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MECHANISTIC STUDIES ON THE ACTIVATION OF BIPHENYL 2-HYDROXYLATION BY GLUCOCORTICOIDS

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Abstract—In order to establish the mechanism by which the selective activation of biphenyl 2-hydroxylation by betamethasone occurs the effect of modifying possible critical factors in the hydroxylation process has been examined. Activation of biphenyl 2-hydroxylation by betamethasone was found in detergent-solubilized rat liver microsomes indicating that intact microsomal membranes are probably not necessary for the activation. Betamethasone had no effect on the spectrally apparent binding of biphenyl or of other type I, type II or reverse type I model substrates. The activation process did not appear to be greatly influenced by changing the ratio of cytochrome P-450 reductase to cytochrome P-450 nor by changing the amount of NADPH. Addition of NADH increased the extent of activation suggesting that betamethasone facilitates transference of the second electron to cytochrome P-450. However, betamethasone also stimulated cumene hydroperoxide supported biphenyl 2-hydroxylation; therefore a step subsequent to cytochrome P-450 reduction is also involved in the activation. Activation did not correlate with increased uncoupling of an active oxygen–cytochrome P-450 complex to form hydrogen peroxide.

A number of examples of activation (or enhancement) of drug-metabolizing enzyme activity have been reported. Activation of the liver microsomal cytochrome P-450 dependent monoxygenases has been reviewed [1–4]. Other drug-metabolizing enzymes that may be activated include the glucuronyl transferases [5] and epoxide hydrolase [6]. Glucuronyl transferase may be activated by detergents and a number of solvents through initiating a change in membrane permeability thereby permitting increased cofactor and/or substrate availability to the enzyme [7]. Suggested mechanisms for activation of "aniline hydroxylase" and "benzo[*a*]pyrene-3-hydroxylase" activity have included the involvement of two or more enzyme forms [8–10] or effects on reduction of cytochrome P-450 [11, 12].

We have previously reported the selective activation of biphenyl 2-hydroxylation, benzo[*a*]pyrene 3-hydroxylation and antipyrine 4-hydroxylation by betamethasone and α-naphthoflavone in rat [13–15] and human [16] liver microsomes and in isolated rat hepatocytes [17]. Activation by betamethasone is very much greater than with the very closely related structure dexamethasone [14]. The present report is concerned with elucidating the mechanism of this biphenyl 2-hydroxylation activation.

MATERIALS AND METHODS

Animals. Male Wistar Albino rats (100–150 g) were supplied by the University of Surrey, Rodent Breeding Unit and were maintained on Sterolit bedding and fed Spillers No. 1 laboratory animal diet and water *ad lib.*, under constant temperature (22°), humidity (56%) and lighting (12 hr darkness).

Chemicals. Biphenyl (BDH Ltd., Poole, U.K.) was recrystallized twice from ethanol to give a product of melting point approximately 70°. α-Naphthoflavone was purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Betamethasone, sodium cholate, dithiothreitol, cytochrome *c* and all cofactors were purchased from Sigma, London (Poole, U.K.). All other reagents, obtained from different sources, were of the highest purity available.

Methods. Washed liver microsomes were prepared as described previously [14] and resuspended in 20 mM Tris–HCl (pH 7.6) containing 0.25 M sucrose and 5.4 mM EDTA to give approximately 20 mg microsomal protein/ml. The microsomes were either used immediately or stored at −80° without loss of basal or activation activity.

Sodium cholate solubilized microsomes were prepared by the method of Lu and Levin [18]. Washed microsomes were suspended in 0.1 M potassium phosphate containing 10 mM EDTA, 0.25 M sucrose and 22% glycerol. Dithiothreitol (1 mM) and sodium cholate (1 mg/mg microsomal protein) were added. The final protein concentration was approximately 10 mg/ml. The suspension was sonicated 3 times for 15 sec in a Kerry sonic bath (50 c/s) and stirred at 4° for 20 min before centrifuging at 105,000 *g* for 60 min to remove insoluble material. Protein [19] and cytochrome P-450 [20] were determined before and after solubilization.

Spectrally apparent cytochrome P-450 binding of biphenyl, phenacetin, aminopyrine and 2-methylbenzimidazole was recorded at 37° by the method of Schenkman *et al.* [21]. Cytochrome *c* reductase

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Table 1. Effect of betamethasone on biphenyl hydroxylation in solubilized rat liver microsomes

Betamethasone (M)	2-Hydroxylation	3-Hydroxylation	4-Hydroxybiphenyl
	pmoles product formed/min/nmole cytochrome P-450		
0	138 ± 32	60 ± 12	955 ± 20
		% control activity	
10^{-8}	121 ± 14	96 ± 5	103 ± 3
10^{-7}	119 ± 11	98 ± 9	102 ± 2
10^{-6}	119 ± 8	94 ± 8	100 ± 1
10^{-5}	144 ± 9*	107 ± 11	105 ± 3
10^{-4}	260 ± 24*	104 ± 11	98 ± 2

Microsomes were solubilized with sodium cholate as described in the text. Results are means of three different livers ± S.E.M.
* Significantly different from control, $P < 0.05$.

was measured according to Williams and Kamin [22].

Biphenyl hydroxylation was measured as previously described [14] using an NADPH-regenerating system. NADH (10^{-3} M) was added when required.

Cumene hydroperoxide supported biphenyl metabolism was measured similarly but the NADPH-generating system was omitted and cumene hydroperoxide was added (0.4 μmoles in 5 μl of methanol to a total incubation volume of 2 ml). Betamethasone and α-naphthoflavone were added to incubation mixtures in dimethylformamide giving a final solvent concentration of 0.5%. The same volume of solvent was also added to control incubations and was found to have no significant effect on the enzyme activities measured.

Hydrogen peroxide was measured by the method of Hildebrandt *et al.* [23]. For these experiments Tween 80 (normally used to solubilize biphenyl) had to be omitted from incubations and biphenyl was added in dimethylformamide (10 μl) to give the same final concentration (1 mM).

Purified NADPH–cytochrome P-450 reductase prepared from liver microsomes of phenobarbitone-treated rats [24] to a specific activity of 8100 units/ml was prepared by Dr. T. Fennell. (One unit of cytochrome P-450 reductase activity is defined as that amount of enzyme catalyzing the reduction of cytochrome *c* at an initial rate of 1 nmole/min at 22°.)

RESULTS

Biphenyl metabolism in solubilised microsomes

The activities for biphenyl hydroxylation in intact rat liver microsomes were 0.29, 0.11 and 0.92 nmoles/min/nmole of cytochrome P-450 for 2-, 3- and 4-hydroxylation respectively. In rat liver microsomes solubilized with sodium cholate (1 mg/mg of protein), biphenyl 4-hydroxylase showed similar activity to that observed in intact microsomes but biphenyl 2- and 3-hydroxylase activities were decreased (Table 1). Betamethasone caused a concentration-related activation of biphenyl 2-hydroxylation (160% increase compared with a 330% increase in non-solubilized microsomes at a concentration of 10^{-4} M betamethasone). In contrast biphenyl 3- and 4-hydroxylation activities were unaffected by the addition of betamethasone.

Substrate binding studies

The spectral changes produced by binding to rat liver microsomes of biphenyl (type I), aminopyrine (type I), phenacetin (reverse type I) and 2-methylbenzimidazole (type II) were measured at 37° in the presence and absence of betamethasone (10^{-4} M). Betamethasone had no significant effect on the K_s or ΔE_{max} of spectral binding of any of these compounds. Betamethasone itself exhibited a modified type II binding spectrum with a maximum at 415 nm and a minimum at 375 nm.

Table 2. Effect of cytochrome P-450 reductase concentration on the activation of biphenyl hydroxylation

Reductase added	Betamethasone (M)	2-Hydroxybiphenyl	3-Hydroxybiphenyl	4-Hydroxybiphenyl
		(pmoles product formed/min/mole cytochrome P-450)		
0	0	102	20	622
0	10^{-4}	354 (347%)	24	682
x 5	0	270	44	1656
x 5	10^{-4}	746 (276%)	38	1502
x 10	0	288	50	1968
x 10	10^{-4}	708 (246%)	46	2024

Sodium cholate solubilized rat liver microsomes were prepared with 90% recovery of cytochrome P-450. Incubations contained 3.5 nmoles of cytochrome P-450, 480 units of endogenous cytochrome P-450 reductase and an excess of NADPH in 2 ml. Purified reductase was added at 5 and 10 times the endogenous concentration. Metabolism was determined as described in the text. Values from a typical experiment are shown.

Table 3. Effects of betamethasone and NADH on rat liver microsomal biphenyl hydroxylation

	2-Hydroxybiphenyl	3-Hydroxybiphenyl	4-Hydroxybiphenyl
	(pmoles product formed/min/mg protein)		
Control	200 ± 34	38 ± 6	722 ± 96
NADH	188 ± 24	44 ± 6	926 ± 94*
Betamethasone	558 ± 48	40 ± 6	710 ± 44
Betamethasone + NADH	942 ± 112*	46 ± 6	974 ± 74*

Metabolism was determined as described in the text with an NADPH-generating system. NADH was added at a concentration of 10^{-3} M, and betamethasone was added at 10^{-4} M. Values are means ± S.E.M. of three different livers.

* Significantly different from the value in the absence of NADH ($P < 0.05$).

Effect of betamethasone on the reduction of cytochrome c *and cytochrome P-450*

Addition of betamethasone (10^{-4} M) had no effect on rat liver microsomal cytochrome *c* reductase activity. Preliminary investigations using cytochrome P-450 as substrate showed a similar lack of effect on cytochrome P-450 reductase activity.

Effect of NADPH concentration on the activation by betamethasone

The effect of changing the NADPH concentration (0.1–2 mM) on the rate of biphenyl 2-hydroxylation by rat liver microsomes was determined. Addition of betamethasone (10^{-4} M) resulted in a large increase in the V_{max} for biphenyl 2-hydroxylation from 0.36 to 2.33 nmoles/min/mg of microsomal protein. However betamethasone had no effect on the K_m for NADPH (0.27 mM), suggesting that betamethasone does not affect the affinity of NADPH for NADPH–cytochrome P-450 reductase.

Effect of cytochrome P-450 reductase concentration on activation

Purified cytochrome P-450 reductase was added to sodium cholate solubilized microsomes to determine if the reductase was the limiting factor involved in the activation of biphenyl 2-hydroxylation. Solubilized microsomes were chosen for this experiment in order to facilitate incorporation of the reductase. Incorporation of 5 and 10 times the endogenous amount of reductase resulted in stimulation of biphenyl hydroxylation with no selective effects on the different routes of biphenyl metabolism, i.e. 2-, 3- and 4-hydroxylation activity (Table 2). Addition of betamethasone (10^{-4} M) caused activation of biphenyl 2-hydroxylation at each reductase concentration and had no significant effect on biphenyl 3- and 4-hydroxylation, as with unsupplemented microsomes. The extent of activation decreased only slightly with increasing reductase concentration.

The effects of added NADH

Because of the possibility that activating agents could produce their effect by facilitating the transfer of the second electron to cytochrome P-450 [25] we investigated the combined effects of NADH and betamethasone on biphenyl hydroxylation (Table 3). Addition of NADH had no effect on the NADPH-supported 2- or 3-hydroxylation of biphenyl but did have a small synergistic effect on 4-hydroxylation. However, in the presence of betamethasone, NADH stimulated the rate of biphenyl 2-hydroxylation by 69%. Thus betamethasone caused a much larger activation in the presence of NADH suggesting that the activation may in part be due to increased transfer of the second electron to cytochrome P-450.

Cumene hydroperoxide supported biphenyl hydroxylation

Preliminary studies revealed that the optimal rate of biphenyl hydroxylation was achieved at a concentration of 0.2 mM cumene hydroperoxide. The relative proportions of hydroxylated products differed considerably from the NADPH-supported system, with greater biphenyl 2- and 3-hydroxylation and much lower 4-hydroxylation activities (0.63 ± 0.05, 0.21 ± 0.02 and 0.31 ± 0.04 nmoles/min/mg microsomal protein respectively). The effects of including betamethasone in the cumene hydroperoxide supported microsomal reaction are shown in Fig. 1. Betamethasone at a concentration of 10^{-6} M caused an increase of 78% in biphenyl 2-hydroxylation which was greater than the increase seen in NADPH-supported 2-hydroxylation at the same concentration [14]. However higher concentrations of betamethasone had a decreasing effect on the

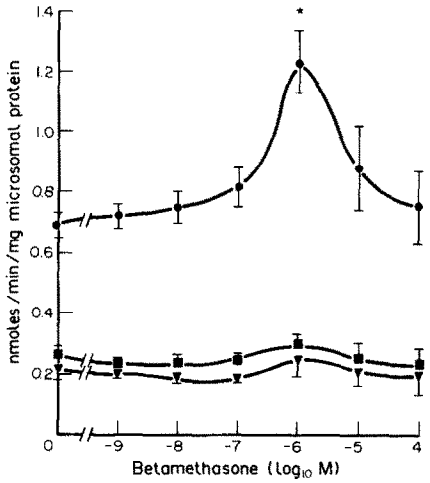

Fig. 1. Effect of betamethasone on cumene hydroperoxide supported biphenyl hydroxylation. Biphenyl (1 mM) was incubated with various concentrations of betamethasone and rat liver microsomes fortified with cumene hydroperoxide (0.2 mM) instead of an NADPH regenerating system. Biphenyl 2-hydroxylation (●), 3-hydroxylation (▼) and 4-hydroxylation (■) were measured. Each point represents the mean of three experiments ± S.E.M. *Significantly different from control (no betamethasone) according to the Student's *t*-test ($P < 0.05$).

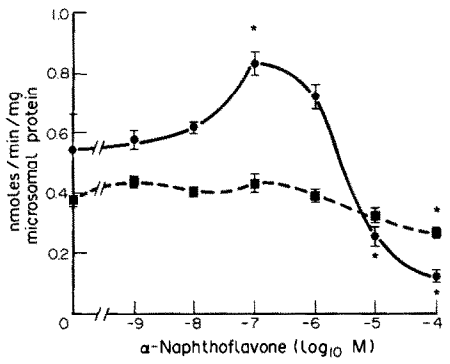

Fig. 2. Effect of α-naphthoflavone on cumene hydroperoxide supported biphenyl hydroxylation. Biphenyl (1 mM) was incubated with various concentrations of α-naphthoflavone and rat liver microsomes fortified with cumene hydroperoxide (0.2 mM) instead of an NADPH-regenerating system. Biphenyl 2-hydroxylation (●) and 4-hydroxylation (■) were measured. Each point represents the mean of three experiments ± S.E.M. *Significantly different from control according to the Student's *t*-test ($P < 0.05$).

cumene hydroperoxide supported hydroxylation (Fig. 1) compared with the increased effect in the presence of NADPH [14].

Similarly, α-naphthoflavone caused a maximum activation of biphenyl 2-hydroxylation of 53% at a concentration of 10^{-7} M using cumene hydroperoxide but as the concentration was increased the extent of activation decreased and then inhibition was seen (Fig. 2). This again contrasts with the observation in the presence of NADPH that activation increased exponentially at concentrations of α-naphthoflavone above 10^{-5} M [15]. High concentrations of α-naphthoflavone also inhibited biphenyl 4-hydroxylation using cumene hydroperoxide.

Hydrogen peroxide generation

The possibility that activation might be due to a decreased uncoupling of an active oxygen–biphenyl–cytochrome P-450 complex was investigated by measuring hydrogen peroxide production in NADPH-fortified rat liver microsomes. The rate of hydrogen peroxide formation in control microsomes in the absence of substrate or activating agent was 1.4 ± 0.2 nmoles/mg microsomal protein/10 min. Addition of biphenyl and/or betamethasone at concentrations producing maximal activation of 2-hydroxylation activity had no effect on the rate of hydrogen peroxide formation.

DISCUSSION

We have previously shown that certain natural and synthetic glucocorticoids, of which betamethasone is the most active, have a selective activation effect on a specific group of cytochrome P-450 related hydroxylation reactions. Of the substrates investigated only biphenyl, benzo[*a*]pyrene and antipyrine were affected. High-pressure liquid chromatographic studies showed that only one route of hydroxylation of each of these substrates is susceptible to activation: namely 2-hydroxylation of biphenyl [14], 3-hydroxylation of benzo[*a*]pyrene [15] and 4-hydroxylation of antipyrine [16]. All these reactions may be considered *o*-hydroxylations but it is unlikely that a single cytochrome P-450 enzyme is involved as the substrates respond differently in different tissues, species and individuals to activation by betamethasone [16, 17].

Accepting that activation involves specific cytochrome P-450 enzymes there are basically three ways in which activation could occur: (a) a membrane effect permitting increased substrate and/or cofactor availability to the enzyme; (b) stimulation of the reductase associated with cytochrome P-450, thereby increasing the flow of electrons to the cytochrome; and (c) a direct effect on cytochrome P-450 itself at one or more of the possible points of interaction (Fig. 3).

A membrane effect is unlikely as biphenyl 2-hydroxylation was inhibited, by detergent [15], and betamethasone did not activate glucuronyl transferase [15], an enzyme that is located deep in the endoplasmic reticulum and is activated by membrane perturbants [5]. In addition, disruption of the endoplasmic reticulum by solubilization of liver microsomes did not stimulate biphenyl 2-hydroxylation or prevent its activation by betamethasone as might be expected if a membrane effect was involved.

Activation does not appear to involve an increase in the extent or affinity of substrate binding to cytochrome P-450 (site 2 in Fig. 3) as the spectral binding of both activateable and non-activateable substrates was unaffected by the presence of betamethasone.

It seems unlikely that betamethasone-mediated activation could be brought about via a change in the activity of NADPH–cytochrome P-450 reductase as this is thought to be a single enzyme [26] and so is not an obvious candidate to produce such selective effects on substrate hydroxylation. In support of this view, betamethasone had no effect on NADPH–cytochrome *c* reductase or NADPH–cytochrome P-450 reductase activity *in vitro* or on the K_m for NADPH which would be expected to decrease if betamethasone increased the affinity of NADPH for the reductase (site 3 in Fig. 3).

However selective effects could be explained by a change in the interaction between cytochrome P-450 reductase and a specific cytochrome P-450 (site 4 in Fig. 3). This step is thought to be rate-limiting for many monooxygenase reactions [26, 27] and therefore may be involved in regulation of enzyme activity. Addition of purified cytochrome P-450 reductase to solubilized microsomes caused a non-selective stimulation of biphenyl hydroxylation. If activation by betamethasone was mediated via this step, addition of excess reductase might be expected to result in a more selective stimulation of 2-hydroxylation and a loss of activation by betamethasone.

Increasing the concentration of reductase by 5–10-fold did not greatly affect the activation by betamethasone although at the highest concentration of reductase used near-maximal levels of hydroxylation of biphenyl in the 3- and 4-positions were achieved. This finding indicates that addition of the first electron is not the crucial limiting factor involved in the activation of biphenyl hydroxylation by betamethasone.

Addition of the second electron to cytochrome

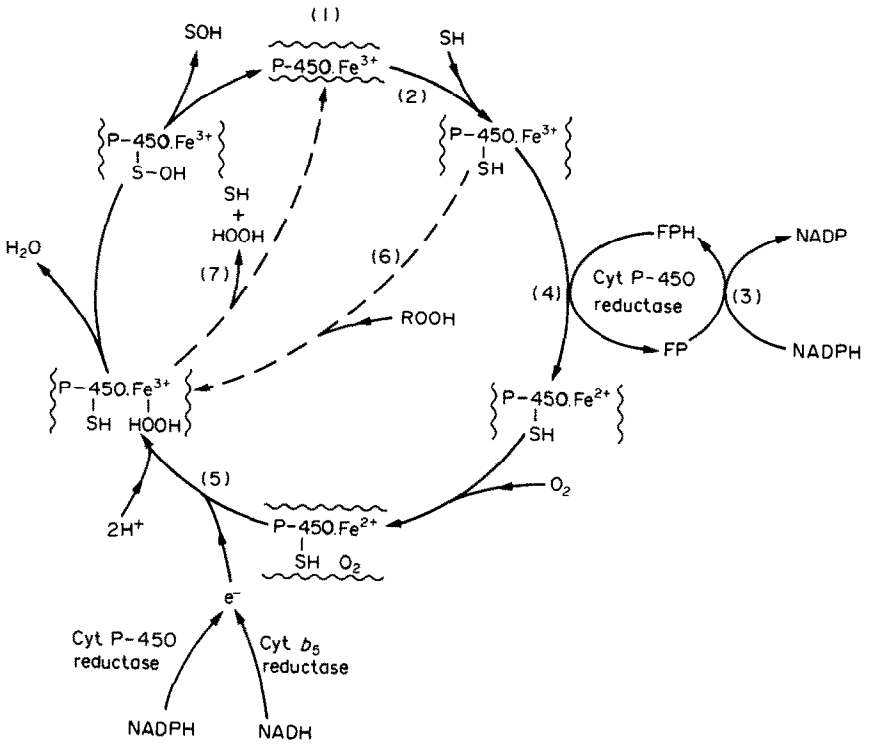

Fig. 3. Cytochrome P-450 cycle showing possible points of interaction with activating agents. (1) Membrane effects (∿∿ = membrane); (2) substrate binding (SH = substrate); (3) interaction between NADPH and cytochrome P-450 reductase (FP = flavoprotein); (4) interaction between cytochrome P-450 reductase and cytochrome P-450; (5) addition of the second electron; (6) organic peroxide (ROOH) supported hydroxylation; (7) uncoupling.

P-450 could be the rate-limiting step. To investigate the possibility of an interaction with betamethasone at this stage (site 5 in Fig. 3), NADH synergism was studied. NADH had a small synergistic effect on biphenyl 4-hydroxylation, but no effect on 2- or 3-hydroxylation in the absence of betamethasone. In the presence of betamethasone NADH did not exert a synergistic effect on 2-hydroxylation, suggesting that betamethasone may facilitate addition of the second electron to cytochrome P-450. However, cumene hydroperoxide supported metabolism, which eliminates both reductase steps in the cytochrome P-450 cycle (site 6 in Fig. 3), still permits activation by betamethasone and α-naphthoflavone to occur so transfer of the second electron cannot be the only site of interaction and a subsequent stage must also be involved.

The activation of cumene hydroperoxide supported biphenyl 2-hydroxylation occurred only at low concentrations of betamethasone and α-naphthoflavone, whereas at higher concentrations activation was decreased and α-naphthoflavone caused marked inhibition. These results are indicative of a multiple enzyme system, as suggested previously [16, 17]. Activation could be mediated via an increase in uncoupling of an active oxygen–cytochrome P-450 complex (site 7 in Fig. 3) resulting in hydrogen peroxide formation leading to lipid peroxidation (e.g. of arachidonic acid) to organic peroxides [28].

As cumene hydroperoxide supported hydroxylation favoured formation of 2-hydroxybiphenyl, this could possibly result in an apparent stimulation of 2-hydroxylation. Flavones have been shown to increase hydrogen peroxide formation in liver microsomes, especially in those mammalian species which are highly susceptible to activation of benzo[*a*]pyrene hydroxylation by flavones, suggesting that enhanced hydrogen peroxide formation may be involved in the activation [28]. However, since cumene hydroperoxide supported metabolism favours production of the quinones, rather than the phenols of benzo[*a*]pyrene [29], this is unlikely to explain the selective increase in 3-hydroxylation seen with betamethasone [15].

In this study, betamethasone had no effect on hydrogen peroxide formation in NADPH-fortified rat liver microsomes, either in the presence or absence of biphenyl. If lipid peroxidation was involved in biphenyl 2-hydroxylation activation, the marked and reproducible concentration-related activation seen with betamethasone [14] would not be expected. The very strict structure–activity relationship of the glucocorticoids [14] for activation is also strong evidence against a general effect of betamethasone, such as stimulation of lipid peroxidation. For example, betamethasone is the most effective activating agent, whereas its stereoisomer dexamethasone, which might be expected to undergo an identical metabolic fate, is only a poor activator

[14]. A high structural specificity for an activator is more consistent with a direct interaction with the cytochrome P-450 apoprotein as, for example, with an allosteric effector.

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