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SHORT COMMUNICATIONS

Absence of an isotope effect in induction of cytochrome P-450 and xenobiotic metabolizing enzyme activities by stable isotope-labelled phenobarbital isotopomers

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The induction mechanism for polycyclic aromatic hydrocarbon-responsive forms of cytochrome P-450 involves the binding of the inducer to an intracellular receptor. Such evidence is lacking for phenobarbital responsive forms [1–3], and "the data to date neither substantiate nor refute the idea that phenobarbital induces cytochrome P-450 gene expression by interacting with specific receptors" [1]. Slight conformational changes such as those resulting from substitution of atoms by stable isotopes could lead to significant changes in physico-chemical properties of the molecules, such as binding to specific receptors [4], and give indications on the structure–activity relationships. It was previously shown that a series of stable isotope-labelled phenobarbital isotopomers has distinctive properties in binding to human serum albumin [5, 6], thus the aim of the present study was to establish if a significant isotope effect occurs in the induction of cytochrome P-450 and related monooxygenase activities by these compounds.

Materials and methods

Stable isotope-labelled phenobarbital isotopomers were synthesized by Commissariat à l'Energie Atomique, Gif-sur-Yvette, France. The drugs were: 5-[phenyl-$(^2H)_5$]-phenobarbital (PB-P; 99% ^2H), [1,3-$(^{15}N)_2$;2-^{13}C]phenobarbital (PB-CN; 90% ^{13}C and 99% ^{15}N) and 5-[ethyl-$(^2H)_5$]phenobarbital (PB-E;99% ^2H). Sodium salts were prepared with NaOH. Unlabelled sodium phenobarbital (PB-NL) was a gift from Laboratoires Specia (France). [^{3}H]TCDD* (52 Ci/mmol) was purchased from KOR isotopes (Cambridge, MA). 7-Alkylresorufins were obtained from Boehringer (France). All other reagents were purchased at the best available grade. Animals were male adult Sprague–Dawley rats (250 g; IFFA-Credo, L'Arbresle, France). They were pretreated with phenobarbital (sodium salt) single daily intraperitoneal administrations (40 mg/kg) for three days and killed 24 hr after the last administration of the inducing agent. Control animals received 0.9% NaCl solution. Hepatic microsomes were prepared by differential centrifugation in HEPES buffer 25 mM, pH 7.6, EDTA Na_2 1.5 mM, DTT 1 mM and glycerol 10%, and stored at −80° in the same medium. The activities of microsomal 7-ethylresorufin, 7-pentylresorufin and 7-benzylresorufin *O*-dealkylases were assayed fluorometrically [7]. 7-Ethoxycoumarin *O*-deethylase activity was determined as described previously [8]. Inhibition of 7-ethoxycoumarin *O*-deethylase activity by metyrapone (10 μM final concentration) was obtained by adding the compound dissolved in dimethylsulfoxide (0.1% final concentration) to the incubation medium 2 min before to start the reaction. Benzphetamine *N*-demethylase activity was measured by the colorimetric procedure of Nash [9]. Cytochrome P-450 and b_5 were measured in microsomes as described by Estabrook and Werringloer [10]. Protein content was determined by the method of Hartree [11], using bovine serum albumin as the standard. The activity of glutathione *S*-transferase was determined spectrophotometrically with 1-chloro-2,4-dinitrobenzene as a substrate [12]. Experiments of [^{3}H]TCDD binding to *Ah* receptor were carried out using hepatic cytosol separated from either control or phenobarbital-treated adult liver exactly as described by Lesca *et al.* [13]. After extraction by chloroform-isopropanol, plasmatic and hepatic concentration of phenobarbital isotopomers were measured by gas chromatography/mass spectrometry according to the method of Benchekroun *et al.* [14]. In all groups five animals were used. Analysis of variance was used to compare the different phenobarbital-treated groups.

Results and discussion

Phenobarbital-type induction occurs after administration of many different compounds [15–17]. Striking differences were found in the potency of the different analogs previously used, but a clear-cut pattern of structure–activity relationships among these different molecules does not exist. Species differences also occur, for example *bis*[2-(3,5-dichloropyridyloxy)]benzene is considerably more potent as an inducer in mice than in rats [18]. In the present study, no significant differences were found in cytochrome P-450 content and monooxygenase activities after administration of the variously labelled phenobarbital isotopomers (Fig. 1). It was expected that sensitive markers of induction by phenobarbital such as 7-pentylresorufin [19] could reflect slight differences in inductive potency, but such an evidence is lacking. The concentration of *Ah* receptor, which regulates the induction process in upper vertebrates, such as rodents or man [20, 21], is generally increased after administration of phenobarbital [13, 22]. In our conditions, its concentration does not change whatever the compound we administered. A correlation being found between the dose of phenobarbital administered and induction [23], plasmatic and hepatic concentrations of phenobarbital were measured (Table 1). Despite large interindividual variations, pharmacokinetic properties of phenobarbital were not modified by stable-isotope labelling. These data are consistent with the observations of Benchekroun *et al.* [24] that no significant isotope effect on kinetic parameters and formation of 4-hydroxylated metabolites occurred after administration of PB-E to man. Studies of the distribution and elimination of PB-CN in man have also shown that kinetic parameters of this substance are identical with those of the unlabelled drug [25]. In the present study, the metabolites of the compounds were not identified, but previous data have shown that the metabolism of phenobarbital is not modified by stable-isotope labelling [26] and that metabolites of the unlabelled compound are not inducers [27]. Given what we know about the mechanism of induction by phenobarbital, the existence of a significant isotope effect should have been an interesting contribution. Unfortunately, we are unable to have such evidence. It is concluded that, if binding to a specific receptor is involved in the induction of cytochrome P-450 and monooxygenase activities by phenobarbital, an isotope effect does not occur

* Abbreviations used: HEPES, 4-(2-hydroxyethyl)-1-piperazone-ethanesulfonic acid; DTT, dithiothreitol; [^{3}H]TCDD, [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

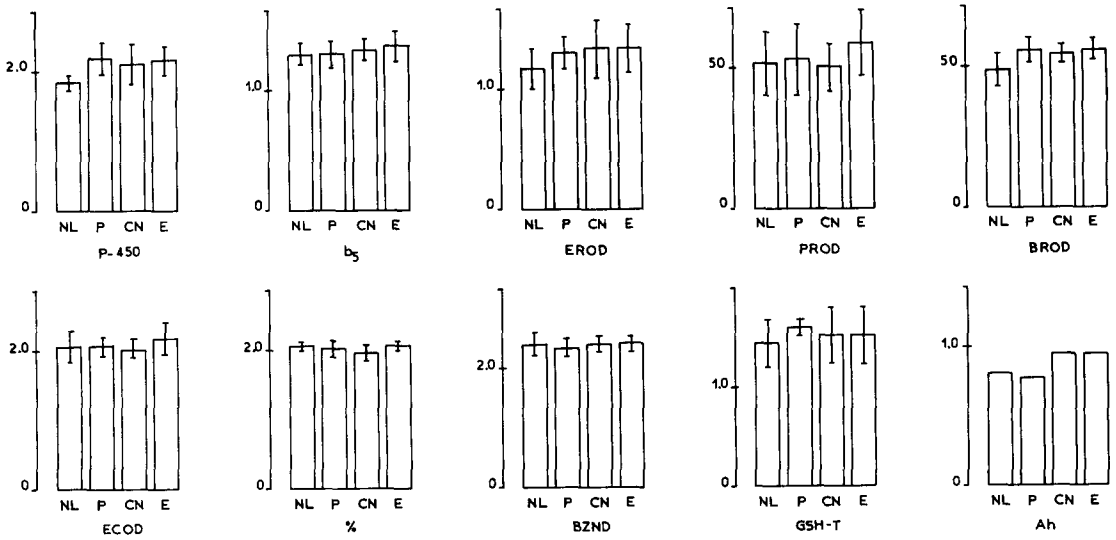

Fig. 1. Induction of monooxygenase activities and some components in rat liver by phenobarbital isotopomers. Rats were killed 24 hr after 3 single daily administrations (40 mg/kg i.p.) of PB-NL (unlabelled phenobarbital), PB-P (5-[phenyl-(^{2}H)$_5$]phenobarbital), PB-CN ([1,3-(^{15}N)$_2$;2-^{13}C]-phenobarbital) or PB-E (5-[ethyl-(^{2}H)$_5$]phenobarbital). Results are expressed as -fold induction related to control activities (N = 5 per group). Control concentrations were: microsomal protein, 22.7 ± 2.3 mg/g liver; cytosolic protein, 86.0 ± 3.2 mg/g liver; cytochrome P-450, 0.89 ± 0.10 nmol/mg protein; cytochrome b_5, 0.24 ± 0.02 nmol/mg protein; receptor *Ah*, 29.2 fmol/mg protein. Control activities were: 7-ethylresorufin *O*-deethylase (EROD), 3.3 ± 0.7 pmol/mg/min; 7-pentylresorufin *O*-dependentylase (PROD), 0.29 ± 0.05 pmol/mg/min; 7-benzylresorufin *O*-debenzylase (BROD), 3.8 ± 1.5 pmol/mg/min; 7-ethoxycoumarin *O*-deethylase (ECOD), 0.45 ± 0.05 nmol/mg/min; benzphetamine *N*-demethylase (BZND), 6.5 ± 0.3 nmol/mg/min; glutathione *S*-transferase (GSH-T), 1730 ± 165 nmol/mg/min. Inhibition of ECOD activity by metyrapone (%) in microsomes from control rats was 32 ± 12%.

Table 1. Plasmatic and hepatic concentrations of phenobarbital isotopomers in rat

	Plasma	Liver
PB-NL	238 ± 55*	556 ± 212**
PB-P	265 ± 150	604 ± 324
PB-CN	293 ± 179	440 ± 192
PB-E	260 ± 64	556 ± 276

Concentrations were measured 24 hr after 3 single daily administrations (40 mg/kg i.p.) of PB-NL (unlabelled phenobarbital), PB-P (5-[phenyl-(^{2}H)$_5$]phenobarbital), PB-CN ([1,3-(^{15}N)$_2$;2-^{13}C]phenobarbital) or PB-E (5-[ethyl-(^{2}H)$_5$]phenobarbital).

* nmol/ml or **nmol/g. Mean ± SD (N = 5).

or is too small to be easily detectable. However, the lack of any isotope effect on the studied mechanism strongly emphasizes the potentiality of labelled isotopomers of phenobarbital for pharmacological studies without any risk of hepatic enzyme induction different from that obtained with the unlabelled analogue.

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REFERENCES

1. Whitlock JP, The regulation of cytochrome P-450 gene expression. *Ann Rev Pharmacol Toxicol* **26**: 333–369, 1986.
2. Eisen HJ, Induction of hepatic P-450 isozymes. Evidence for specific receptors. In: *Cytochrome P-450: Structure, Mechanism and Biochemistry* (Ed. Ortiz de Montellano PR), pp. 315–344. Plenum Press, New York, 1986.

3. Fonne R and Meyer UA, Mechanisms of phenobarbital-type induction of cytochrome P-450 isozymes. *Pharmacol Ther* **33**: 19–22, 1987.
4. Browne TR, Stable isotopes in pharmacology studies: present and future. *J Clin Pharmacol* **26**: 485–489, 1986.
5. Brazier JL, Ribon B, Falconnet JB, Cherrah Y and Benchekroun Y, Etude et utilisation des effets isotopiques en pharmacologie. *Thérapie* **42**: 445–450, 1987.
6. Cherrah Y, Etude de l'effet isotopique sur la fixation protéique plasmatique et la distribution tissulaire. *Thèse Doctorat Etat Sciences Pharmaceutiques*, Lyon, 1987.
7. Rifkind AB and Muschick H, Benoxaprofen suppression of polychlorinated biphenyl toxicity without alteration of mixed oxidase function. *Nature* **303**: 524–526, 1983.
8. Rivière JL, Grolleau G and Bach J, Hepatic biotransformation in the buzzard (*Buteo buteo*) and the Japanese quail (*Coturnix coturnix*): effect of PCBs. *Comp Biochem Physiol* **82C**: 439–443, 1985.
9. Nash T, The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem J* **55**: 416–421, 1953.
10. Estabrook RW and Weringloer J, The measurement of difference spectra: application to the cytochromes of microsomes. In: *Methods in Enzymology* (Eds. Fleischer S and Packer L), Vol 52C, pp. 212–220. Academic Press, New York, 1978.
11. Hartree EF, Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal Biochem* **48**: 422–427, 1972.
12. Habig WH, Pabst MJ and Jakoby WB, Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**: 7130–7139, 1974.
13. Lesca P, Fernandez N and Roy M, The binding components for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and polycyclic aromatic hydrocarbons. Separation from the rat and mouse hepatic cytosol and characterization of a light density component. *J Biol Chem* **262**: 4827–4835, 1987.
14. Benchekroun Y, Ribon B, Desage M and Brazier JL, Simultaneous quantitation of phenobarbitone and p-hydroxybarbitone and their perdeuteroethyl analogues in biological fluids by combined gas chromatography-mass spectrometry. *J Chromatogr* **420**: 287–296, 1987.
15. Pelkonen O and Karki T, Effect of physicochemical and pharmacokinetic properties of barbiturates on the induction of drug metabolism. *Chem Biol Interact* **7**: 93–99, 1973.
16. Denomme MA, Bandiera S, Lambert I, Copp L, Safe L and Safe S, Polychlorinated biphenyls as phenobarbitone-type inducers of microsomal enzymes. Structure–activity relationships for a series of 2,4-dichloro-substituted congeners. *Biochem Pharmacol* **32**: 2955–2963, 1983.
17. Menez JF, Souhaili H, Vignier V, Batt AM and Siest G, Modulation of induction of cytochrome P-450 in rat liver microsomes using *O*- and *N*-isopropyl derivatives of barbiturates. In: *Xth European Drug Metabolism Workshop, Guildford, UK*, July 1986 (Eds. Bendford DJ, Bridges JW and Gibson GG), p. 115. Taylor and Francis, London, 1986.
18. Poland A, Mak I, Glover E, Boatman RJ, Ebetino FH and Kende AS, 1,4-bis(2-(3,5-dichloropyridyloxy))-benzene, a potent phenobarbital-like inducer of microsomal mono-oxygenase activity. *Mol Pharmacol* **18**: 571–580, 1980.
19. Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T and Mayer RT, Ethoxy-, pentoxy-and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem Pharmacol* **34**: 3337–3345, 1985.
20. Durrin LK, Jones PBC, Fischer JM, Galeazzi DR and Whitlock JP, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin receptors regulate transcription of the cytochrome P-450 gene. *J Cell Biochem* **35**: 153–160, 1987.
21. Poland A and Glover E, Variation in the molecular mass of the *Ah* receptor among vertebrate species and strains of rats. *Biochem Biophys Res Commun* **149**: 1439–1449, 1987.
22. Okey AB and Vella LM, Elevated binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3-methylcholanthrene to the *Ah* receptor in hepatic cytosols from phenobarbital-treated rats and mice. *Biochem Pharmacol* **33**: 531–538, 1984.
23. Tavoloni N, Jones MJ and Berk PD, Dose-related effects of phenobarbital on hepatic microsomal enzymes. *Proc Soc Exp Biol Med* **174**: 20–27, 1983.
24. Benchekroun Y, Ribon R, Falconnet JB, Cherrah Y and Brazier JL, Pharmacokinetic equivalence of 5-[ethyl-(^{2}H)$_5$]- and unlabelled phenobarbital. *J Clin Pharmacol* **29**: 168–173, 1989.
25. Browne TR, Van Langenhove A, Costello CE, Biemann K and Greenblatt DJ, Pharmacokinetic equivalence of stable-isotope-labelled and unlabeled drugs. Phenobarbital in man. *J Clin Pharmacol* **22**: 309–315, 1982.
26. Perel JM, Dayton PG, Tauriello CL, Brand L and Mark LC, Metabolic studies with deuterated phenobarbital. *J Med Chem* **10**: 371–374, 1967.
27. Cresteil T, Mahu JL, Dansette PM and Leroux JP, *In vivo* administration of hydroxylated phenobarbital metabolites: effect on rat hepatic cytochromes P-450, epoxide hydrolase and UDP-glucuronosyltransferase. *Biochem Pharmacol* **29**: 1127–1133, 1980.

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Combined effects of AY9944 and plasma LDL (or whole plasma) on lymphocyte blastic transformation

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During blastic transformation of lymphocytes stimulated by lectins, a peak in cholesterol synthesis occurs in phase G1 of the cell cycle [1, 2]. Such cholesterol synthesis is indispensible for cells to enter into phase S. Various inhibitors of cholesterol synthesis, 20, α-hydroxycholesterol, 25, α-hydroxycholesterol, 7-cetocholesterol, acting at the step of HMGCoA reduction, are capable of inhibiting the incorporation of [^{3}H]thymidine into cell DNA and the occurrence of blastic forms in stimulated lymphocytes [1–3]. Kay and Wilce [4] have further observed that AY9944 (*trans* 1,4bis(2-chlorobenzylaminomethyl)cyclohexane), an inhibitor of the ultimate stage of cholesterol synthesis,