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INTERACTION OF AROMATIC HYDROCARBONS AND DRUGS WITH ADRENAL MICROSOMAL CYTOCHROME P-450 IN THE GUINEA PIG

J. W. GREINER, R. E. KRAMER, D. A. ROBINSON, W. J. CANADY and H. D. COLBY

Departments of Physiology and Biophysics and Biochemistry, West Virginia University Medical Center, Morgantown, West Virginia 26506, U.S.A.

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Abstract—Addition of simple aromatic hydrocarbons (benzene, ethylbenzene, naphthalene) to guinea pig adrenal microsomes produced typical Type I difference spectra ($\Delta OD_{385-420}$). Spectral dissociation constants (K_s) for each indicated a far higher affinity for adrenal than hepatic cytochrome P-450. Hydrocarbon affinities for adrenal cytochrome P-450 were similar to that for progesterone, an endogenous steroid substrate. Ethylmorphine and aniline produced Type I and Type II spectral changes respectively in adrenal microsomes. The K_s and magnitude of spectrum for each in adrenals was similar to that in livers. Nonetheless, demethylation of ethylmorphine proceeded far more rapidly in adrenal than hepatic tissue. The Michaelis constants (K_m) for ethylmorphine metabolism in both tissues were similar. Although the aniline-induced difference spectra in adrenal and hepatic microsomes did not differ substantially, aniline hydroxylase activity was far greater in liver. Pretreatment of guinea pigs with phenobarbital or 3-methylcholanthrene increased hepatic but not adrenal ethylmorphine metabolism. Spironolactone pretreatment, in contrast, did not affect hepatic metabolism, but significantly lowered adrenal demethylase activity. The results indicate a relative non-specificity of guinea pig adrenal microsomal cytochrome P-450 and suggest that the adrenal cortex may represent a significant site for the extra-hepatic metabolism of foreign compounds in the guinea pig.

In recent years considerable attention has been focused upon the metabolism of foreign compounds by extra-hepatic tissues. Varying amounts of drug- or hydrocarbon-metabolizing activity have been demonstrated in lung, intestine, kidney, skin, spleen, placenta, testis and adrenals [1–11]. Generally, enzyme activity in liver far exceeds that in other tissues. Cytochrome P-450-dependent enzyme activity in steroidogenic organs in particular, has been found to be highly specific for steroid substrates. Wattenberg and Leong [1, 2] noted that benzpyrene (BP) was hydroxylated by rat adrenal glands, but in relatively limited quantities. Similarly, Kupfer and Orrenius [12] demonstrated demethylase activity in guinea pig adrenal microsomes but far less than in liver.

In contrast to the results obtained in lower species, adrenal metabolism of foreign compounds in the primate fetus has been shown to equal or exceed hepatic oxidation [13–17]. The specific activities of aryl hydrocarbon hydroxylase, aromatic nitro reduction, azo linkage reduction and aniline hydroxylation were all found to be greater in human fetal adrenals than livers. Since comparable adrenal activity has yet to be demonstrated in other species, no suitable animal model is readily available to study adrenal metabolism of xenobiotics. However, we have recently noted maturational changes in adrenal microsomes in guinea pigs, resulting in increasing rates of hydroxylation of endogenous steroid substrates with increasing age (unpublished observations). Studies have now been carried out to examine the interactions of various exogenous compounds with adrenal cytochrome P-450 in mature guinea pigs. The results indicate a relative non-specificity of adrenal microsomal mixed function oxidases in the adult guinea pig, particularly in relation to substrate-induced spectral changes.

METHODS

Adult (650–800 g) male guinea pigs of the English Smooth Hair variety were obtained from Hilltop Farms, Scottdale, PA. Animals were maintained under standardized conditions of light (6 a.m.–6 p.m.) and temperature 22 ± 1° on a diet of Purina Laboratory Chow and water *ad lib*. Sodium phenobarbital (75 mg/kg/day for 7 days), 3-methylcholanthrene (20 mg/kg/day for 4 days) and spironolactone (100 mg/kg/day for 3 days) were administered as i.p. injections in saline, cotton seed oil and propylene glycol respectively. In all experiments controls received the appropriate vehicle only.

Animals were sacrificed by decapitation between 9:00 and 10:00 a.m. Adrenals and livers were quickly removed and placed in cold 1.15% KCl. Tissues were homogenized in 1.15% KCl containing 0.05 M Tris–HCl, pH 7.4. Homogenates were centrifuged at 9000 *g* for 20 min in a Sorvall Refrigerated Centrifuge. Aliquots of the supernatants were taken for enzyme assays and the remainder centrifuged at 105,000 *g* for 60 min in a Beckman preparative centrifuge. Microsomal pellets were resuspended in buffered KCl (pH 7.4) at a concentration of 2–3 mg protein/ml. Substrate-induced spectral changes in hepatic and adrenal microsomes were obtained using a Cary 17 recording spectrophotometer at room temperature. Adrenal microsomes were essentially free of mitochondrial contamination as indicated by an absence of significant 11β-hydroxylase activity and of any 11-deoxycortisol-induced spectral change. Spectral dissociation constants (K_s) were calculated by the method of Schenkman *et al.* [18]. Cytochrome P-450 was measured as described by Omura and Sato [19], using a molar extinction coefficient of 91 mM^{-1} cm^{-1} and microsomal protein was determined by the method of Lowry *et al.* [20].

Table 1. Drug and hydrocarbon-induced spectral changes in guinea pig adrenal and hepatic microsomes

Compound (Type spectrum)	$\Delta OD(10^{-2})$/mg protein Liver	Adrenal	K_s(M) Liver	Adrenal
Benzene (I)	2.4 (2.1–2.8)	1.2 (1.0–1.4)	4.1×10^{-4}	1.1×10^{-6}
Ethylbenzene (I)	4.5 (4.1–4.8)	3.9 (3.5–4.3)	6.2×10^{-5}	4.0×10^{-7}
Naphthalene (I)	3.2 (2.9–3.5)	1.7 (1.4–2.4)	2.4×10^{-5}	6.1×10^{-8}
Ethylmorphine (I)	1.3 (1.0–1.6)	2.5 (2.3–2.7)	2.3×10^{-4}	1.9×10^{-4}
Aniline (II)	1.9 (1.6–2.1)	2.2 (1.8–2.6)	4.5×10^{-4}	2.4×10^{-4}
Metyrapone (II)	2.9 (2.8–3.3)	4.3 (4.1–4.7)	3.2×10^{-6}	1.0×10^{-6}
Progesterone (I)	1.1 (0.8–1.4)	10.5 (8.2–12.7)	2.7×10^{-6}	7.8×10^{-7}

Values represent means (range) of 2–4 determinations.

Reaction mixtures for enzyme assays contained either aniline (15 μmoles) or ethylmorphine (10 μmoles) and 0.5 ml 9000 *g* supernatant from liver (200 mg/ml) or adrenal (100 mg/ml), glucose-6-phosphate (9 μmoles), $MgSO_4$ (24.2 μmoles), NADP (2.08 μmoles) and Tris–HCl buffer (0.02 M) pH 7.4, in a final vol of 3.0 ml. Semicarbazide HCl (25 μmoles) served as a trapping agent for formaldehyde produced from ethylmorphine. Incubations were carried out in a Dubnoff Metabolic Incubator at 37° for 15 min under air. Formaldehyde [21] and *p*-aminophenol [22] were assayed by methods previously described. All samples were read against appropriate tissue and substrate blanks and standards. Values obtained by incubating heat inactivated tissue did not differ from unincubated controls.

RESULTS

As previously noted [23], addition of benzene, ethylbenzene or naphthalene to hepatic microsomes produced typical Type I ($\Delta OD_{385-420}$) difference spectra (Table 1). The same aromatic hydrocarbons also produced Type I spectral changes of similar magnitude in adrenal microsomes. In fact, the spectral dissociation constants (K_s) calculated for each indicated a far greater affinity for adrenal than hepatic microsomal cytochrome P-450 (Table 1). The affinities of the hydrocarbons for adrenal cytochrome P-450 as determined spectrally were similar to that for progesterone (Table 1). Binding of simple aromatic hydrocarbons to adrenal cytochrome P-450 might, as in the liver [23], be the result of hydrophobic interaction. The increasing affinity observed with increasing hydrocarbon size (Table 1) is consistent with that hypothesis.

Ethylmorphine and aniline, foreign compounds that produce classical Type I and Type II difference spectra respectively in hepatic microsomes, produced the same spectral changes in adrenal microsomes (Table 1, Figure 1). For both substances the magnitudes of the induced spectra and the spectral dissociation constants were similar in hepatic and adrenal microsomes. McIntosh and Salhanick [24] previously reported that aniline produced a Type II difference spectrum when added to bovine adrenal mitochondria, but did not inhibit 11β-hydroxylation. In addition, metyrapone, an inhibitor of hepatic oxidative metabolism [25], but in the adrenal previously found to be a relatively specific inhibitor of mitochondrial hydroxylases [26, 27], interacted with guinea pig adrenal microsomal cytochrome P-450 to produce its characteristic spectral change ($\Delta OD_{425-405}$, Table 1). Metyrapone-induced spectra were similar (K_s and ΔOD) in adrenal and hepatic microsomes. The magnitude of spectral change produced by metyrapone in adrenal microsomes was far too great to be explained by mitochondrial contamination.

Since substrate-induced spectral changes cannot necessarily be equated with oxidative metabolism,

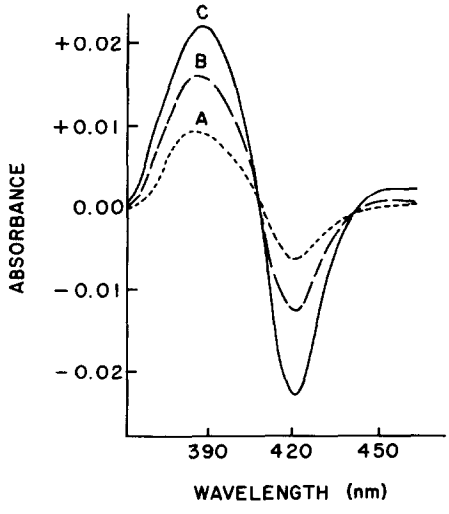

Fig. 1. Ethylmorphine-induced spectral changes in guinea pig adrenal microsomes. Ethylmorphine was added to the sample cuvette in final concentrations of 1.7×10^{-4} M (A); 3.5×10^{-4} M (B); 6.0×10^{-4} M (C). Cuvettes contained 2.8 mg protein/ml and 2.1 nmoles cytochrome P-450/mg protein. Equal volumes of water were added to the reference cuvette with each addition of ethylmorphine.

Table 2. Adrenal and hepatic metabolism of ethylmorphine and aniline in male guinea pigs

	Liver	Adrenal
Organ wt (g)	27.3 ± 0.7	0.81 ± 0.09
Microsomal protein (mg/g tissue)	26.1 ± 1.9	46.2 ± 2.2*
Cytochrome P-450 (nmoles/mg prot)	0.9 ± 0.1	2.0 ± 0.2*
Ethylmorphine metabolism (nmoles/min/g tissue)	45.2 ± 3.3	266.4 ± 30.1*
K_m	1.0×10^{-4} M	4.5×10^{-4} M
Aniline metabolism (nmoles/min/g tissue)	75.1 ± 4.0	8.2 ± 2.1*
K_m	5.3×10^{-3} M	ND

Values expressed as mean ± S.E.M.; 6–10 values/tissue. K_m values are means of duplicate determinations.
* $P < 0.05$ (vs. liver) ND—not determined.

further studies were carried out to compare drug-metabolizing activity in guinea pig adrenal and hepatic tissue (Table 2). Ethylmorphine (EM) demethylation by adrenal microsomes proceeded far more rapidly than hepatic metabolism. Hepatic EM metabolism in guinea pigs, as previously noted by several investigators [10, 28], is low when compared to other commonly studied species, but adrenal activity is comparable to that in male rat liver. The K_m (Table 2) for adrenal EM demethylation is also similar to that in hepatic tissue and closely approximates the spectral dissociation constants for EM in both adrenal and hepatic microsomes (Table 1). Both adrenal and hepatic EM metabolism were inhibited by metyrapone, with K_i's of 4.8×10^{-5} M and 6.2×10^{-6} M, respectively.

In contrast to the rapid metabolism of ethylmorphine by guinea pig adrenal microsomes, little aniline hydroxylase activity was demonstrable. Aniline metabolism by liver far exceeded that by adrenal tissue. However, the K_m for hepatic aniline metabolism was far higher than for most other substrates including ethylmorphine (Table 2). Accurate determination of the K_m for aniline hydroxylation in the adrenal was made impossible by the extremely low activity. Thus, despite producing similar spectral changes in adrenal and hepatic microsomes, aniline was not metabolized to any significant degree by adrenal tissue.

Administration of phenobarbital or 3-methylcholanthrene to guinea pigs increased hepatic but not adrenal EM metabolism (Table 3). Others [1, 7, 13] had previously noted that adrenal benzpyrene metabolism also was not inducible. Treatment with spironolactone, however, markedly reduced adrenal EM demethylation (Table 3) without significantly affecting hepatic metabolism. As reported by Menard *et al.* [29], spironolactone produced a large decline in adrenal microsomal cytochrome P-450 concentration.

DISCUSSION

Previous investigations suggested that adrenal microsomal cytochrome P-450 was relatively specific for endogenous steroid substrates. Few reports of substantial adrenal metabolism of foreign compounds have appeared. Only in the primate fetus has adrenal drug metabolizing activity previously been found to exceed that in liver [13, 14]. Even when adrenal metabolism of exogenous substances was observed, however, substrate-induced Type I spectral changes could not be demonstrated. Zachariah and Juchau [17] found that neither ethylmorphine nor aminopyrine, in concentrations up to 10^{-3} M, produced spectral changes in human fetal adrenal microsomes. Aniline and nicotinamide produced Type II spectra, but only at very high concentrations (K_s of about 10^{-2} M).

Table 3. Effects of pre-treatment with phenobarbital, 3-methylcholanthrene or spironolactone on adrenal and hepatic ethylmorphine metabolism in guinea pigs

Pre-treatment	Effects of pretreatment (% of control) Liver	Adrenal
Phenobarbital		
Cytochrome P-450	152 ± 12*	103 ± 13
Ethylmorphine metabolism	312 ± 13*	92 ± 22
3-Methylcholanthrene		
Cytochrome P-450	125 ± 15	101 ± 10
Ethylmorphine metabolism	151 ± 7*	84 ± 16
Spironolactone		
Cytochrome P-450	75 ± 18	23 ± 10*
Ethylmorphine metabolism	80 ± 19	32 ± 8*

Values expressed as per cent of control values ± S.E.M.; 4–6 animals/group. Mode of expression of parameters as in Table 2.
* $P < 0.05$ (vs. controls).

Kupfer and Orrenius [12], using younger guinea pigs than employed in the present studies, demonstrated adrenal demethylation of aminopyrine and *p*-chloro-*N*-methylaniline, but less than in liver. Aminopyrine and hexobarbital produced Type I spectral changes in hepatic microsomes but none in adrenal preparations. However, both compounds diminished the magnitude of the cortisol-induced Type I spectrum in adrenal microsomes, suggesting some interaction with adrenal cytochrome P-450. Estabrook *et al.* [30], using bovine adrenals, found that various drugs including ethylmorphine, aminopyrine and hexobarbital, upon addition to mitochondria and/or microsomes produced 'inverted Type I' spectral changes. The authors suggested that the drug-induced spectra resulted from the displacement of endogenous steroid substrates from cytochrome P-450. Moreover, both ethylmorphine and hexobarbital were found to inhibit adrenal 11β-hydroxylation, a mitochondrial cytochrome P-450 catalyzed reaction.

The present studies provide further evidence for the non-specificity of adrenal microsomal mixed function oxidases, at least in the guinea pig. Simple aromatic hydrocarbons (benzene, ethylbenzene, naphthalene) interacted with adrenal microsomes, producing typical Type I spectra. Spectral dissociation constants (K_s) indicated a far greater hydrocarbon affinity for adrenal than hepatic cytochrome P-450. In fact, the apparent affinities in adrenal microsomes were similar to that for progesterone, an endogenous steroid substrate. The significance of hydrocarbon interactions with adrenal microsomes is presently unclear. Simple aromatics can serve as substrates for hepatic mixed function oxidases [31–33]. Although various investigators have reported benzpyrene metabolism by adrenal microsomes [1, 2, 7, 9], oxidation of simple aromatic hydrocarbons by adrenals has never been demonstrated. Studies are now in progress to consider the possibility. Adrenal metabolism of hydrocarbons may be of particular importance if, as in the liver, toxic metabolites are produced, resulting in local or peripheral tissue damage. Even if little adrenal oxidation of hydrocarbons occurs, however, the high affinity of aromatics for adrenal microsomal cytochrome P-450 could produce inhibition of normal corticosteroidogenesis.

Ethylmorphine and aniline also produced spectral changes in guinea pig adrenal microsomes. Spectral dissociation constants and the magnitude of the spectra for both compounds were similar in hepatic and adrenal preparations. Nonetheless, catalytic activity differed significantly in adrenals and liver. Ethylmorphine was metabolized far more rapidly by adrenal tissue whereas the rate of aniline hydroxylation was greater in liver. These observations demonstrate a marked divergence of spectral and catalytic activities and illustrate the danger inherent in assuming the two to be identical. The ratio of adrenal to hepatic aminopyrine demethylation noted by Kupfer and Orrenius [12] and confirmed by us (unpublished observations) was intermediate between that for aniline and ethylmorphine metabolism (Table 2). Thus, relative adrenal activity varies with the substrate employed, making further studies necessary to determine the catalytic specificity of adrenal mixed-function oxidases. Such studies may also serve to identify those foreign compounds most likely to interfere with normal adrenal steroid hormone synthesis.

The apparent broad specificity of adrenal cytochrome P-450 in the guinea pig suggests that the adrenal cortex may represent a significant site of extra-hepatic metabolism in that species. In addition, the guinea pig adrenal gland may provide a suitable model for the study of extra-hepatic drug metabolism in a steroidogenic organ. Little is presently known about the relationship between exogenous and endogenous substrate interactions with cytochrome P-450 in steroidogenic tissues. In hepatic microsomes, steroids and drugs have been shown to act as competitive substrates [34]. If the relationship in the adrenal is similar, exposure to foreign compounds like simple aromatic hydrocarbons, for example, could affect adrenal steroidogenesis. Estabrook *et al.* [30] have demonstrated inhibition by drugs of mitochondrial but not microsomal steroid hydroxylation in bovine adrenals. Further insight into the relationship between exogenous and endogenous substrate metabolism by adrenal microsomes may be obtained by examining the factors affecting each. The present results indicate that spironolactone decreases adrenal but not hepatic EM metabolism. Previous studies [29] demonstrated a similar decline in adrenal 17-hydroxylase activity after spironolactone pre-treatment. Of particular interest for further study are the effects of various hormones, particularly ACTH, which contribute to the regulation of adrenal steroid secretion, on the interaction of steroids and foreign compounds with adrenal and hepatic cytochrome P-450. Such comparative studies may contribute valuable information concerning the substrate specificities and catalytic components of various cytochrome P-450-containing mixed function oxidases.

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