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CHARACTERIZATION OF THE ACTIVATION OF HEPATIC MICROSOMAL HYDROXYLATION BY BETAMETHASONE AND α NAPHTHOFLAVONE

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Abstract—Biphenyl 2-hydroxylation is selectively activated *in vitro* by incubation of betamethasone or α naphthoflavone with control male rat liver microsomes. Biphenyl 3- and 4-hydroxylation activities are unchanged or marginally inhibited. The nature of the enzymes involved in the activation has been investigated. Metyrapone (1 mM) completely inhibited the expression of the activation but had a lesser effect on the basal 2-, 3- and 4-hydroxylation activities. SKF525A (1 mM)† inhibited both basal and betamethasone-activated enzyme activities by 25–35 per cent. Of other drug metabolizing enzymes investigated, only benzo[*a*]pyrene hydroxylation activity was increased by betamethasone and α naphthoflavone. Acetone (0.6 M) caused a small activation (40 per cent) of biphenyl 2-hydroxylation but inhibited 4-hydroxylation. The non-ionic detergent Brij 35 inhibited biphenyl 2-, 3- and 4-hydroxylation. It was concluded that activation of biphenyl 2-hydroxylation differs from activation of aromatic amine hydroxylation and glucuronyl transferase but may be related to activation of benzo[*a*]pyrene hydroxylation by naphthoflavones.

There are a number of examples of *in vitro* enhancement of drug metabolizing enzymes which, unlike induction, does not involve *de novo* protein synthesis. The hydroxylation of certain aromatic amines can be activated by various agents including acetone [1, 2], ethyl isocyanide [3], 2,2′-bipyridine [4], metyrapone [5] and diethylmaleate [6]. It has been suggested that these may either be due to a change in equilibrium between two forms of cytochrome P-450 [1] or to a direct effect on the rate-limiting step in the cytochrome P-450 sequence [7]. Glucuronyl transferases are activated by a wide range of chemical and physical means [8]. This is thought to be mediated by effects on the phospholipid and possibly protein of the endoplasmic reticulum membrane, increasing the availability of the substrate and/or cofactors to the active site of the enzyme [9]. Activation of benzo[α]pyrene hydroxylation by α naphthoflavone has been reported in rat [10] and human [11] liver. Again the existence of two different forms of the enzyme has been suggested.

Certain steroids, especially betamethasone, selectively activate 2- but not 3- or 4-hydroxylation of biphenyl [12, 13]. The objective of the present study was to characterize the types of cytochrome P-450 involved in basal and betamethasone-activated biphenyl hydroxylation by performing inhibition studies. The activation of biphenyl 2-hydroxylation was also compared with other known activations and a preliminary investigation made to discover alternative substrates for the enzyme.

MATERIALS AND METHODS

Animals. Control male Wistar albino rats (80–100 g) were used. All animals were maintained on Sterolit bedding and fed on Spiller No. 1 laboratory animal diet and water *ad lib.*

Chemicals. Biphenyl (BDH Chemicals Ltd., Poole, U.K.) was twice recrystallized from ethanol to give a product of melting point 70°. Betamethasone, NADP and DL-trisodium isocitrate were purchased from Sigma, London (Poole, U.K.). Benzo[*a*]pyrene and α naphthoflavone (αNF) were from Aldrich Chemical Co. (Dorset, U.K.). Isocitrate dehydrogenase (EC. 1.1.1.42) was obtained from Boehringer Mannheim Co. (F.R.G.). Standard 3-hydroxybenzo[*a*]pyrene was obtained from the Carcinogenesis Research Program, National Cancer Institute (Bethesda, MD). All other chemicals were of at least analytical reagent grade.

Enzyme assays. Preparation of tissues and the biphenyl hydroxylation assay were performed as described previously [13]. For the inhibition studies, metyrapone, SKF525A and αNF were added to the incubation mixtures as solutions in DMF prior to preincubation, as for betamethasone. Biphenyl metabolism was measured in the presence of inhibitor, betamethasone, inhibitor plus betamethasone, and solvent alone. The DMF concentration was 1% in all incubations and was shown to have no significant effect on biphenyl metabolism. In experiments studying the effect of the non-ionic detergent, Brij 35, on biphenyl hydroxylation, Tween 80 (the detergent used to solubilize biphenyl in other experiments) was omitted from the incubations. Biphenyl was added in DMF solution to give the same final concentration (3.25 mM). Again DMF concentration did not exceed 1%.

Other enzyme assays included aniline 4-hydroxy-

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† Abbreviations used: αNF, α naphthoflavone; SKF525A,2-diethylaminoethyl-2,2-diphenylvalerate; DMF, dimethyl formamide; h.p.l.c., high pressure liquid chromatography.

lation [14], glucuronyl transferase [15] using Brij 35 as activator, benzo[*a*]pyrene hydroxylation by an adaption of the fluorimetric method of Dehnen *et al.* [16] and an adaption of the high-pressure liquid chromatographic (h.p.l.c.) method of Selkirk *et al.* [17], ethoxyresorufin *O*-deethylation [18], 7-ethoxycoumarin *O*-deethylation [19] and ethylmorphine *N*-demethylation [20]. Microsomal protein was determined by the method of Lowry *et al.* [21].

In experiments planned to investigate alternative substrates for the activatable enzyme, the biphenyl concentration was reduced to 1 mM and test substrates (also 1 mM) were added before incubation.

RESULTS

Inhibition studies. Metyrapone had only a small effect on biphenyl 4-hydroxylation in microsomes from male rat livers, 7 per cent inhibition being observed at 10^{-3} M (Fig. 1). Biphenyl 2- and 3-hydroxylation were increasingly inhibited by metyrapone concentrations of greater than 10^{-5} M. At 10^{-3} M the levels of inhibition were 57 and 68 per cent for 2- and 3-hydroxylation, respectively. Betamethasone (10^{-4} M) activated biphenyl 2-hydroxylation 3- to 5-fold with no effect on 3- and 4-hydroxylation [13]. The betamethasone-activated 2-hydroxylation activity was inhibited more markedly and commenced at a lower metyrapone concentration (10^{-6} M) than in controls. Activation was completely prevented by 10^{-3} M metyrapone. In this experiment betamethasone and metyrapone were both added to the reaction mixture before preincubation. Altering the sequence of addition did not significantly alter the activation.

SKF525A (10^{-3} M) inhibited biphenyl 2-, 3- and 4-hydroxylation by 20, 25 and 25 per cent, respectively. The betamethasone activated biphenyl 2-hydroxylation was inhibited 35 per cent by 10^{-3} M SKF525A. Lower concentrations of SKF525A (10^{-7}–10^{-4} M) had no significant effect (data not shown).

αNF (5×10^{-4} M) had no effect on biphenyl 4-hydroxylation but activated biphenyl 2-hydroxylation in male rat hepatic microsomes by 370 ± 75 per cent (Fig. 2). The effects of αNF and betamethasone (10^{-4} M) were not additive but resulted in an activation equal to that of betamethasone alone. αNF had little or no effect on biphenyl 2-hydroxylation of liver microsomes from mature female rats or male hamsters (results not shown).

Effects of betamethasone on other enzymes. To ascertain whether this activation is unique to biphenyl 2-hydroxylation, the effect of betamethasone on other drug metabolizing enzymes was examined. Aniline 4-hydroxylation, ethymorphine *N*-demethylation and 7-ethoxycoumarin-*O*-deethylation were unaffected, whereas ethoxyresorufin-*O*-deethylation and glucuronyl transferase (with 4-nitrophenol as substrate) activities were inhibited by 10–15 per cent at the concentrations of betamethasone tested (10^{-7}–10^{-4} M) (data not shown). Benzo[*a*]pyrene hydroxylation as determined fluorimetrically [16] was subject to a small but significant activation (13 per cent) when betamethasone (10^{-6}) was included in the incubation. This was confirmed by use of [^{3}H]benzo[*a*]pyrene, metabolites of which were separated by h.p.l.c. and fractions collected for liquid scintillation counting. A 15 per cent increase in the formation of a tritiated metabolite

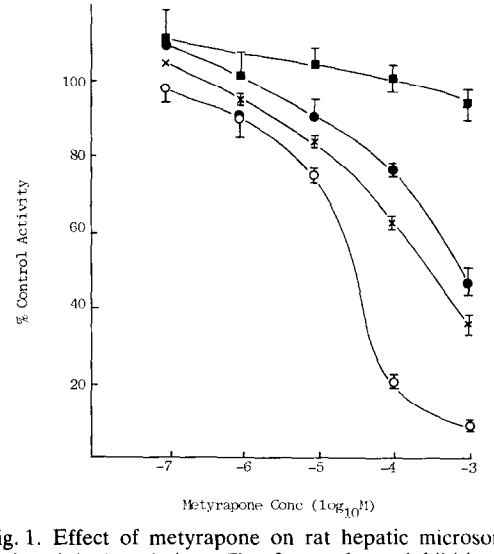

Fig. 1. Effect of metyrapone on rat hepatic microsomal biphenyl hydroxylation. The figure shows inhibition of 2-hydroxylation (●), 3-hydroxylation (×), 4-hydroxylation (■) and 2-hydroxylation in the presence of betamethasone (10^{-4} M) (○). 3- and 4-hydroxylation are unchanged by betamethasone. Each point represents the mean of three experiments ± S.E.M. The control values were: ●, 0.28 ± 0.03; ×, 0.09 ± 0.01; ■, 0.78 ± 0.06; ○, 0.89 ± 0.06 nmoles/min/mg microsomal protein.

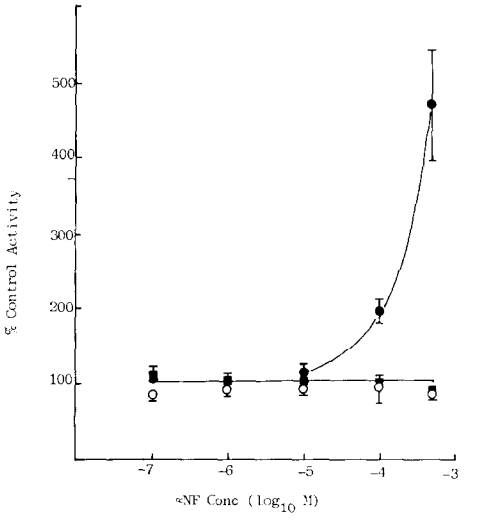

Fig. 2. Effect of αNF on rat hepatic microsomal biphenyl hydroxylation. The figure shows the effect of αNF on 2-hydroxylation (●), 4-hydroxylation (■) and 2-hydroxylation in the presence of betamethasone (10^{-4} M) (○). 4-Hydroxylation is unchanged by betamethasone. Low solubility prevented the use of higher concentrations of αNF. Each point represents the mean of three experiments ± S.E.M. The control values were: ●, 0.28 ± 0.03; ■, 0.78 ± 0.06; ○, 0.89 ± 0.06 nmoles/min/mg microsomal protein.

with the same elution time as standard 3-hydroxybenzo[*a*]pyrene was found with betamethasone (10^{-6} M). The formation of other metabolites was largely unaffected. The selective effect of betamethasone on certain P-450 activities only suggests that the interaction of the corticosteroid is at P-450 itself and not at the level of electron flow to P-450. αNF (10^{-6} M) also caused similar activations to betamethasone when benzo[*a*]pyrene metabolism was measured both fluorimetrically and by h.p.l.c.

Activation of biphenyl 2-hydroxylation was also compared with other known activations: of aniline 4-hydroxylation by acetone [1] and of glucuronyl transferase by the detergent Brij 35 [15]. Aniline 4-hydroxylation was found to be activated maximally by 0.68 M acetone, which gave an increase of 115 per cent. The effects of acetone on biphenyl hydroxylation can be seen in Fig. 3. An increase in 2-hydroxylation of about 40 per cent was seen with acetone concentrations of 0.4–0.8 M. Other concentrations of acetone had no significant effect on biphenyl 2-hydroxylation. Biphenyl 3-hydroxylation was marginally increased (16 per cent) by 0.34 M acetone, but inhibited above 0.68 M. Biphenyl 4-hydroxylation was inhibited most strongly and was 40 per cent of control activity at 1 M acetone. Brij 35 (0.1%) produced an increase of 245 per cent in glucuronyltransferase activity to 4-nitrophenol but had no effect, or marginally inhibited biphenyl hydroxylation in the 2-, 3- and 4-positions (data not shown).

Cortisol, corticosterone, oestradiol, testosterone, tyrosine, tryptophan and vitamin D all had no effect on activation of biphenyl 2-hydroxylation by betamethasone when included in incubations in equimolar concentrations with biphenyl (data not shown) and were thus thought unlikely to be natural substrates for the activatable enzyme.

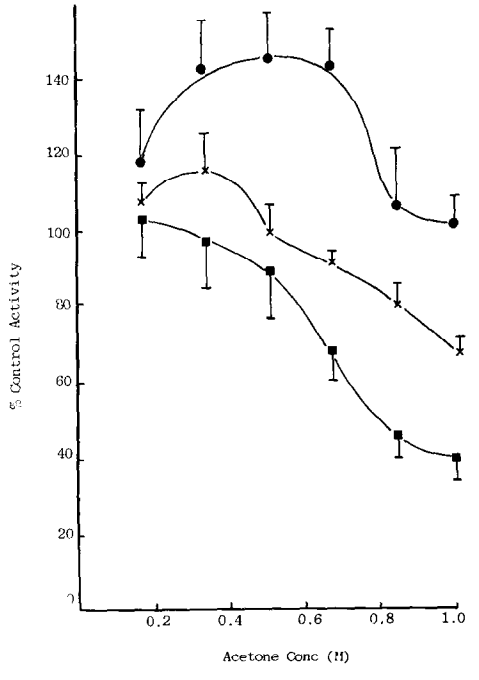

Fig. 3. Effect of acetone on rat hepatic microsomal biphenyl hydroxylation. The figure shows the effect of acetone on 2-hydroxylation (●), 3-hydroxylation (×) and 4-hydroxylation (■). Each point represents the mean of four experiments ± S.E.M. The control values were: ●, 0.28 ± 0.03; ×, 0.09 ± 0.01; ■, 0.78 ± 0.06 nmoles/min/mg microsomal protein.

DISCUSSION

Metyrapone is a potent inhibitor of adrenal steroid hydroxylation and of a number of cytochrome P-450 related drug metabolizing enzymes in liver microsomes from both control and phenobarbitone treated rats [5]. It appears to inhibit metabolism of a number of substrates showing type 1 binding spectra whereas metabolism of some type II compounds such as acetanilide are enhanced [1]. Metyrapone had only a small effect on biphenyl 4-hydroxylation, contrary to previous reports [22], but inhibited 2- and 3-hydroxylation more markedly. The similarity between inhibition of 2- and 3-hydroxylation seen in Fig. 1 was unexpected as 3-hydroxylation resembles 4-hydroxylation in its induction characteristics [23] and also in its lack of sensitivity to glucocorticoids. It has been suggested that conversion of cytochrome P-450 to P-448 may be responsible for the activation of biphenyl 2-hydroxylation [24]. However, we have found that the activation is completely inhibited by addition of metyrapone with betamethasone before incubation, suggesting that a species of cytochrome P-450 more sensitive than P-448 to metyrapone is involved. Metyrapone appears to inhibit the activated enzyme rather than inhibiting the activation process as it has similar effects when added to the incubation before, after or concomitantly with betamethasone. These results are consistent with our suggestion that two enzymes may be involved in the activation [13].

SKF525A is another inhibitor of cytochrome P-450 related enzymes from livers of control and phenobarbitone-treated rats [1]. However, it was less effective than metyrapone as an inhibitor of biphenyl 2- and 3-hydroxylation. It has a similar effect on hydroxylation activity in all three positions with only slightly greater inhibition of betamethasone-activated 2-hydroxylation. The results with these two inhibitors suggest the form of the enzyme that is activated by glucocorticoids may be similar to that which is responsible for steroid hydroxylation, as these are often more sensitive to metyrapone inhibition [25].

αNF is a potent inhibitor of liver microsomal drug metabolizing enzymes from animals treated with 3-methylcholanthrene but has less effect on control and phenobarbitone-treated animals [10, 22]. If enhancement of biphenyl 2-hydroxylation reflected a change from cytochrome P-450 to P-448 as previously suggested [24], then it should have been inhibited by αNF. However, αNF had no effect on the activation by betamethasone when added concomitantly to the incubation, but when added alone it caused an activation equal to that produced by betamethasone. Betamethasone has little or no effect on biphenyl 2-hydroxylation of liver microsomes from mature female rats or male hamsters [13]. Similar results were found with αNF, suggesting that αNF resembles betamethasone in its effect on control

and phenobarbitone-induced microsomes. However, betamethasone also activated biphenyl 2-hydroxylation in liver microsomes from 3-methylcholanthrene-treated rats whereas αnapthaflavone produced a marked inhibition. This difference may be simply due to the marked inhibition of the 3-methylcholanthrene-induced enzyme activity by αNF, masking rather than preventing activation of the basal enzyme activity.

Glucuronyl transferase is a membrane-bound enzyme that is activated by many different agents including non-ionic detergents [8]. This is thought to be due to a membrane permeability change permitting increased substrate and/or cofactor access to the enzyme which is deep in the endoplasmic reticulum [9]. As glucuronyltransferase was unaffected by betamethasone and Brij 35 inhibited biphenyl 2-hydroxylation, this type of membrane effect cannot explain the activation reported here.

Ethylmorphine *N*-demethylation and 7-ethoxycoumarin *O*-deethylation are, like biphenyl 4-hydroxylation, largely cytochrome P-450 dependent. As expected, they were unaffected by betamethasone. Acetone caused slight activation of biphenyl 2- (and 3-) hydroxylation. In contrast, aniline 4-hydroxylation was unaffected by betamethasone. It is thus unlikely that betamethasone has a similar action to acetone but the effect of acetone on biphenyl hydroxylation cannot be explained at present.

Benzo[*a*]pyrene hydroxylation, ethoxyresorufin *O*-deethylation and aniline 4-hydroxylation have been suggested to be cytochrome P-448 dependent but only the first of these was activated by betamethasone. Analysis of metabolites by h.p.l.c. suggested the increase was in the formation of 3-hydroxybenzo[*a*]pyrene only. In contrast, induction of cytochrome P-448 by treatment with 3-methylcholanthrene results in 3 to 4-fold increases in 3- and 9-hydroxylation of benzo[*a*]pyrene but much larger increases (20 to 25-fold) in formation of the 7,8- and 9,10-diols [26]. Thus an increase in cytochrome P-448 activity cannot explain the specificity of the activation caused by betamethasone. αNF caused a similar activation of benzo[*a*]pyrene 3-hydroxylation. It has been shown previously that αNF stimulates benzo[*a*]pyrene hydroxylation in liver microsomes from control and phenobarbitone-treated rats but inhibits in liver microsomes from 3-methylcholanthrene-treated rats [10, 22]. These effects appear to be dependent on age and sex; activation is high in young male and female control rats and decreases with age [27]. In mature control male rats αNF has a slight inhibitory effect whereas in female rats the enzyme is inhibited by 50 per cent [27]. A similar age and sex dependence was found for the enhancement of biphenyl 2-hydroxylation by betamethasone except that inhibition does not occur [28]. Enhancement was found with adult male but not female rat liver microsomes. Thus αNF appears to have a biphasic effect in that it both inhibits and activates drug metabolizing enzymes. Betamethasone resembles αNF in the enhancing action only. Inhibition of biphenyl hydroxylation has not been found at any concentration of betamethasone [13]. αNF has been shown to stimulate metabolism of aflatoxin B_1, benzo[*a*]pyrene, zoxazolamine and antipyrine in human liver homogenates [11, 29]. Betamethasone also activates biphenyl 2-hydroxylation and benzo[*a*]pyrene hydroxylation in human liver microsomes (D. J. Benford, A. R. Boobis, D. S. Davis and J. W. Bridges; unpublished data). The structural relationship between betamethasone and αNF which is not immediately apparent is being investigated. Because betamethasone appears to be very selective in its activating effects on P-450 dependent enzymes, is free from significant obfuscating inhibitory properties and its effects are preserved when endoplasmic reticulum membrane modifiers are added, it should provide a very valuable and subtle probe for studying the properties of cytochrome P-450.

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