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TISSUE AND SEX DIFFERENCES IN THE ACTIVATION OF AROMATIC HYDROCARBON HYDROXYLASES IN RATS

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Abstract—Betamethasone and α-naphthoflavone produced similar activation of biphenyl 2-hydroxylase and benzo[*a*]pyrene 3-hydroxylase in control male rat liver microsomes. In small intestinal epithelial microsomes, betamethasone had no effect whereas α-naphthoflavone caused a pronounced activation of benzo[*a*]pyrene hydroxylation and a lesser activation of biphenyl 2-hydroxylation. In lung microsomes, betamethasone had no effect on either enzyme activity whereas α-naphthoflavone had no effect on biphenyl 2-hydroxylase but inhibited benzo[*a*]pyrene hydroxylase. In kidney cortex microsomes from male rats both compounds caused inhibition or had no effect whereas in kidney cortex microsomes from female rats betamethasone activated whereas α-naphthoflavone had no effect.

Activation also occurred in isolated viable hepatocytes from male rats. The response of biphenyl 2-hydroxylase was very similar to that found in male rat liver microsomes but benzo[*a*]pyrene hydroxylase was more sensitive to activation and less sensitive to inhibition than in microsomes. The findings are interpreted as demonstrating the presence of more than one 'latent' aromatic hydrocarbon hydroxylase in rodents.

In previous studies certain cytochrome P-450 mediated hydroxylations were found to be activated rapidly in male rat liver microsomes by naturally occurring and synthetic glucocorticoids and by α-naphthoflavone. Metabolic reactions which have been shown to be activated include: 2-hydroxylation of biphenyl, 3-hydroxylation of benzo[*a*]pyrene and 4-hydroxylation of antipyrine [1–3]. Metabolism at other sites of these molecules was unaffected [1, 3] as were *O*-deethylation of ethoxycoumarin and ethoxyresorufin and 4-hydroxylation of aniline [1]. The synthetic glucocorticoid betamethasone was the most potent activating agent [2]. The effects were dependent on age and sex in rats, with activation being high in immature animals and adult males but declining rapidly to no effect in maturing female rats [4]. This sex difference was not however apparent in human liver microsomes as activation occurred in samples from either sex [3]. Betamethasone and α-naphthoflavone appeared similar in their activation effects on rat liver microsomes but α-naphthoflavone is a potent inhibitor of 3-methylcholanthrene-inducible cytochrome P-450 [5] whereas inhibition by betamethasone has not been observed in liver microsomes from either control or pretreated rats [2].

The present study was undertaken: (a) to investigate activation in extraheptic microsomes, and (b) to ascertain whether activation occurs in intact hepatocytes as well as cell homogenates and fractions.

METHODS

Animals. Untreated Wistar albino rats (100–150 g unless otherwise stated) as supplied by the University of Surrey breeding unit were used. All animals were acclimatized for a minimum of 3 days on 'Sterolit' bedding and allowed access to food (Spillers No. 1 Laboratory Diet) and water *ad lib*. For all tissue preparations the animals were killed between 8.30 and 9.30 a.m.

Microsomal assays. Small intestinal epithelial microsomes were prepared from 250–300-g male rats by the method of Shirkey *et al.* [6]. Lung microsomes were prepared essentially as described previously for the liver [2] except that it was necessary to scissor-chop the tissue very finely before homogenisation with 10 strokes of a Potter–Elvehjeim (size B, 2950 rpm). Kidney cortex microsomes were prepared by the method of Jakobsson [7].

Biphenyl hydroxylation in extrahepatic microsomes were measured as described previously for liver microsomes [2] except that an increased incubation period of 15 min was used. Dimethylformamide was used as solvent for the activating agents and was added alone to control incubations. The final concentration of 0.5% had no significant effect on the enzyme activities measured. Metabolites were detected by high-pressure liquid chromagraphy [8], or by fluorimetry [9] for experiments with α-naphthoflavone because a metabolite of α-naphthoflavone co-elutes with 2-hydroxybiphenyl in the high-pressure liquid chromatography method. Benzo[*a*]pyrene hydroxylation was assayed by the fluorimetric method of Dehnen *et al.* [10] also using a 15-min incubation period for extrahepatic microsomes. α-Naphthoflavone and its metabolites did not cause fluorescence interference with either biphenyl or benzo[*a*]pyrene metabolites in these assays.

Metabolism in hepatocytes. Hepatocytes were isolated by collagenase/hyaluronidase treatment of liver slices from 60–80-g male rats [11] giving a yield of 15×10^6–20×10^6 viable cells/g of liver with a viability index of 92–96% as determined by exclusion of trypan blue. Cells were suspended to 2×10^6 viable cells/ml in Leibovitz L-15 medium containing

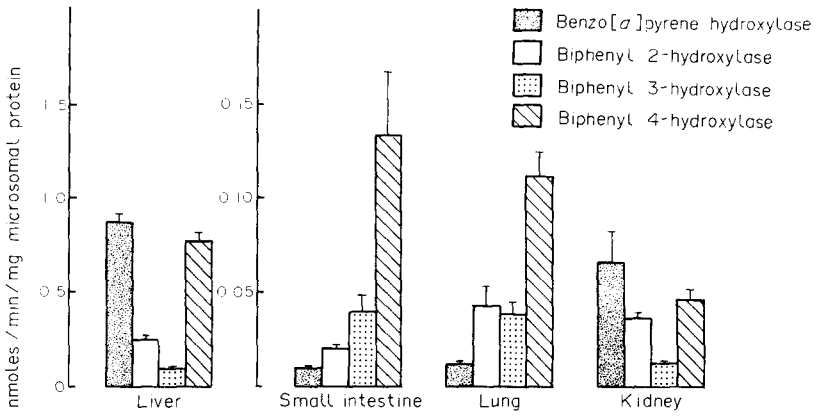

Fig. 1. Tissue differences in microsomal hydroxylase activities of male rats. Each value is the mean of at least four experiments ± S.E.M.

10% foetal calf serum and incubated with biphenyl (70 μM) or benzo[*a*]pyrene (80 μM) according to the method of Wiebkin *et al.* [12]. Substrates and activating agents were added in dimethylformamide; the final solvent concentration of 0.4% in all incubations had no effect on the enzyme activities measured. All additions had no effect on cell viability during the period of incubation. Reactions were stopped after 30 min by immersing the flasks in ice.

Aliquots of the cell suspensions were then subjected to different deconjugation procedures. To obtain total biphenyl metabolites, aliquots (1-ml in duplicate) were boiled with an equal volume of 10 M HCl for 1 hr and then extracted and assayed as for microsomal incubations. This hydrolysis could not be used for benzo[*a*]pyrene metabolites because of their instability. For both substrates unconjugated metabolites were extracted and the remaining aqueous phase was incubated for 16 hr with 'Ketodase' [2500 U (William Warner & Co. Ltd, Eastleigh, U.K.)] to hydrolyse the glucuronides. The released free metabolites were extracted and the remaining sulphate conjugates in the aqueous phase were subsequently hydrolysed with aryl sulphatase [375 U, type H1 (Sigma Chemical Co., London, U.K.)]. Biphenyl metabolites were extracted and assayed as earlier, benzo[*a*] pyrene metabolites (predominantly 3-hydroxybenzo[*a*]pyrene) were extracted and assayed by the method of Nebert and Gelboin [13] using a standard plot of authentic 3-hydroxybenzo[*a*]pyrene with quinine sulphate (0.1 μg/ml in 0.05 M H_2SO_4) as the fluorescence standard.

RESULTS

Studies in extrahepatic microsomes

A comparison of biphenyl and benzo[*a*]pyrene hydroxylation activities in microsomes from liver, small intestine, lung and kidney cortex microsomes of adult male rats is shown in Fig. 1. In small intestine and lung microsomes biphenyl 4-hydroxylase activity was predominant whereas activity towards benzo[*a*]pyrene was very low. In liver and kidney microsomes benzo[*a*]pyrene hydroxylase and biphenyl 4-hydroxylase activities were similar but all liver activities were at least an order of magnitude greater than in extrahepatic microsomes. There was no significant sex diffference in the extrahepatic microsomal metabolism of benzo[*a*]pyrene or biphenyl (data not shown).

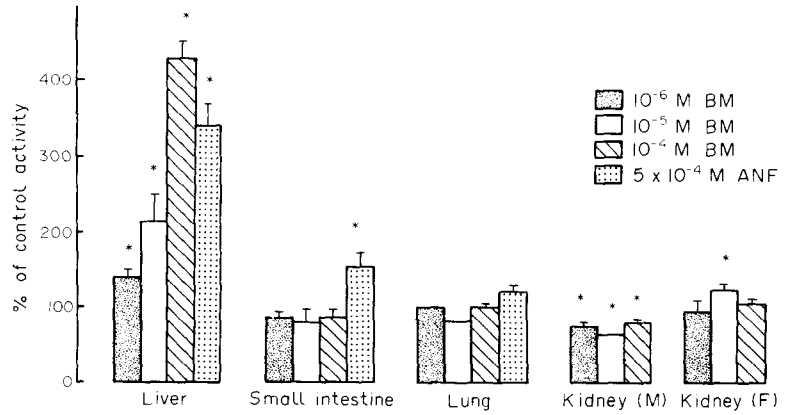

Fig. 2. Effects of betamethasone (BM) and α-naphthoflavone (ANF) on biphenyl 2-hydroxylation in rat microsomes. Microsomes were from male animals except for the kidney where male (M) and female (F) rats were compared. Each value is the mean of at least four experiments ± S.E.M. *Significantly different from control ($P < 0.05$, Students *t*-test).

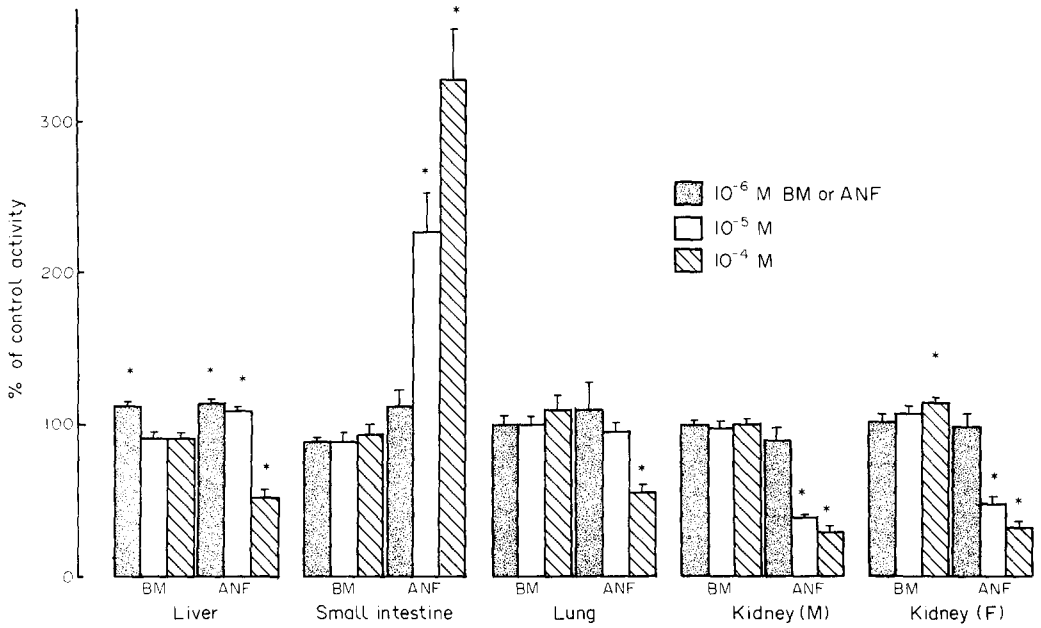

Fig. 3. Effects of betamethasone (BM) and α-naphthoflavone (ANF) on benzo[*a*]pyrene hydroxylation in rat microsomes. Microsomes were from male animals except for the kidney where male (M) and female (F) rats were compared. Each value is the mean of at least four experiments ± S.E.M. *Significantly different from control ($P < 0.05$, Student's *t*-test).

As reported previously [1] betamethasone and α-naphthoflavone both caused a large activation of biphenyl 2-hydroxylase (Fig. 2) and a small but consistent activation of benzo[*a*]pyrene hydroxylase (Fig. 3) in liver microsomes from adult male rats. The effect of α-naphthoflavone on benzo[*a*]pyrene hydroxylation was biphasic, with activation at 10^{-6} and 10^{-5} M but inhibition at 10^{-4} M. Betamethasone also activated at 10^{-6} M in liver microsomes but had no significant effect at higher concentrations. In contrast betamethasone (10^{-6}–10^{-4} M) had no apparent effect on biphenyl or benzo[*a*]pyrene hydroxylation activities in small intestine microsomes. The effects of α-naphthoflavone were the converse of those in liver microsomes with a marked activation of benzo[*a*]pyrene hydroxylase (228% increase at 10^{-4} M) and a smaller increase in biphenyl 2-hydroxylation (54% at 5×10^{-4} M) with no change in 4-hydroxylation.

In lung microsomes betamethasone (10^{-6}–10^{-4} M) had no effect on either enzyme activity while α-naphthoflavone (10^{-4} and 5×10^{-4} M) had no effect on biphenyl 2-hydroxylation but inhibited benzo[*a*]pyrene hydroxylation at 10^{-4} M (Figs. 2 and 3).

In kidney cortex microsomes of both male and female adult rats, benzo[*a*]pyrene hydroxylation was markedly inhibited by α-naphthoflavone (10^{-5} M and above). Betamethasone had no effect of benzo[*a*]pyrene hydroxylation in male rat kidney microsomes but a 10^{-4} M concentration caused a small but significant activation of benzo[*a*]pyrene hydroxylase in female rat kidney cortex microsomes (Fig. 3). There was also a sex difference in the effect of betamethasone on biphenyl 2-hydroxylation in kidney cortex microsomes with 20–30% inhibition in microsomes from male rats but either activation or no effect in microsomes from female rats (Fig. 2).

Biphenyl 3- and 4-hydroxylations were inhibited by 20–30% by betamethasone (10^{-6}–10^{-4} M) in male rat kidney cortex microsomes whereas these reactions were unaffected by either betamethasone or α-naphthoflavone in other tissues (data not shown).

Activation in isolated rat hepatocytes

The metabolite profile of biphenyl in isolated rat hepatocytes is shown in Table 1. The relative proportions of the total metabolites hydroxylated in the three positions were similar to those observed with control rat liver microsomes (Fig. 1). Little or no unconjugated metabolites could be detected after 30 min incubation. The major conjugates of 3- and 4-hydroxybiphenyl were sulphates whereas equal proportions of sulphate and glucuronide were detected with 2-hydroxybiphenyl. However a major proportion (72%) of the further metabolites of 2-hydroxybiphenyl could not be hydrolysed by either 'Ketodase' or sulphatase but was hydrolysable by acid treatment. A similar discrepancy was observed by Wiebkin *et al.* [12]. The most likely explanation is that 2-hydroxybiphenyl forms a stable conjugate that is resistant to enzymic treatment.

Betamethasone (10^{-4} M) caused a similar activation of biphenyl 2-hydroxylation in isolated hepatocytes to that observed in liver microsomes but caused an unexpected but small inhibition of biphenyl 3- and 4-hydroxylation (Fig. 4). The activation of biphenyl 2-hydroxylation is accounted for by increases in unconjugated 2-hydroxybiphenyl and in the formation of its glucuronide and acid hydrolysable conjugate but 2-hydroxybiphenyl sulphate formation was unchanged by the presence of betamethasone. The inhibition of biphenyl 3- and 4-

Table 1. Biphenyl and benzo[*a*]pyrene metabolism in isolated rat hepatocytes*

Metabolite produced	2-Hydroxybiphenyl	3-Hydroxybiphenyl	4-Hydroxybiphenyl	Hydroxybenzo[*a*]pyrene
	(nmoles product formed/30 min/10^7 cells)			
Free	0.01	<0.01	0.21 ± 0.09	0.90 ± 0.23
Glucuronide	0.88 ± 0.25	0.54 ± 0.44	9.12 ± 1.18	1.80 ± 0.60
Sulphate	1.18 ± 0.57	4.20 ± 0.42	16.16 ± 1.94	2.70 ± 0.60
Total	2.07 ± 0.44	4.74 ± 0.42	25.49 ± 1.05	5.40 ± 0.67
Total by acid hydrolysis	7.26 ± 1.52	4.39 ± 0.43	25.50 ± 3.33	†

* Results are means of six (biphenyl) or three (benzo[*a*]pyrene) investigations on different hepatocyte preparations ± S.E.M.
† Not determined.

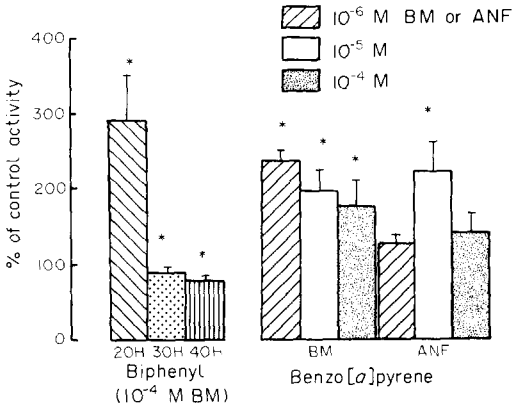

Fig. 4. Effects of betamethasone (BM) and α-naphthoflavone (ANF) on biphenyl 2-, 3- and 4-hydroxylation (20H, 30H and 40H) and benzo[*a*]pyrene hydroxylation in male rat hepatocytes. Results are means of six (biphenyl) or three (benzo[*a*]pyrene) investigations on different hepatocyte preparations ± S.E.M. *Significantly different from control ($P < 0.05$, Student's *t*-test).

hydroxylation affected sulphate and glucuronide formation equally.

The phenolic metabolites of benzo[*a*]pyrene were metabolised to approximately equal proportions of sulphate and glucuronide in isolated rat hepatocytes (Table 1). Betamethasone and α-naphthoflavone both increased the formation of total metabolites as shown in Fig. 4 but unlike the situation for biphenyl there were no apparent selective effects on the levels of sulphate and glucuronic acid conjugates. Maximum activation was observed with 10^{-5} M α-naphthoflavone (119% increase) and 10^{-6} M betamethasone (135% increase). Higher concentrations of either compound did not inhibit the metabolism of benzo[*a*]pyrene in contrast to the effects in hepatic microsomes.

DISCUSSION

Biphenyl and benzo[*a*]pyrene hydroxylation in microsomes of extrahepatic tissues was an order of magnitude lower than in liver microsomes and showed different metabolic profiles in each tissue. Betamethasone did not appear to activate biphenyl 2-hydroxylation in any of the extrahepatic tissues investigated except the kidney cortex of female rats and inhibited biphenyl metabolism in kidney microsomes from male rats. Inhibition of biphenyl metabolism by betamethasone has not been described previously [1–4].

α-Naphthoflavone caused a small activation of biphenyl 2-hydroxylation and a very marked increase in benzo[*a*]pyrene hydroxylation in intestinal microsomes. Stohs *et al.* [14] observed a similar degree of activation by α-naphthoflavone of benzo[*a*]pyrene hydroxylation in isolated rat intestinal epithelial cells. It is of interest that the degree of activation by α-naphthoflavone of the two enzyme activities in intestinal microsomes is the converse of that found in liver microsomes. In agreement with Wiebel *et al.* [5], benzo[*a*]pyrene hydroxylation in lung and kidney cortex microsomes was found to be more susceptible to inhibition by α-naphthoflavone than in liver microsomes.

No sex differences have been reported in the microsomes of rat lung or intestine but rat kidney microsomal benzo[*a*]pyrene and zoxazolamine hydroxylase activities have been shown to be higher in female than in male rats [15, 16] which is the converse situation to that in liver microsomes. In the present study, no significant difference was found between male and female rat kidney cortex microsomes in control biphenyl and benzo[*a*]pyrene hydroxylase activities or in the response to α-naphthoflavone. However betamethasone had a small activating effect in kidney microsomes from female rats only. It is interesting that in adult liver microsomes activation by betamethasone occurs in male but not in female rats [4].

The earlier findings support the view that the difference in cytochrome P-450 species between tissues in the rat are both quantitative and qualitative.

At identical concentrations betamethasone caused a similar activation of biphenyl 2-hydroxylase in isolated rat hepatocytes to that in adult male rat liver microsomes. It is interesting that formation of free 2-hydroxybiphenyl, its glucuronide and an unidentified acid-hydrolysable conjugate were all increased whereas formation of the sulphate was unchanged. Wiebkin *et al.* [17] observed that at low concentrations of 2- and 4-hydroxybiphenyl, sulphate conjugation was predominant and with increasing concentration glucuronidation became more important. It was suggested that this might be due to the limited availability of sulphate within the cells. In betamethasone-activated hepatocytes there is more 2-hydroxybiphenyl produced but it seems unlikely that this is sufficient to render the sulphate levels limiting. It is also unlikely that betamethasone or its

Table 2. Tissue and sex differences in susceptibility of aromatic hydrocarbon hydroxylases to betamethasone and α-naphthoflavone*

			Biphenyl 2-hydroxylation	Benzo[a]pyrene hydroxylation
Liver	M	BM	+++	+
		ANF	+++	+
Liver	F	BM	0	0
		ANF	−	−
Kidney	M	BM	−	0
		ANF		−−−
Kidney	F	BM	+	+
		ANF		−−−
Lung	M	BM	0	0
		ANF	0	−−
Intestine	M	BM	0	0
		ANF	+	+++

* M, male; F, female; BM, betamethasone; ANF, α-naphthoflavone; +, activation; 0, no effect; −, inhibition.

metabolites could compete for the sulphate as betamethasone is only metabolised very slowly and sulphate metabolites have not been reported.

Biphenyl 3- and 4-hydroxylation in isolated rat hepatocyes were inhibited by betamethasone. This result was unexpected as these activities were not affected by betamethasone in rat liver microsomes. Betamethasone did not have a direct effect on the hepatocytes as the viability of neither control nor treated hepatocytes changed during the incubations.

Benzo[a]pyrene hydroxylation in isolated rat hepatocytes was much less susceptible to inhibition by α-naphthoflavone than in liver microsomes from rats of the same age. Thus 10^{-5} M α-naphthoflavone caused an activation of 119% and 10^{-4} M did not inhibit. In contrast, in liver microsomes, a maximum activation of 15% was obtained in the presence of 10^{-6} M α-naphthoflavone and higher concentrations caused increasing inhibition. Betamethasone also caused a much greater activation of benzo[a]pyrene hydroxylation in isolated rat hepatocytes than in liver microsomes.

Isolated hepatocytes are thought to bear more relationship to the whole animal than subcellular fractions. But whereas betamethasone treatment of hepatocytes and liver microsomes caused a similar activation of biphenyl 2-hydroxylase, betamethasone pretreatment of rats caused only a small increase after 2–4 hr [2]. This difference may be due to differences in the bioavailability of betamethasone to the hepatocytes *in vivo* and *in vitro*. The occurrence of the activation in the whole cell and *in vivo* indicates that activation is not simply an artefact of microsomal preparations but may have some physiological relevance. It has been suggested that biphenyl and benzo[a]pyrene are metabolised by similar enzymes [18]. The tissue and sex differences in susceptibility of these enzymes to betamethasone and α-naphthoflavone (Table 2) emphasize that there are some similarities but many differences in the response to these compounds.

Since the activatable 'benzo[a]pryene hydroxylase' and 'biphenyl 2-hydroxylase' appear to utilise the same cofactors and reductases [19] these differences in response to α-naphthoflavone and betamethasone are presumably ascribable to at least two different forms of 'latent' cytochrome P-450, the levels of each varying from tissue to tissue. A similar conclusion has been reached for human liver [3].

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