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EFFECTS OF PHENOBARBITONE AND β-NAPHTHOFLAVONE ON HEPATIC MICROSOMAL DRUG METABOLISING ENZYMES OF THE MALE BEAGLE DOG

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Abstract—Hepatic microsomes, prepared from male beagle dogs treated with phenobarbitone or β-naphthoflavone, were compared with microsomes from control dogs and from control, phenobarbitone and β-naphthoflavone treated rats with respect to various microsomal enzyme activities and for protein profiles generated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The concentration of total cytochrome P-450 (0.46 nmoles/mg protein) and aldrin epoxidase (0.44 nmoles/mg/min) was lower in control dogs than in the rat, although ethoxycoumarin *O*-deethylase (ECOD-1.03 nmoles/mg/min) was 2 times and ethoxyresorufin *O*-deethylase (EROD-0.10 nmoles/mg/min) 5 times higher in the control dogs examined. Possibly as a result of this difference, β-naphthoflavone induced ECOD 10-fold and EROD 100-fold in the rat, while these enzymes were only increased 3-fold and 5-fold respectively in the two β-naphthoflavone-treated dogs. Consistent with this, control dog microsomes were found to contain a 58,000 mol. wt protein band that was not present in the SDS–PAGE of control rat microsomes but which was induced in both species by β-naphthoflavone. Although not identical, the effects of each inducer on the protein profiles were similar in both species. β-Naphthoflavone produced a marked increase in relative liver weight and, in contrast to published work in the rat, also increased NADPH–cytochrome *c* reductase levels in the dog. In general, the effects of phenobarbitone were qualitatively and quantitatively similar in both the dog and the rat.

The metabolism of a wide variety of exogenous and endogenous substrates is catalysed by the hepatic microsomal mixed function oxidase system, which contains cytochrome P-450 as the terminal oxidase [1, 2]. This haemoprotein exists in multiple forms [3], which are responsible both for the broad substrate specificity and for the way in which metabolism varies according to species, age, sex, tissue, hormonal status and exposure of the animal to foreign compounds [4]. Differences in the constitutive levels of cytochrome P-450 isozymes together with differences in substrate specificity of each isozyme may be implicit in producing pronounced species differences in the pharmacology and toxicity of a given substrate, since the drug metabolising system is capable of catalysing the activation as well as inactivation of various compounds [4]. Therefore, characterisation of some of the molecular mechanisms which influence the pharmacological or toxic response in one species, would assist in the extrapolation of experimental findings to other species and to man.

The albino rat and the beagle dog are probably the most important mammalian species routinely used in major toxicity studies. However, whereas the hepatic microsomal drug metabolising systems of the rat have been well characterised, and the effects of various enzyme inducers extensively studied [4–8], very little work of this nature has been done in the dog [9–12]. The limited *in vitro* work carried out in the dog has shown that while the concentration of total cytochrome P-450 is lower, some enzyme activities are substantially higher than those found in the rat [10, 11], indicating differences either in the constitutive levels of different cytochrome P-450 isozymes or in their substrate specificities. These findings of higher total levels of cytochrome P-450 are consistent with species differences observed in the oxidative metabolism *in vivo* of many compounds [13–16], which show that, in general foreign compounds are metabolised less extensively or less rapidly in the dog than in rodents. Similarly, the effects of the classical enzyme inducers, phenobarbitone and β-naphthoflavone [7, 17, 18], on drug metabolism in the dog have been limited to *in vivo* investigations using such markers as antipyrine [19], nitrazepam [20] and caffeine [21]. The aim of this study, therefore, was to characterise more fully the hepatic microsomal drug metabolising enzyme system of the dog and the resultant effects of induction with phenobarbitone or β-naphthoflavone, and compare the findings with those from the extensively studied rat.

MATERIALS AND METHODS

Chemicals. Sodium phenobarbitone was obtained from BDH (Poole, Dorset), and β-naphthoflavone and 7-ethoxycoumarin from the Aldrich Chemical Co. (Gillingham, Dorset). 7-Hydroxycoumarin and NADPH were obtained from the Sigma Chemical Co. (Poole, Dorset) and aldrin and dieldrin from Applied Sciences (Pennsylvania, U.S.A). 7-Ethoxyresorufin was purchased from Pierce and Warriner Ltd. (Chester, Cheshire) and resorufin from Pfaltz and Bauer Inc. (Conn, U.S.A.). All materials for electrophoresis were obtained from Bio-Rad Laboratories (Watford, Herts).

Animals. Adult male beagle dogs, weighing

between 13 and 22 kg and aged between 1 and 4 years, were obtained from the Alderley Park Dog Breeding Unit. Originally derived from English hunting stock, a colony of beagles has been maintained in this unit since 1950, producing the Alderley Park strain which has now been close bred for over 30 years. These dogs have a normal lifespan of between 11 and 13 years and have no strain characteristics notably different from those in other colonies. A control group of three dogs was used untreated. Three dogs were scheduled to receive seven daily intraperitoneal doses of phenobarbitone (100 mg/ml in saline) at 20 mg/kg. However, since they showed signs of ataxia on the third morning, the dose was reduced to 10 mg/kg for 2 days before returning to 20 mg/kg for the final 3 days. A further two dogs received β-naphthoflavone (50 mg/ml in arachis oil) as daily intraperitoneal doses of 10 mg/kg. One dog (BNF-8) received seven daily doses, while the other (BNF-7) was not dosed on day 7 due to abdominal tenderness. Twenty-four hours after the scheduled final dose, the dogs were killed by overdosing with Euthatal (sodium pentobarbitone; May and Baker, Essex, U.K.).

Preparation of microsomes. Liver microsomes were prepared essentially as described by Orton and Parker [35], except that homogenising and washing buffers contained 15% glycerol to help stabilise cytochrome P-450. The animals were exsanguinated and the livers removed into ice-cold KCl buffer (0.15 M KCl, 0.1 M phosphate buffer, pH 7.4, containing 15% glycerol). All further operations were carried out at 4°. To overcome any enzyme localisation effects, the whole liver, after removal of the gall bladder, was minced in an equivalent volume of sucrose buffer (0.25 M sucrose, 0.1 M phosphate buffer, pH 7.4, containing 15% glycerol) using a large electric mincer. Aliquots of the mince were then homogenised in 2 vol. of sucrose buffer using a Potter–Elvejhem glass homogeniser with a Teflon pestle. The homogenate was centrifuged at 24,000 *g* for 10 min, and the supernatant removed and further centrifuged at 230,000 *g* for 45 min to prepare the microsomal fraction. The microsomal pellet was washed by resuspending in KCl buffer and harvested by centrifugation at 230,000 *g* for 45 min. The washed microsomal pellet was resuspended in 0.1 M phosphate buffer, pH 7.4 (1 ml/g liver) and stored in liquid nitrogen until assayed.

Assays. Protein concentration was determined by the method of Lowry *et al.* [22]. Total cytochrome P-450 concentrations were determined from the dithionite-reduced CO-difference spectra using an extinction coefficient of 91/mM/cm as described by Omura and Sato [1]. NADPH-cytochrome *c* reductase levels were measured according to the method of Phillips and Langdon [23] using an extinction coefficient of 19.1/mM/cm. Aldrin epoxidase was determined by measuring the amount of dieldrin formed using the gc-electron capture method described by Wolff *et al.* [24]. 7-Ethoxycoumarin *O*-deethylase was assayed fluorimetrically by following the production of 7-hydroxycoumarin [25] and 7-ethoxyresorufin *O*-deethylase was measured similarly by monitoring the production of resorufin [26]. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially by the method of Laemmli [27] using microsomes solubilised in mercaptoethanol and SDS. A 1.5-mm slab gel apparatus was used as described by Dent *et al.* [28] with the upper gel consisting of 3% acrylamide and the lower gel of 10% acrylamide. The gels were stained for protein using Coomassie brilliant blue R, and stored in 3% glycerol prior to photography. Molecular weights were determined from the electrophoretic mobilities of the protein bands compared to the mobilities of protein standards: carbonic anhydrase, 31,000; ovalbumin, 45,000; bovine serum albumin, 66,200; phosphorylase B, 92,500, obtained from Bio-Rad Laboratories.

RESULTS

Effects on indices of microsomal enzyme levels and relative liver weight

Administration of phenobarbitone and β-naphthoflavone to the dog produced an increase in relative liver wt when compared to the ratio found in control dogs (Table 1). The liver to body wt ratio was consistent within each group, with β-naphthoflavone producing a more pronounced increase than phenobarbitone. Stimulation of liver growth has been widely observed in the rat following treatment with

Table 1. The effect of phenobarbitone and β-naphthoflavone on relative liver wt and hepatic microsomal enzyme levels of the dog

Treatment	Dog no.	Liver/body wt (%)	Cytochrome P-450 (nmoles/mg protein)	(λmax)	NADPH-cyt. *c* reductase (nmoles/mg protein/min)
Control	1	2.9	0.49	450	200
	2	2.9	0.36	450	200
	3	2.8	0.52	450	204
Mean		2.9	0.46	450	201
Phenobarbitone	4	3.3	1.24	450	324
	5	3.1	0.99	450	272
	6	3.6	1.12	450	256
Mean		3.3	1.12	450	284
β-Naphthoflavone	7	4.5	0.72	449	252
	8	4.5	1.18	447.5	452
Mean		4.5	0.95	448	352

Animals of differing weights (13–22 kg) were randomly spread between the groups.

phenobarbitone, polycyclic aromatic hydrocarbons and also many other chemicals [7].

The mean concentration of cytochrome P-450 of 0.46 nmoles/mg of protein found in control dog liver microsomes was slightly lower than the range (0.6–0.8 nmoles/mg) normally found in adult Alderley Park male rats. Phenobarbitone produced about a 2.5-fold increase over the control level of cytochrome P-450 in the dog and, although the wavelength maximum of the reduced-CO spectrum remained at 450 nm, the spectral peak was much sharper than the control. The total cytochrome P-450 concentration was increased 2-fold by β-naphthoflavone treatment and the peak maximum of the reduced-CO spectrum was shifted downfield to 448 nm (Table 1). The dog (BNF-8) having no interruption in treatment had a greater haemoprotein concentration and showed a larger wavelength shift than the dog (BNF-7) where dosing was stopped 48 hr before termination. The effects of these inducers on the concentration of total cytochrome P-450 are proportionally similar to those found in the rat [7, 8].

Both inducers also increased the level of NADPH–cytochrome *c* reductase in dog liver microsomes (Table 1). This is in contrast to their effects in the rat, where cytochrome P-448 inducers, such as β-naphthoflavone, produce no change in the reductase level [6].

Effects on microsomal enzyme activities

Aldrin epoxidase has been shown to be a fairly specific marker for cytochrome P-450-dependent activity in the rat, being induced by phenobarbitone but reduced by β-naphthoflavone [24] (Table 2). Under optimal conditions of incubation, the level of aldrin epoxidase in control dog microsomes was found to be about 40% of that in the rat, although phenobarbitone treatment produced about a 4-fold induction of enzyme activity in both species. β-Naphthoflavone treatment, however, produced about a 30% increase in microsomal aldrin epoxidase activity in the dog but resulted in a 40% decrease in the rat enzyme (Table 2).

In contrast to aldrin epoxidase and total cytochrome P-450, the level of 7-ethoxycoumarin *O*-deethylase found in dog hepatic microsomes (Table 2) was about twice that found in the rat, as reported previously by Oesch and co-workers [10]. This enzyme is induced by both phenobarbitone and β-naphthoflavone in the rat [25], being increased 4-fold and 10-fold respectively. In dog microsomes, 7-ethoxycoumarin *O*-deethylase was increased only 3-fold by both inducers, such that β-naphthoflavone produced a much lower effect proportionally on this enzyme in the dog than in the rat.

7-Ethoxyresorufin *O*-deethylase is a fairly specific marker for cytochrome P-448-mediated activity, inducible by polycyclic aromatic hydrocarbons in several species [26]. The level of this enzyme in control dog liver microsomes (Table 2) was 5 times higher (and up to 10 times higher in control dogs of other studies—unpublished results) than the very low level of activity found in control rat microsomes, an observation also made by Gregus *et al.* [10]. In both the dog and the rat, phenobarbitone had a slight stimulatory effect on 7-ethoxyresorufin *O*-deethylase, but β-naphthoflavone had a marked species difference in its effects on this enzyme. In rat hepatic microsomes, 7-ethoxyresorufin *O*-deethylase is induced about 100-fold by β-naphthoflavone, whereas the enzyme activity in dog microsomes is increased from its higher control value by only about 5- or 6-fold (Table 2).

Qualitative differences in microsomal proteins on SDS–PAGE

The electrophoretic pattern on SDS gels of protein-staining bands from the dog hepatic microsomal preparations are shown in Fig. 1, which includes, for comparison, similarly treated microsomal preparations from the rat. Control dog microsomes produced three dense bands (designated 1, 2 and 3) in the mol. wt region of 45,000–60,000, typical of cytochrome P-450 isozymes [3, 29–31], although it is recognised that other microsomal proteins also have minimum mol. wts in this range. These protein bands had mol. wts of 49,500, 50,500 and 52,000 respectively, while some less distinct bands (5–7) were also observed with higher mol. wts (54,000–58,500). Microsomes from the phenobarbitone-treated dog showed a slight increase in the density of bands 1 and 2, a marked increase in band 3 (mol. wt, 52,000),

Table 2. The effects of phenobarbitone and β-naphthoflavone on hepatic microsomal enzyme activities of the dog

Treatment	Dog no.	Aldrin epoxidase	Ethoxycoumarin *O*-deethylase	Ethoxyresorufin *O*-deethylase
Control	1	0.43	0.98	0.13
	2	0.42	1.17	0.06
	3	0.47	0.94	0.11
Mean		0.44 (1.10)	1.03 (0.52)	0.10 (0.02)
Phenobarbitone	4	2.05	3.67	0.13
	5	1.63	2.64	0.13
	6	1.94	2.92	0.16
Mean		1.87 (3.85)	3.08 (2.00)	0.14 (0.03)
β-Naphthoflavone	7	0.50	2.42	0.45
	8	0.65	3.82	0.68
Mean		0.58 (0.66)	3.12 (5.17)	0.56 (2.22)

Enzyme activities are expressed as nmoles product formed/min/mg protein.

The figures in parentheses are the mean values obtained using microsomes from four rats (male Alderley Park albino 180–200 g) either untreated, dosed i.p. with phenobarbitone at 80 mg/kg/day for 3 days, or dosed i.p. with β-naphthoflavone at 25 mg/kg/day for 3 days.

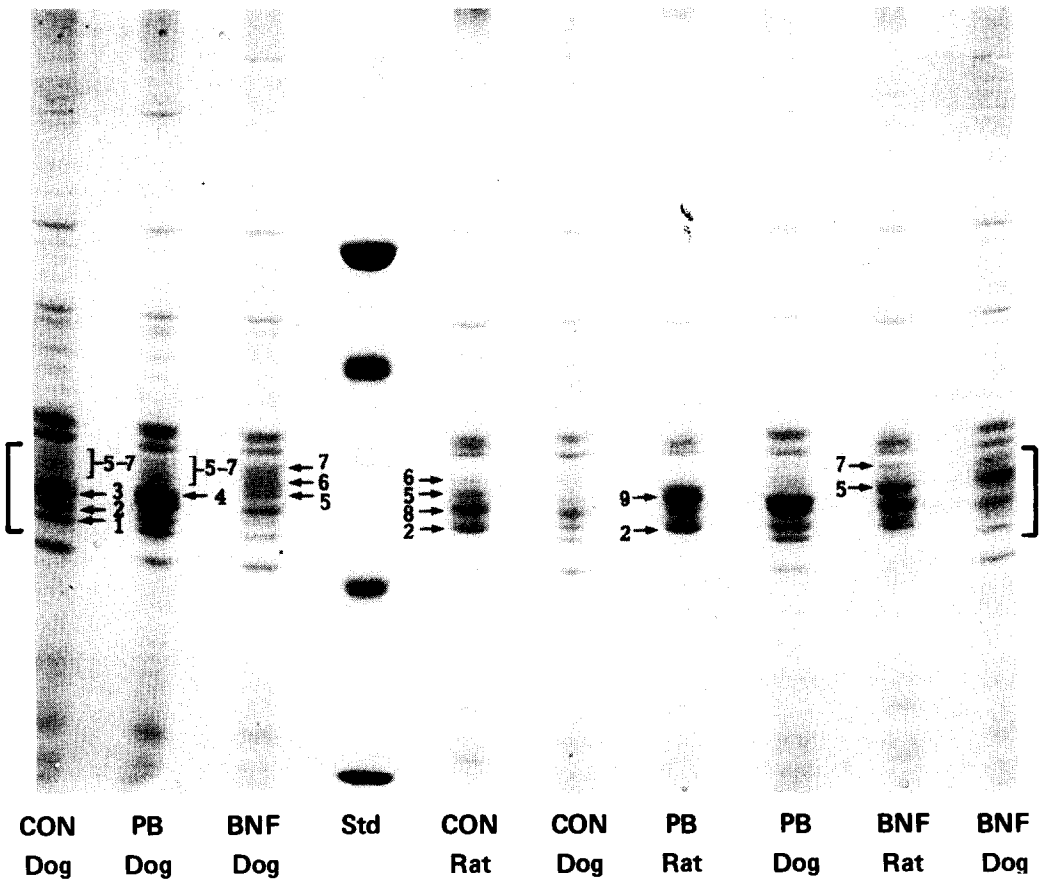

Fig. 1. Electrophoresis of liver microsomes from dogs and rats, either untreated (CON), phenobarbitone-treated (PB), or β-naphthoflavone-treated (BNF). Ten micrograms of solubilised microsomal protein were applied to each channel. Proteins of known molecular weights (Std) were used for comparing mobilities, and the gel was stained for protein using Coomassie brilliant blue R.

and greatly enhanced band 4 (mol. wt, 53,500), which was very faint in control microsomes. No change was observed in the higher mol. wt proteins. In contrast, β-naphthoflavone treatment produced marked increases in bands 5, 6 and 7, the high mol. wt proteins, making them quite distinct and comparable in intensity to the other bands. Little change was apparent in the other protein bands, although band 2 was slightly less intense. In comparison to the dog, control rat microsomes had no protein band 1 or 7, but had three major bands (2, 8 and 5) with mol. wts of 50,500, 53,000 and 55,000. In the rat, phenobarbitone treatment induced a major protein (band 9) of mol. wt 54,000, slightly heavier than that increased in dog microsomes. The effects of β-naphthoflavone treatment in both rat and dog microsomes were qualitatively similar, namely increasing the higher mol. wt proteins of bands 5, 6, and 7. In the rat, however, this represents a vast increase in the relative proportion of band 7 compared to control microsomes. While the electrophoretic patterns observed in the rat microsomes are similar to those found by other workers, the subunit mol. wts of the major inducible proteins in this study are slightly higher than those published for both phenobarbitone-inducible (52,000) and 3-methylcholanthrene- or β-naphthoflavone-inducible (56,000) cytochrome P-450 [3, 31, 32].

DISCUSSION

The present study to examine the *in vitro* effects of enzyme induction in the dog has several limitations. In order to use a minimum number of animals, the dose level, route of administration, number of doses and time of necropsy after the final dose were not varied to maximise induction but, rather, were chosen on the basis of published work *in vivo* [20, 21] with a view to ensuring substantial levels of induction. In any future studies, improvements could be made in the dosing schedule. Intraperitoneal administration was used to overcome the possibility of incomplete oral absorption. While this route was suitable for the aqueous phenobarbitone

dose, the arachis oil used in the β-naphthoflavone formulation was only slowly absorbed from the abdominal cavity and apparently caused some peritoneal irritation. Such a problem was not reported by Aldridge and Neims [21], who used a similar dose level and formulation, but a shorter exposure time. However, the arachis oil may be suitably replaced by using small volumes of ethanol as solvent, without toxic effects. The phenobarbitone dosing regimen could also be improved by using a lower initial dose level of 10 mg/kg/day for 2 or 3 days, before increasing the level to 20, or possibly higher, to achieve maximal induction. Despite all the shortcomings of the dosing procedure, substantial levels of enzyme induction were observed in all the treated dogs.

Comparison of the various enzyme markers in the untreated rats and dogs indicated a marked difference in the constitutive levels of the different cytochrome P-450 isozymes contributing to these enzyme activities. The cytochrome P-448-dependent enzyme, ethoxyresorufin *O*-deethylase [26], had a much higher activity in the dog than in the rat, and ethoxycoumarin *O*-deethylase, which is also catalysed, at least in part by cytochrome P-448 [25], was also higher in the dog, as reported previously by other workers [10, 11]. These enzymic differences were supported by the microsomal protein patterns observed following SDS–polyacrylamide gel electrophoresis, which showed that the higher mol. wt proteins (55,000–58,000) of the cytochrome P-450 region were more pronounced in the dog than in the rat. These proteins are thought to include the cytochrome P-448 isozyme(s) [3, 30–32], which are inducible in several species by β-naphthoflavone and 3-methylcholanthrene, and were induced by β-naphthoflavone in both the rat and dog in this study. These findings suggest, therefore, that in control animals, dogs have a much higher constitutive level of the cytochrome P-448 isozyme than the very low level known to be present in the liver microsomes of the rat [33]. This obviously does not hold for all the cytochrome P-450 isozymes, since the levels of total haemoprotein and of aldrin epoxidase, which is cytochrome P-450-dependent and has little or no contribution from cytochrome P-448 [34], were found to be higher in the rat than in the dog. The higher level of total cytochrome P-450 in the rat is consistent with *in vivo* metabolic activity, which is generally higher than that found in the dog [16], although this could also be due to the rat having a relative liver wt about twice that found in the dog [16].

With these marked species differences in the levels of constitutive enzymes, it was not entirely unexpected, therefore, that enzyme inducers, particularly of the cytochrome P-448 isozyme, would produce different effects in the rat and dog, since enzyme induction has been found to show species dependence [4, 7]. In this respect, β-naphthoflavone produced a quantitatively lesser effect in the dog than in the rat, although qualitatively the effects were similar. In both species, the cytochrome P-448 isozyme was induced, as were the relative activities of ethoxycoumarin *O*-deethylase and ethoxyresorufin *O*-deethylase; and these findings were supported by an increase in the higher mol. wt proteins observed on SDS–PAGE, typical of cytochrome P-448 induction by polycyclic aromatic hydrocarbons [3, 30–32]. As well as producing a much lower induction of the cytochrome P-448-dependent enzymes in the dog, β-naphthoflavone produced a marked increase in NADPH-cytochrome *c* reductase levels, effects normally associated in the rat with phenobarbitone-like induction rather than the more specific effects generated by polycyclic aromatic hydrocarbons [6, 7]. In contrast, the effects produced by phenobarbitone were similar both quantitatively and qualitatively in the dog and the rat.

The results shown in Table 2, together with the total cytochrome P-450 levels, provide a typical *in vitro* screen for enzyme induction but also illustrate the difficulty in the extrapolation of findings from one species to another. The effects of these classical enzyme inducers on the hepatic microsomal drug metabolising systems of the dog now provide a data base with which the effects produced by new compounds may be compared. This will allow the inducing potential of any new compound to be assessed as an integral part of the regulatory toxicity studies rather than using extra animals to carry out a separate *in vivo* induction study.

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