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THE EFFECT OF LIPID COMPOSITION ON THE METABOLISM OF LIGNOCAINE BY A RECONSTITUTED MIXED FUNCTION OXIDASE SYSTEM FROM RAT LIVER

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Abstract—Hepatic microsomal preparations from male and female rats were delipidated by column chromatography following cholate solubilisation. The enzyme activities were reconstituted using known lipids and the vesicle reconstitution method. Enzyme activity was assayed using lignocaine as the substrate for the mixed function oxidase. This substrate gives two products; one which is predominantly found in the male (*N*-deethylated derivative) and one that is found in approximately equal amounts in both sexes (3-hydroxylated product). Reconstitution of male- but not female-derived enzyme in mixed dilauroylphosphatidylcholine (DLPC)/dilauroylphosphatidylethanolamine (DLPE) vesicles gave a higher *N*-deethylase activity than if the same enzyme was reconstituted in DLPC vesicles. 3-Hydroxylase activity, which is not sex-dependent, was not affected by DLPE. Microsomal lipids from male animals were more efficient than female-derived lipids in reconstituting *N*-deethylase activity from both male- and female-derived enzymes. Microsomal lipids were more efficient than DLPC in reconstituting *N*-deethylase activity from both male- and female-derived enzymes but 3-hydroxylase activity was similar in the two lipids. There is, thus, a sex- and pathway-dependent effect of the lipids: the male-specific *N*-deethylase pathway is more affected by lipid composition and then more so in the male-derived enzyme. It is possible, therefore, that some of the sex differences in drug metabolism may be related to changes in lipid composition.

The early work on the mixed function oxidase system in liver indicated that there were three essential components, i.e. cytochrome P-450, NADPH-cytochrome P-450 reductase and a heat-stable factor [1]. This factor was subsequently found to be phospholipid and phosphatidylcholine was found to be the most effective in reconstituting enzyme activity [2]. This fact holds true for the metabolism of fatty acids, drugs, steroids and carcinogens [1, 3]. Work with phospholipase C also indicates the essential role of phospholipids in microsomal oxidation [4, 5]. The requirement for lipid has been further refined when dilauroylphosphatidylcholine (DLPC) was found to stimulate drug metabolism in a reconstituted system [6] although Parke [7] suggested that this was an oversimplification of the endoplasmic reticulum and misrepresented the physiological situation as DLPC does not exist *in vivo*. Taniguchi *et al.* [8] have suggested that protein insertion and interaction is affected by this artificial phospholipid as compared to the natural lipids.

The idea is still prevalent, however, that the microsomal phospholipids are simply structural components to hold the enzyme in place. This view is challenged by findings of correlations between changes in lipid composition and drug metabolism. Decreased drug metabolism is seen in pregnancy in the rat [9] and this correlates with altered phospholipid content [10]. Indeed, Turcan *et al.* [11] have shown that the decreased drug metabolism in pregnancy is *not* related to decreased cytochrome P-450 but rather to changes in lipid content of the microsomes. Similarly sex differences in drug metabolism have been shown to correlate to sex-dependent lipid differences [12]. Early work in this laboratory has shown that all three essential components of the mixed function oxidase system are required for the full expression of sex-dependent drug and steroid metabolism [13]. This work showed a very complex interaction of proteins and lipids and proved difficult to interpret due to the three possible variables. An experimental design involving fewer variables was, therefore, needed to ascertain the exact role of the lipid and reductase independently.

Although correlations of drug metabolism with phospholipid content have been seen, it is certainly the case that many effects on drug and steroid metabolism are related to changes in the protein components and, in particular, cytochrome P-450. For instance, sex-dependent cytochrome P-450 species have been isolated and shown to metabolise drugs and steroids in a manner which would explain the sexual dimorphism of the metabolism of particular substrates [14–16]. Preliminary work, however, has indicated that the lipid environment may affect the activity of these sex-dependent enzymes and lead to a disappearance of their sex differences [17].

In order to define more closely the role of the phospholipids, it was necessary to design a study in which the other components were kept constant. The preferential removal of the microsomal lipid (thus leaving the cytochrome P-450 and reductase unaltered) and subsequent reconstitution with known lipids is one such system and has been employed in this study. The substrate, lignocaine, was chosen as it has a sex-dependent (*N*-deethylation) and a sex-independent (3-hydroxylation) pathway of metabolism [18].

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MATERIALS AND METHODS

NADPH, dilauroylphosphatidylcholine, dilauroylphosphatidylethanolamine and cytochrome *c* were obtained from the Sigma Chemical Co. Ltd. (Dorset, U.K.). Bio-Gel P-30 was supplied by Bio-Rad Ltd. (Watford, U.K.). All other reagents were of the highest purity available commercially.

All rats used throughout the study were mature Wistar stock bred in the department (males 300–350 g and females 225–250 g body weight). The animals were kept in a controlled environment (temperature 19 ± 1°; lights on 0700–1900) and allowed free access to food (CRM Nuts, Labsure, Croydon, U.K.) and tap water.

The animals were killed by CO_2 asphyxiation followed by cervical dislocation and the livers quickly excised and rinsed in ice-cold 0.25 M sucrose to remove excess blood and other foreign material. The liver was finely chopped and homogenised in 4 volumes of 0.25 M sucrose using a Potter-Elvehjem homogeniser with loose-fitting Teflon pestle. The microsomal fraction of the liver was prepared by ultracentrifugation as described by Berg and Gustafsson [19]. The microsomal pellet was resuspended in a solution containing 1.15% (w/v) potassium chloride and 10 mM EDTA at pH 7.4 and recentrifuged at 105,000 *g* for 60 min at 4° in a Beckman L8-55M ultracentrifuge. This removes haemoglobin as described by Mihara and Sato [20]. The subsequent pellet was resuspended in buffer A (containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol in 0.1 M potassium phosphate buffer at pH 7.25) at a protein concentration of approx. 10 mg/ml. This was used as the microsomal preparation in subsequent experiments.

The microsomal membrane proteins were solubilised by the careful addition of 10% (w/v) sodium cholate solution in a dropwise manner to the cleaned microsomal suspension while stirring the mixture on ice and under a constant flow of oxygen-free nitrogen. The sodium cholate was added to a concentration of 1.8% (w/v) and the mixture was left for a further 30 min. The solubilised preparation was centrifuged at 105,000 *g* for 60 min at 4° to remove unsolubilised material. The supernatant is referred to as the solubilised preparation. If required, the solubilised preparation was concentrated by precipitating the proteins by the addition of ammonium sulphate to 80% saturation and redissolving the pellet in buffer A containing 1.8% sodium cholate.

The solubilised preparation was delipidated by applying 1 ml of sample to a Bio-Gel P-30 column (1.5 × 50 cm) and eluting with buffer A containing 0.7% sodium cholate at a flow rate of 0.1 ml/min. One millilitre fractions were collected and assayed for microsomal proteins and lipid phosphorus as described below. Proteins pass out of the column in the void volume and lipids are retained. Fractions containing the proteins but no lipid were combined and used in the reconstitution experiments described below.

Microsomal lipids were extracted from the haemoglobin-free microsomes by the organic solvent extraction method of Overturf and Dryer [21] and stored in 1 mg portions at −20° under nitrogen.

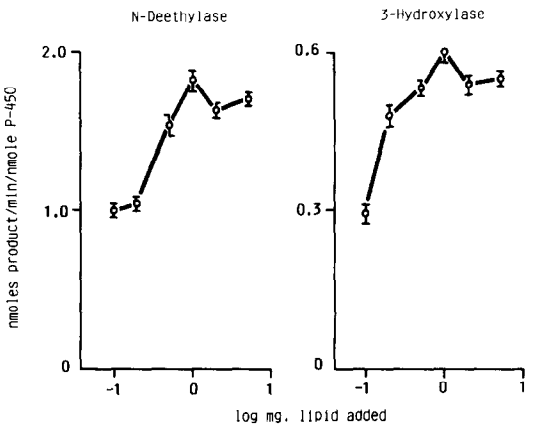

Fig. 1. The effect of differing lipid:protein ratios on the metabolism of lignocaine in a reconstituted lipid vesicle system using microsomal lipids and enzymes derived from a male rat. 0.1 nmole of cytochrome P-450 was used in each reconstitution.

The delipidated microsomal proteins were reconstituted into lipid vesicles of known composition by the method of Ingelman-Sundberg and Glaumann [22] using either a single phospholipid (dilauroylphosphatidylcholine (DLPC)) a mixture of pure phospholipids (DLPC and dilauroylphosphatidylethanolamine (DLPE)) or the mixture of lipids extracted from the microsomes as described above. Differing protein:lipid ratios (from 1 to 50 mg lipid per nmole cytochrome P-450) were tested to ascertain the optimal amount of lipid to be used in subsequent experiments.

The drug-metabolizing activity of the reconstituted enzymes was assayed using lignocaine as substrate in a modification of the method of Skett *et al.* [18]. Reconstituted enzyme or microsomal preparation (containing approx. 0.1 nmole cytochrome P-450) was incubated with ^{14}C-labelled substrate in the presence of excess NADPH and the metabolites and unchanged substrate extracted and assayed according to Skett *et al.* [18]. Results were expressed as nmoles product formed/min/nmole cytochrome P-450.

The mixed function oxidase components were assayed by the methods of Omura and Sato [23] (cytochrome P-450), Philips and Langdon [24] (NADPH-cytochrome P-450 reductase) and Rouser *et al.* [25] (phospholipid phosphorus). Total protein content was assayed by the method of Lowry *et al.* [26] using bovine serum albumin as standard.

Enzyme activities were calculated from the radioactivity present in the metabolites using a custom-made computer program and expressed as nmoles product formed/min/nmole cytochrome P-450 and as mean ± SD of at least six determinations of the same reconstituted preparation. Statistical significance was tested using Student's *t*-test for unpaired samples and the level of significance set at $P < 0.05$.

RESULTS

Solubilisation and delipidation of the microsomal enzymes did not lead to the preferential loss of any

Table 1. The effect of reconstitution with diauroylphosphatidylcholine (PC) and dilauroylphosphatidylethanolamine (PE)/PC mixture (1/3, w/w) on metabolism of lignocaine by enzymes derived from male and female rat liver

Enzyme derived from	Lipid	*N*-Deethylase	3-Hydroxylase
Male	PC	1.48 ± 0.17	0.48 ± 0.01
Male	PC/PE	1.72 ± 0.02*	0.47 ± 0.03
Female	PC	0.98 ± 0.09	0.53 ± 0.05
Female	PC/PE	0.91 ± 0.10	0.46 ± 0.07
Microsomes			
Male		1.93 ± 0.47	0.49 ± 0.13
Female		0.97 ± 0.21†	0.59 ± 0.08

Results expressed as nmoles product formed/min/nmole cytochrome P-450 and as mean ± SD of at least six values.

* $P < 0.01$ as compared to PC value; † $P < 0.05$ as compared to male value; PC = dilauroylphosphatidylcholine; PE = dilauroylphosphatidylethanolamine.

of the components measured except for the lipid which was reduced by over 80% in all experiments (i.e. up to 20%, but more typically 3–5% of native lipid remained). Cytochrome P-450:reductase ratios were typically 0.8 (calculated as nmoles of cytochrome P-450/unit of reductase) in microsomes and 0.75 in final vesicle preparation (a unit of reductase is defined as the amount of enzyme which reduced 1 μmole of cytochrome *c* per minute at 20°). As is seen in Fig. 1, the addition of 0.1 nmole of cytochrome P-450 to 1 mg of lipid (in this case microsomal lipids obtained from the male rat) gave the maximum enzyme activity, indicating that this is the optimal amount of lipid to use for the reconstitution. This amount of lipid is more than was removed by the delipidation procedure. A similar result was seen for all lipids used. The above ratio of protein to lipid was therefore used for the rest of the study.

Following the delipidation and reconstitution procedures outlined above and analysing the protein and lipid contents of the various fractions, then it can be calculated that the lipid added for optimal reconstitution (which is in excess) accounts for more than 97% of the lipid in the vesicles. The NADPH-cytochrome P-450-reductase activity was similar in comparison groups but varied somewhat between experiments.

Table 1 shows the effect of incorporating the delipidated enzymes into DLPC and DLPC/DLPE (3/1) vesicles. It is seen that DLPE had no effect on the enzymes isolated from female liver but significantly increased the *N*-deethylating activity of the enzymes isolated from the male animal without having any effect on the 3-hydroxylating activity. It is also noted that *N*-deethylating activity was higher in the male-derived vesicles than the female-derived vesicles whereas the 3-hydroxylating activity was similar. This should be compared to the sex differences in metabolism noted for the microsomal preparation (given at the foot of Table 1). This sex difference in the activity of the vesicles was maintained throughout the study.

Table 2 shows the effects of preparing vesicles from microsomal lipids derived from male and female livers. It is seen that the enzymes derived from the male showed a lower *N*-deethylating activity in female-derived lipids while the 3-hydroxylating activity of the same preparation was not significantly affected. The enzymes derived from the female also showed a decreased *N*-deethylating activity in female-derived lipids.

Table 3 shows the effects of incorporating enzymes derived from male and female animals into vesicles prepared from DLPC or male-derived microsomal lipids. It is seen that the *N*-deethylating activity of the enzymes is higher in microsomal lipids than in DLPC but that 3-hydroxylating activity is unaffected by the nature of the lipid. This is equally the case for enzymes derived from male and female animals.

DISCUSSION

The methods employed in this study allowed the study, in isolation of the role of lipids in the control of drug metabolism.

It is seen (Table 1) that there is both a sex- and pathway-dependent effect of DLPE when microsomal enzymes are incorporated into a membrane containing DLPC and DLPE in the proportions found in the microsomes (3:1 (w/w) DLPC:DLPE) [27]. An effect of incorporation of phosphatidylethanolamine (PE) is well documented: Ingelman-Sundberg and Glaumann [28], working with the rabbit cytochrome P-450, LM_2, showed that PE was better than phosphatidylcholine in maintaining 7-ethoxycoumarin *O*-dealkylation. These authors suggested that this effect was due to a charge difference between the different lipids [29]. It is interesting to note that the only effect seen of DLPE was in the male-derived system with the male-specific enzyme (the *N*-deethylase) [18]. This indicates that the male-specific enzymes are more susceptible to alteration by lipid composition. This could be due to a sex-specific enzyme in the male being dependent on membrane charge or membrane fluidity, both of which are altered by the incorporation of DLPE [2, 30]. The existence of a sex-specific cytochrome P-450 species in the male has been reported [16, 31]. Another explanation is an interaction with other proteins contained in the protein fraction of the microsomes. The most likely candidate is cytochrome b_5 which is present in the incubations and can influence metabolism catalysed by cytochrome P-450 [28]. Further work involving the isolation of

Table 2. The effect of reconstitution with microsomal lipids from male and female rat liver on the metabolism of lignocaine by enzymes derived from male and female rat liver

Enzyme derived from	Lipid derived from	*N*-Deethylase	3-Hydroxylase
Male	Male	1.89 ± 0.03	0.48 ± 0.04
Male	Female	0.57 ± 0.08‡	0.43 ± 0.08
Female	Male	1.07 ± 0.03	0.54 ± 0.09
Female	Female	0.89 ± 0.08*	0.46 ± 0.04

Results expressed as nmoles product formed/min/nmole cytochrome P-450 and as mean ± SD of at least six values.
* $P < 0.01$, ‡ $P < 0.001$.

Table 3. The effect of reconstitution with DLPC and microsomal lipids derived from male rat liver on the metabolism of lignocaine by enzymes derived from male and female rat liver

Enzyme derived from	Lipid	*N*-Deethylase	3-Hydroxylase
Male	PC	1.45 ± 0.04	0.48 ± 0.03
Male	ML	2.31 ± 0.14‡	0.50 ± 0.04
Female	PC	0.90 ± 0.09	0.44 ± 0.06
Female	ML	1.68 ± 0.08‡	0.47 ± 0.02

Results expressed as nmoles product formed/min/nmole cytochrome P-450 and as mean ± SD of at least six values.
‡ $P < 0.001$; PC = dilauroylphosphatidylcholine; ML = microsomal lipid.

the cytochrome P-450 species responsible for the *N*-deethylation in the male is in progress to clarify these points.

The decreased *N*-deethylase activity of male-derived enzymes in female-derived lipids (Table 2) is similar to the effect seen by Barr and Skett [13] using partially purified cytochrome P-450 and reductase preparations. This latter preparation gives lower activities indicating that reconstitution of isolated enzymes is not an optimal system to investigate. As in the previous experiment only the sex-dependent (male-specific) enzyme is affected. Using the female-derived enzymes, *N*-deethylase activity is also seen to be decreased by the use of female-derived lipids. It is noted that the sex difference seen for the *N*-deethylase in the microsomal preparation (Table 1) is only fully expressed if the correct lipid preparation is used (i.e. male enzymes and male lipid mixture compared to all female reconstitution).

Microsomal lipid was more effective than DLPC in reconstituting *N*-deethylase activity (Table 3). This agrees with earlier work with partially purified enzymes [17] and with previous findings with rabbit cytochrome P-450 LM_2 [30]. The microsomal lipids, as prepared, contain a complex mixture of phospholipids as well as trigylcerides and cholesterol. Any of these components could account for the differences seen. Indeed, cholesterol has been reported to affect the metabolism of drugs when incorporated into DLPC vesicles [32]. Sex differences also exist in the proportions and fatty acid composition of the hepatic microsomal phospholipids [12] and these differences could lead to the effects seen. Further work is in progress to separate and analyse the microsomal lipids used in these experiments and use the separated fractions to reconstitute the enzyme activities. This should enable us to ascertain the role of sex dependent lipids in the modulation of microsomal enzyme activity.

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