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The influence of acute phenytoin administration on biotransformation and lipid peroxidation in the liver of rats of different ages

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Phenytoin is a frequently used antiepileptic. Its influence on biotransformation of xenobiotics in liver is well known. The metabolism of benzphetamine [1], dicoumarol and hexobarbital [2] increases after administration of phenytoin.

Whereas Eling *et al.* [2] have shown that phenytoin increases the biotransformation activities which are inducible by phenobarbital we have found enhanced activities inducible by 3-methylcholanthrene or β-naphthoflavone in 60-day-old male rats [3]. In this study additionally other age groups were investigated to reveal whether the induction type is age dependent.

Furthermore the influence of phenytoin on lipid peroxidation in the liver of the same animals of different ages was determined to detect any interaction between induction and lipid peroxidation. Acute intoxication by several drugs leads to enhanced lipid peroxidation and to liver damage [4–7] and the monooxygenase system may play an important role by formation of compounds which can act as initiators of lipid peroxidation [4].

Finally we tried to find possible interactions of microsomal lipid peroxidation and the different isozymes of cytochrome P-450 by adding various substrates for different isozymes of cytochrome P-450 to the incubation mixtures.

Materials and methods

Male Wistar rats (up to an age of 10 days, males and females) of the Institute's colony breed (Uje: Wist) were used. They were raised and housed under controlled conventional conditions [room temperature 22–26°, humidity >50%, natural day–night light circle, soft wood bedding, pellet diet (cubed diet, VEB Versuchstierproduktion, Berlin) and tap water *ad lib*; litters restricted to 6, weaning at 28–30 days] [8]. The rats received phenytoin (diphenylhydantoin sodium, DPH) for three days prior to being killed at various doses i.p. One control group was injected with 0.9% NaCl-solution, a second control group with 12% ethanol. Ethanol was used in this concentration for the dilution of phenytoin. On the 10th, 30th, 60th and 120th day of life the animals were killed in ether anaesthesia by decapitation, and the livers were removed for preparation of the 9000 *g* supernatant. One part of the supernatant

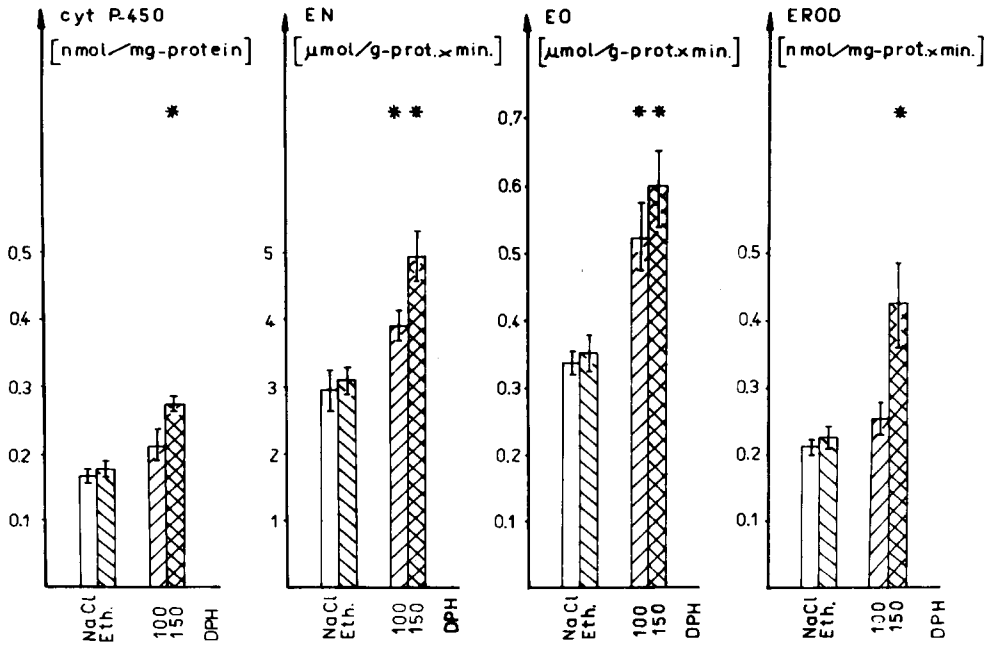

Fig. 1. The influence of a 3-day-pretreatment with phenytoin (DPH, dose in mg/kg body wt) on cytochrome P-450 concentration (cyt P-450), ethylmorphine N-demethylation (EN, formaldehyde release), ethoxycoumarin O-deethylation (EO, coumarin release), and ethoxyresorufin O-deethylation (EROD, resorufin release) in the 9000 *g* liver supernatant of 60-day-old male rats (N = 6). Controls received the same volumes of either saline (NaCl) or 12% ethanol (Eth). (*) Significant differences from ethanol treated controls ($P \leq 0.05$).

was used for the determination of the biotransformation parameters, from the remaining supernatant microsomes were prepared. For the characterization of biotransformation the cytochrome P-450 concentration [9], ethylmorphine N-demethylation (EN) [10], 7-ethoxycoumarin O-deethylation (EO) [11] and 7-ethoxyresorufin O-deethylation (EROD) [12] were determined.

The non-enzymatic ascorbate-dependent peroxidation was determined by the same method as the enzymatic NADPH-dependent lipid peroxidation [13]. The substrates were added to the incubation mixtures in the following concentrations: ethylmorphine (EM) 6 μmol in 0.1 ml, 7-ethoxycoumarin (EC) 50 nmol in 0.1 ml and 7-ethoxyresorufin (ERF) 1.8 nmol in 0.1 ml phosphate buffer.

Results and discussion

Influence of phenytoin treatment on hepatic monooxygenase activities in rats of different ages. After a 3 day treatment of 10-day-old female and male rats with 100 mg/kg body wt phenytoin significant increases in cyt P-450 concentration, EN, EO and EROD were observed (not shown). The dose of 150 mg/kg body wt was not tolerated by this age group.

In Fig. 1 the results on the 60-day-old male rats are given. An increase in cyt P-450, EN, EO and EROD could be observed. This increase is always significant if the animals received 150 mg/kg body wt phenytoin.

For the 30- and 120-day-old male rats also an increase in all biotransformation parameters investigated was found (not shown here). Ethanol treatment was without influence on the biotransformation parameters investigated.

Influence of phenytoin treatment on hepatic microsomal lipid peroxidation in rats of different ages. The lipid peroxidation in microsomes from 10-day-old rats is significantly increased after treatment with 100 mg/kg body wt phenytoin (Fig. 2). Ethanol causes a slight decrease in lipid peroxidation in comparison with the saline treated controls. Addition of different substrates used for the investigation of biotransformation shows the following picture: whereas EM inhibits the lipid peroxidation in the saline and ethanol-treated controls almost completely, EC was without influence on the extent of lipid peroxidation. Addition of ERF completely blocks lipid peroxidation (not shown).

After phenytoin treatment the inhibition caused by addition of EM is diminished significantly. ERF inhibits the lipid peroxidation completely also after phenytoin treatment (data not shown).

The lipid peroxidation is also increased in microsomes of 30-day-old male rats if the animals were pretreated with phenytoin. Ethanol slightly decreases the lipid peroxidation (not shown). Addition of the different substrates to the microsomes shows the same picture as for microsomes from 10-day-old animals.

In contrast to the 10- and 30-day-old animals the phenytoin and also the ethanol treatment were without influence on the lipid peroxidation in microsomes from 60- and 120-day-old rats (not shown).

The addition of different substrates caused the same changes as in microsomes of the younger animals.

To investigate the mechanism by which ERF completely inhibits lipid peroxidation this compound was added after the incubation of microsomes. In this case no influence could be observed so that a trapping of lipid peroxidation products is unlikely (results not shown).

With diminished concentrations of ERF in the incubation mixture also a decrease of the inhibitory effect on lipid peroxidation could be demonstrated. ERF (0.2 nmol) in the incubation mixture was ineffective in the inhibition of lipid peroxidation (results not shown).

The ascorbate-dependent lipid peroxidation in microsomes is also inhibited by ethoxyresorufin, but to a lower extent than the NADPH-dependent lipid peroxidation (Fig. 3). Fe^{2+} has no influence on the ethoxyresorufin induced inhibition of lipid peroxidation (Fig. 3). Without any addition of Fe^{2+} the inhibition by ethoxyresorufin is complete.

Resorufin, the product of ethoxyresorufin O-deethylation, in different concentrations including the concentrations which are formed during the biotransformation reaction caused no changes in microsomal lipid peroxidation (not shown).

Pentoxyresorufin, the dealkylation of which characterizes different subtypes of cyt P-450 than ethoxyresorufin also leads to a complete inhibition of lipid peroxidation (not shown).

Hepatic monooxygenase activities are catalyzed by different isozymes of cytochrome P-450, according to Thomas *et al.* [14] cytochrome P-450a–j. Among these isozymes cytochrome P-450b is inducible by phenobarbital and cytochrome P-450c is the major 3-methylcholanthrene inducible isozyme [15, 16]. The different monooxygenase activities are catalyzed by diverse isozymes with partially overlapping substrate specificity. Ethylmorphine N-demethylation (EN) is mainly catalyzed by the phenobarbital inducible

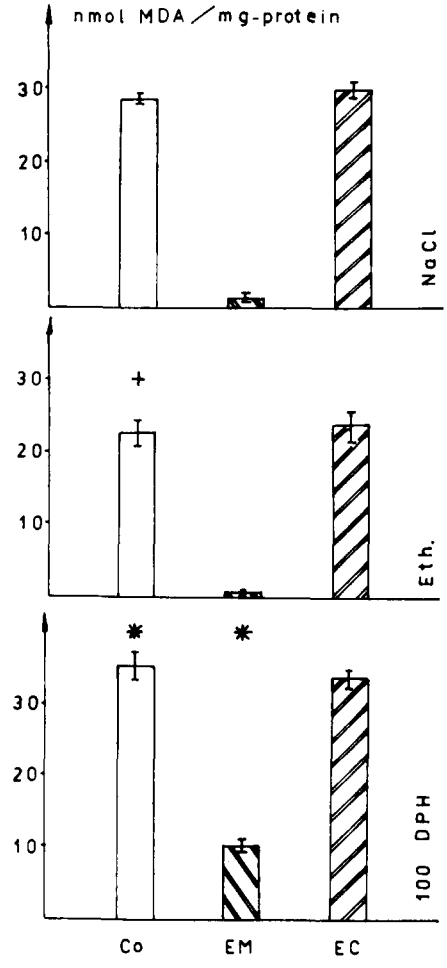

Fig. 2. The influence of a 3-day-pretreatment with phenytoin (DPH) on NADPH-dependent lipid peroxidation (MDA = malondialdehyde) in microsomes of 10-day-old male and female rats with addition of the substrates ethylmorphine (EM) and ethoxycoumarin (EC). (N = 5–6). (+) Significant differences from saline-treated controls. (*) Significant differences from ethanol-treated controls ($P \leq 0.05$).

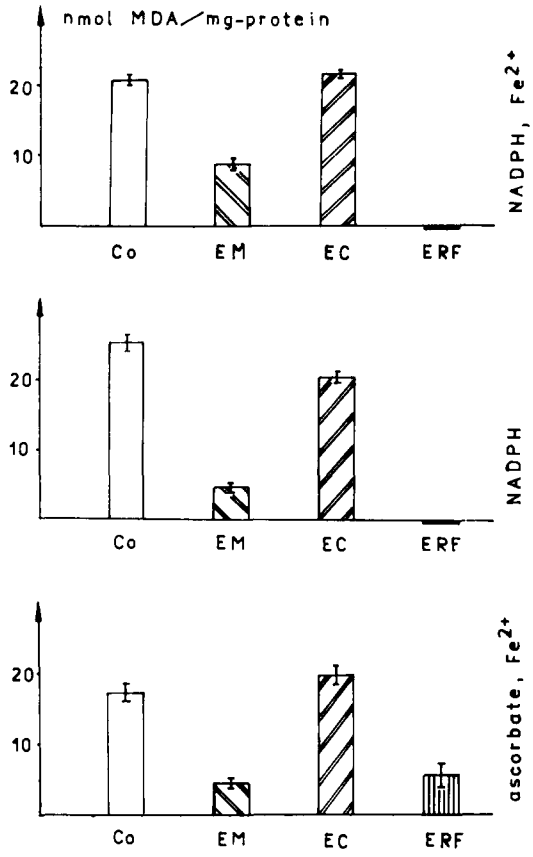

Fig. 3. The ascorbate-dependent lipid peroxidation with exogenous Fe^{2+}, the NADPH-dependent lipid peroxidation without and with exogenous Fe^{2+} in microsomes of untreated 60-day-old male rats with addition of the substrates ethylmorphine (EM), ethoxycoumarin (EC) and ethoxyresorufin (ERF) ($N = 4$; $P \leq 0.05$).

cyt P-450b, but also by the cyt P-450 forms inducible by pregnenolone-16-carbonitrile (PCN), as demonstrated by Elshourbagy and Guzelian [17], 7-ethoxyresorufin O-deethylation (EROD) almost exclusively by the 3-methylcholanthrene inducible cyt P-450c. 7-Ethoxycoumarin O-deethylation (EO) represents both isozymes, but the activity is more inducible by inducers of the 3-methylcholanthrene-type [16].

Phenytoin administered over 3 days enhanced the total amount of cyt P-450 and the activities of the isozymes characterized by the different biotransformation reactions in all age groups investigated. Young animals are more sensitive to the inducers than older animals. This is also evident in the case of phenytoin treatment, for cytochrome P-450, EN, EO and EROD. Not only the activities are enhanced which are inducible by phenobarbital but also the activities inducible by 3-methylcholanthrene. Previous results with 60-day-old rats [3] are complemented by the same results in the other age groups.

As compared with the phenobarbital and 3-methylcholanthrene induction pattern phenytoin is a medium-type inducer as is e.g. phenylbutazone [18]; the structures of both compounds are similar.

It is possible that phenytoin acts by a more common mechanism than the inducers phenobarbital or 3-methylcholanthrene which have different induction mechanisms. The inducing effect of 3-methylcholanthrene is mediated by a cytosolic receptor [15]. The NADPH-generating system is not affected by phenytoin [2].

After short term treatment with phenytoin the lipid peroxidation is also increased. But this increase could be found only in 10- and 30-day-old rats. The increase in lipid peroxidation can be provoked by a decrease in hepatic glutathione levels as a consequence of diminished activities of glutathione peroxidases and glutathione transferases. A similar mechanism is described for the carbon tetrachloride induced lipid peroxidation [7]. Moreover it is possible that the effect of phenytoin on lipid peroxidation is connected with metabolites of phenytoin itself. Hassell *et al.* [19] describe the formation of a reactive epoxide during the metabolism of phenytoin and it is well known that the detoxification mechanisms including conjugation reactions are fully developed in adult animals only.

The higher rate of lipid peroxidation in microsomes from young rats can be explained by a change in the substrate availability for lipid peroxidation: so the rate of unsaturated fatty acids in the membranes is increased after induction by phenobarbital. In this way the lipid peroxidation can be enhanced [20].

Investigations concerning changes in membrane composition after phenytoin administration are necessary.

P-450 concentration and lipid peroxidation activities are not inversely related as suggested by Högberg *et al.* [21]. Our own study shows an increase of both P-450 concentration and lipid peroxidation in liver microsomes from young, though not from adult, rats after phenytoin treatment. Bast and Haenen [22] demonstrated that the interrelationship between P-450 and lipid peroxidation is complicated by the several functions of P-450, which acts as oxygenase, oxidase and peroxidase.

With different substrates like EM, EC and ERF we tried to get data on the relationship of different P-450 isozymes and lipid peroxidation.

After addition of these substrates which are known to be only poor or inefficient uncouplers for the oxidative pathway of P-450 [23] a possibly selective action of a given isozyme either as monooxygenase or initiator of lipid peroxidation can be checked.

ERF binds almost selectively to P-450c and inhibits lipid peroxidation almost completely. The conclusion that P-450c is predominantly active in initiating lipid peroxidation cannot be drawn, as EC proved to be ineffective and pentoxyresorufin, dealkylated by phenobarbital-inducible P-450 isozymes, is a strong inhibitor of lipid peroxidation.

Resorufin, the main metabolite after dealkylation of both ethoxy- and pentoxyresorufin, was not effective.

Taken together it can be concluded: the inhibition of lipid peroxidation is not related to the binding to any P-450 isozyme, it could be due to the specific structure of 7-alkoxyresorufin. The lower inhibitory action in ascorbate-mediated lipid peroxidation shows that the electron transport chain including NADPH-cytochrome P-450-reductase could play an important role.

Whereas the changes in lipid peroxidation after addition of EC and ERF are independent of pretreatment of the animals with phenytoin, the action of EM was modified. These findings can be explained by changes in the NADPH-generating system. But it is not proven so far that phenytoin does not influence the NADPH-generating system.

Institute of Pharmacology and Toxicology
Friedrich Schiller University
Jena
German Democratic Republic
**Institute of Physiology*
Bulgarian Academy of Sciences
Sofia
Bulgaria

F. JAHN
M. KADIISKA*
E. SERBINOVA*
W. KLINGER
T. STOYTCHEV*

REFERENCES

1. Gut J and Becker BA, Effects of diphenylhydantoin (DPH) on hepatic drug metabolism in rabbits. *Fed Proc* **30**: 225, 1971.
2. Eling TE, Harlisan RD, Becker BA and Fouts JR, Diphenylhydantoin effect on neonatal and adult rat hepatic drug metabolism. *J Pharmacol Exp Ther* **171**: 127–134, 1970.
3. Jahn F, Ulrich U and Klinger W, The influence of prenatal administration of phenytoin on postnatal development and inducibility of hepatic monooxygenases in rats. *Pharmacol Toxicol*, in press.
4. Jaeschke H, Kleinwaechter C and Wendel A, The role of Acrolein in allylalcohol-induced lipid peroxidation and liver cell damage in mice. *Biochem Pharmacol* **36**: 51–57, 1987.
5. Uysal M, Keyer-Uysal M, Kocak-Toker N and Aykac G, The effect of chronic ethanol ingestion on brain lipid peroxide and glutathione levels in rats. *Drug Alcohol Depend* **18**: 73–75, 1986.
6. Uysal M, Yalvin AS, Kocak-Toker N, Keyer-Uysal M and Aykac G, Lipid peroxidation in liver and plasma of rats treated with benzene. *IRCS Med Sci* **14**, 772, 1986.
7. Yalcin AS, Kocak-Toker N, Uysal M, Aykac G, Sivas A and Öz H, Stimulation of lipid peroxidation and impairment of glutathione-dependent defense system in the liver of rats repeatedly treated with carbon tetrachloride. *J Appl Toxicol* **6**: 303–306, 1986.
8. Klinger W, Müller D, Danz M, Kob D and Madry M, Influence of impairment of the immune system on hepatic biotransformation reactions, their postnatal development and inducibility. *Exp Pathol* **24**, 219–225, 1983.
9. Matsubara T, Koike M, Touchi A, Tochino Y and Sugero K, Quantitative determination of cytochrome P-450 in rat liver homogenate. *Anal Biochem* **75**: 569–603, 1976.
10. Klinger W and Müller D, Ethylmorphine N-demethylation by liver homogenate of newborn and adult rats, enzyme kinetics and age course of V_{max} and K_m. *Acta Biol Med Germ* **36**: 1149–1159, 1977.
11. Aitio A, A simple and sensitive assay for ethoxycoumarin O-deethylation. *Anal Biochem* **85**: 488–491, 1978.
12. Pohl RJ and Fouts JR, A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Anal Biochem* **107**: 150–155, 1980.
13. Ohkawa H, Ohiski N and Yagi K, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* **95**: 351–358, 1979.
14. Thomas PE, Reidy J, Reik LM, Ryan DE, Koop DR and Levin M, Use of monoclonal antibody probes against rat hepatic cytochrome P-450c and P-450d to detect immunochemically related isozymes in liver microsomes from different species. *Arch Biochem Biophys* **235**: 239–253, 1984.
15. Goldstein JA, Mechanism of induction of hepatic drug metabolizing enzymes: recent advances. *Trends Pharmacol Sci* **5**: 290–293, 1984.
16. Conney AH, Induction of microsomal cytochrome P-450 enzymes: the first Bernard B. Brodie Lecture at Pennsylvania State University. *Life Sci* **36**: 2493–2518, 1986.
17. Elshourbagy NA and Guzelian PS, Separation, purification, and characterization of a novel form of hepatic cytochrome P-450 from rats treated with pregnenolone-16α-carbonitrile. *J Biol Chem* **255**: 1279–1285, 1980.
18. Klinger W, Elger J, Franke H, Reinicke C, Traeger A, Volkmann H, Wahrenberg J and Ankermann H, Untersuchungen zum Mechanismus der Enzyminduktion. XV. Induktionsmuster und submikroskopische Veränderungen in der Leber nach Applikation von Phenylbutazon bei unterschiedlich alten Ratten. *Acta Biol Med Germ* **24**: 463–482, 1970.
19. Hassell TM, Maguire JH, Cooper CG and Johnson PT, Phenytoin metabolism in the cat after long-term oral administration. *Epilepsia* **25**: 556–563, 1984.
20. Dhami MSC, Parke DV and Feuer G, Evidence for variations in membrane phospholipids following the induction of P-450 and P-448. *Abstracts of the 5th International Conference on Biochemistry, Biophysics and Ind. of cyt P-450.* Budapest, 1985.
21. Högberg J, Bergstrand A and Jacobson SV, Lipid peroxidation of rat liver microsomes, its effect on the microsomal membrane and some membrane-bound microsomal enzymes. *Eur J Biochem* **37**: 51–59, 1973.
22. Bast A and Haenen GRM, Cytochrome P-450 and glutathione: what is the significance of their interrelationship in lipid peroxidation. *Trends Biochem Sci* **9**: 510–513, 1984.
23. Freitag A and Schmitt W, Die Oxidasefunktion von Cytochrom P-450 unter dem Einfluß eines Typ I. und eines Typ II-Substrates im Altersgang bei männlichen Ratten. Dissertation an der Medizinischen Fakultät der Universität Jena, 1986.

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Eicosanoid production and cell accumulation induced by intrapleural injection of sodium arachidonate in the rat. Characterization of the model

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The proinflammatory effects of arachidonic acid (AA*) have been evaluated *in vivo*. Although AA lacks intrinsic chemotactic properties [1], high doses injected into rabbit skin significantly increase leukocyte infiltration [2]. Topically applied AA produces an edema in the ears of mice that can be modulated by mixed inhibitors of the 5-lipoxygenase (LO)/cyclooxygenase (CO) pathway including nor-

* Abbreviations: AA, arachidonic acid; NaAA, sodium arachidonate; CO, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; LT, leukotriene; LO, lipoxygenase; RIA, radioimmunoassay; PMNL, polymorphonuclear leukocytes; and i.p., intraperitoneal.