isoniazid or PB+DXM can be explained if we suppose that CYP2E1 is the only P450 form active in p-nitrophenol hydroxylation in isoniazid-induced rat liver microsomes, while there may

Table II. Effects of inhibitors on the induction of microsomal p-nitrophenol hydroxylase enzyme in PB+DXM-treated rats. Rats were treated with PB+DXM and the subgroups also received actinomycin D (2 mg/kg i.p.) or cycloheximide (5 mg/kg i.p.). Liver microsomes (1.5 mg protein /ml) were assayed with 0.2 mM p-nitrophenol.

Treatment	p-nitrophenol hydroxylation (nmol product / mg protein / min)
PB+DXM	1.006 ±0.06
+ actinomycin D	0.410 ±0.02
+ cycloheximide	0.178 ±0.04

be some other P450(s) in microsomes from PB+DXM-induced rats that can keep the ability of p-nitrophenol hydroxylation in the presence of 3-amino-1,2,4-triazole or of anti CYP2E1 IgG. These findings are in good agreement with the Western blot results obtained after immunochemical staining with anti CYP2E1 IgG which showed the same intensity of CYP2E1 in microsomes treated with PB and DXM alone or in combination comparing to microsomes from untreated rats; PB+DXM-treatment did not change the amount of CYP2E1 in hepatic microsomes.

In order to examine whether the PB+DXM-enhanced p-nitrophenol hydroxylase activity was due to the induction of a P450 form involving de novo mRNA and P450 protein synthesis, the effects of actinomycin D (an inhibitor of RNA synthesis) and cycloheximide (an inhibitor of protein synthesis) were studied (Table II). Actinomycin D caused decrease in p-nitrophenol hydroxylation activity of PB+DXM-treated rats. The protein synthesis inhibitor, cycloheximide also lowered hydroxylation of p-nitrophenol in liver microsomes from rats treated with PB+DXM. The results in this series of experiments suggest that the biosynthesis of mRNA and protein of a P450 form is required for the enhanced p-nitrophenol hydroxylation.

From these results it may be presumable that one (or more) P450 form(s) different from CYP2E1 is induced by PB+DXM coadministration. Moreover, considering the mechanism of induction biosynthesis of mRNA and P450 protein is required for the enhanced activity of p-nitrophenol hydroxylation.

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