The role of microsomal phospholipids and their fatty acid composition in the control of hepatic metabolism of lignocaine

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1 Sex differences exist in the metabolism of lignocaine by the rat liver. Microsomal phospholipids have been implicated in the control of these sex differences. Induction of diabetes in the male rat abolishes these sex differences. The difference in drug metabolism between the male and female rat is, thus, the same as that between the control and diabetic male rat.

2 By using reconstitution of delipidated male microsomal proteins with male-, female- and diabetic-derived phospholipids as well as synthetic phospholipids, it should be possible to delineate the role of phospholipids in the control of drug metabolism.

3 Female- and diabetes-derived phospholipids decrease the activity of the male-specific lignocaine N-deethylase specifically by between 35 and 52%.

4 Analysis of the phospholipid classes and fatty acid content of the various fractions indicated that stearic acid content was increased and arachidonic acid content decreased in both female- and diabetic-derived lipids as compared to control males. Linoleic acid content was decreased in female- but increased in diabetic-derived lipids as compared to control males. Subsequent correlation to N-deethylase activity, however, rules out all but the arachidonic acid content of the phospholipids as a controlling factor of lignocaine metabolism.

5 Use of synthetic phosphatidylcholine (PC) species for reconstitution indicates that diarachidonyl-PC is the most efficient at activating the N-deethylase and indicates that the degree of unsaturation of the fatty acyl side-chains of PC is of major importance in the regulation of this enzyme activity.

6 The presence of unsaturated fatty acids, and especially arachidonic acid, in the phospholipids is, thus, a major controlling influence on the specific activation of lignocaine N-deethylase in the rat liver.

Introduction

There are three essential components of the cytochrome P-450-dependent hepatic mixed function oxidase enzyme system; cytochrome P-450, NADPH-cytochrome P-450 reductase and phospholipid (Lu & Coon, 1968) and of these, the amount and type of cytochrome P-450 has long been considered the major controlling influence.

Indeed, if sex differences in drug metabolism in the rat are examined, it is seen that there are sex-dependent isozymes of cytochrome P-450 which could explain most of the sexual differentiation seen. For instance, a female-specific cytochrome P-450 (isozyme 15 β -hydroxylase, MacGoeck *et al.*, 1984),

P-450-female (Kato & Kamataki, 1982) or UT-I/2d (Waxman, 1984; now designated cytochrome P-450IIC12) has been purified as has a male-specific isoform (variously termed cytochrome P-450 2c/UT-A or RLM5 (Waxman, 1984; Morgan *et al.*, 1985); now designated cytochrome P-450IIC11).

However, Barr & Skett (1984) provided evidence that, for at least one substrate, sex-dependent cytochrome P-450 was not the whole answer. In this study it was seen that the activity of the male-specific lignocaine N-deethylase could be controlled by the microsomal lipid. This finding was confirmed and extended by Meftah (Meftah & Skett, 1987a,b; Meftah, 1988) to show that the isoenzyme of cytochrome P-450 performing the N-deethylation was

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the male-specific isoform previously isolated (2c above) and that this isoenzyme specifically interacted with microsomal phospholipid to modify the activity of the enzyme.

The sex-dependent enzyme activities in the male rat are also susceptible to modulation by the diabetic state as previously described (Skett *et al.*, 1984; Skett & Joels, 1985; Skett, 1986), with the male-specific enzyme activities moving towards the female level (a feminisation). The N-deethylation of lignocaine was significantly and markedly reduced in the diabetic state without any effect on the non-sex-dependent 3-hydroxylation of the same substrate (Skett & Joels, 1985).

It was, thus, of interest to examine how the phospholipid fraction of the hepatic endoplasmic reticulum could modulate the male-specific N-deethylation of lignocaine by comparing the effects of female- and diabetic-derived phospholipids and the analysis of the type of phospholipid present and its fatty acid content.

Methods

Experimental animals

Mature rats of Wistar stock, bred in the Department, were used throughout the study. Males weighed 300–350 g and females 225–250 g at the start of the experiment. The animals were kept in a light- and temperature-controlled environment (lights on 07 h 00 min–19 h 00 min; temperature $19 \pm 1^\circ\text{C}$) and were allowed free access to food (CRM Nuts, Labsure, Croydon, U.K.) and tap water. A single intravenous dose of streptozotocin (90 mg kg^{-1} in distilled water) was used to induce diabetes mellitus as described previously (Skett, 1986).

Preparation of the delipidated microsomal protein

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. The microsomal fraction of the tissue was prepared by the ultracentrifugation method of Berg & Gustafsson (1973) and haemoglobin was removed from this fraction as described by Mihara & Sato (1978). The cleaned microsomal preparation was subsequently dissolved by the addition of sodium cholate and delipidated by elution from a Bio-Gel P-30 column as described previously (Meftah & Skett, 1987a). The protein fraction from this column is the delipidated microsomal protein and has been shown to contain no measurable phospholipid (Meftah & Skett, 1987a).

Reconstitution of an active enzyme system

The delipidated proteins were reconstituted with lipids of known composition by the method of Ingelman-Sundberg & Glaumann (1980) using extracted microsomal lipids, prepared as below, or synthetic phospholipids. The optimum ratio of lipid to protein, previously tested (Meftah & Skett, 1987a), was used in all cases. Assay of drug-metabolizing capacity was assessed by use of lignocaine as substrate, by a modification of the method of Skett *et al.* (1980), as described by Meftah & Skett (1987a). The cytochrome P-450 content of the reconstituted preparation was assayed by the method of Omura & Sato (1964) and the results are expressed as nmol product formed $\text{min}^{-1}\text{ nmol}^{-1}$ cytochrome P-450.

Separation and assays of phospholipids

Microsomal lipids were extracted from the cleaned microsomal fraction by the organic solvent extraction method of Overturf & Dryer (1969) and stored at -20°C in the dark under a nitrogen atmosphere until used. The phospholipid fraction was prepared by silica gel column chromatography as described by Rouser *et al.* (1970) and the phospholipids further separated into their respective groups (based on head group analysis) by two-dimensional thin-layer chromatography on silica gel as previously described (Yavin & Zutra, 1977). This method can separate distinctly phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol from each other and from phosphatidic acid. The amount of phospholipid in each spot was assayed by determining the phosphorus content by the method of Rouser *et al.* (1970).

Preparation and assay of fatty acid content of microsomal phospholipids

The fatty acid composition of the microsomal phospholipids was assayed by purifying the phospholipid fraction as outlined above and converting the mixture to fatty acid methyl esters as described by Kates (1964). This method involved refluxing the lipids in the presence of methanolic sodium hydroxide solution and subsequent extraction of the methyl esters formed. Fatty acid methyl esters were stored at -20°C in the dark under a nitrogen atmosphere until assayed. The fatty acid methyl esters were analysed by capillary gas chromatography on a 1% OV-1 column (film thickness 250 nm, length 12 m) in a Perkin-Elmer 8420 Gas Chromatograph at 190°C (isothermal) by use of an FID detector. Authentic standard fatty acid methyl esters were also run through the column for positive identification of the

peaks obtained. Quantitation of the peaks was by triangulation.

Statistics

Results are calculated as the mean $\pm$ standard deviation (s.d.) of at least six independent samples from different microsomal preparations (actual number of samples given in the tables). Statistical analysis was by the use of Student's *t* test for unpaired samples (for comparing two groups) or Duncan's multiple range test (for three or more groups). The level of significance was set at $P < 0.05$.

Chemicals

Bio-Gel P-30 was purchased from Bio-Rad Laboratories, Watford, U.K., Sephadex G-50 from Pharmacia Fine Chemicals, Milton Keynes, U.K. and [carbonyl- ^{14}C]-lignocaine from Amersham Intl. plc, Aylesbury, U.K. Unlabelled lignocaine was the kind gift of Astra Läkemedel AB, Södertälje, Sweden. NADPH, ammonium molybdate, streptozotocin, the free fatty acids and the phosphatidylcholine species were all obtained from Sigma Chemical Co. Ltd., Poole, U.K. All other reagents were of the highest purity available commercially.

Results

Effect of different microsomal phospholipids on metabolism of lignocaine

As can be seen in Table 1, reconstruction of microsomal proteins derived from the male rat liver with microsomal phospholipids derived from the same source gave a pattern of metabolism with a greater preponderance of monoethylglycylxylidide (MEGX). This is similar to the metabolism seen for the untreated microsomal preparation from the male rat

Table 1 The effect of reconstitution with lipids from different sources on the metabolism of lignocaine by male delipidated microsomal proteins

Lipid source		3-Hydroxylase	N-deethylase
Male	(A)	0.50 $\pm$ 0.03	1.86 $\pm$ 0.02
Female	(B)	0.49 $\pm$ 0.02	1.04 $\pm$ 0.02
3 d diabetic male	(C)	0.48 $\pm$ 0.02	1.20 $\pm$ 0.15
21 d diabetic male	(D)	0.50 $\pm$ 0.03	0.90 $\pm$ 0.06
		<u>C B A D</u>	<u>D B C A</u>

Results are expressed as nmol product formed $\text{min}^{-1} \text{nmol}^{-1}$ P-450 and are means $\pm$ s.d.

$n = 12$ for control animals and 6 for diabetic animals.

Data analysed by Duncan's multiple range test — letters refer to groups and those underlined are not significantly different ($P > 0.05$).

liver (Skett *et al.*, 1980). Reconstitution with female phospholipids or phospholipids derived from the diabetic male animals gave a significantly lower N-deethylase activity without any effect on the 3-hydroxylase activity. Indeed, statistical analysis of the results indicated that the phospholipids derived from the chronic (21 days) diabetic animals were poorer than the female-derived lipids in reconstituting N-deethylase activity ($P < 0.05$).

Analysis of phospholipids in microsomal fraction

As the source of the phospholipid had such a marked effect on the N-deethylation of lignocaine, it was of interest to analyse the composition of the added phospholipid fraction. Table 2 shows the results of this analysis (based on phosphorus content of the separated lipids). It is seen that there is a significant ($P < 0.05$) decrease in the total lipid content (expressed as g^{-1} liver) of all groups compared to the control male. This lowering is reflected in the decreased amount of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in both the female- and chronic diabetic-derived lipids. The lipids

Table 2 Phospholipid content of microsomal lipids derived from male, female and diabetic male rats

Phospholipid class	Male	Female ($\mu\text{mol g}^{-1}$ wet weight liver)	3 d STZ	21 d STZ
Total	11.5 $\pm$ 0.5	9.0 $\pm$ 0.6*	10.5 $\pm$ 0.3*	7.8 $\pm$ 0.5***
PC	6.5 $\pm$ 0.2	5.5 $\pm$ 0.2*	6.0 $\pm$ 0.4	4.5 $\pm$ 0.3**
PE	3.9 $\pm$ 0.2	2.5 $\pm$ 0.4**	3.5 $\pm$ 0.2	2.5 $\pm$ 0.2**
PS	0.3 $\pm$ 0.2	0.3 $\pm$ 0.2	0.3 $\pm$ 0.1	0.3 $\pm$ 0.2
PI	0.8 $\pm$ 0.1	0.7 $\pm$ 0.1	0.7 $\pm$ 0.1	0.5 $\pm$ 0.1
PC/PE	1.67	2.20	1.71	1.80

Results are expressed as mean $\pm$ s.d. of 6 independent samples for each group.

Data analysed by Student's unpaired *t* test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to male control.

PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, PI = phosphatidylinositol.

STZ = streptozotocin and denotes a diabetic animal. 3 d = 3 days after STZ; 21 d = 21 days after STZ.

Table 3 Analysis of fatty acyl side-chain content in phospholipids from male, female and diabetic male hepatic microsomes

Fatty acid	Male	Female	3 d STZ	21 d STZ
Palmitic	30 ± 3	29 ± 2	30 ± 3	30 ± 2
Palmitoleic	14 ± 2	14 ± 2	15 ± 1	11 ± 1*
Stearic	10 ± 1	25 ± 1***	10 ± 1	14 ± 1*
Oleic	10 ± 2	10 ± 1	10 ± 1	15 ± 2*
Linoleic	12 ± 1	8 ± 1**	11 ± 1	14 ± 1*
Arachidonic	12 ± 1	5 ± 1**	6 ± 1**	3 ± 1***
Others	12 ± 1	9 ± 1	18 ± 2	13 ± 1
% unsaturated	48	37	42	42

Results are expressed as mean ± s.d. of 6 independent samples.

Statistical analysis was by means of Student's *t* test for unpaired samples; * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001 compared to the control male.

For key to abbreviations used see Table 2.

derived from the acute (3 day) diabetic rats showed no significant decrease in PC or PE as compared to the intact males. There were no significant differences in phosphatidylserine (PS) or phosphatidylinositol (PI) between any of the groups. There appeared to be a graded difference from the male-derived lipids with the highest content of each phospholipid through the acute diabetic- and female-derived lipids to the lipids derived from the chronically diabetic animals, which showed the lowest content of each phospholipid class on each occasion. The PC/PE ratio was markedly higher in female-derived microsomes than any of the other groups (2.2 compared to 1.67–1.8).

Analysis of fatty acid content of phospholipid fraction

A further analysis of the total phospholipid fraction to determine its fatty acid content resulted in the data shown in Table 3. It is seen that there were some marked differences between male- and female-derived phospholipids, with the male-derived lipids exhibiting a higher proportion of polyunsaturated fatty acids (linoleic (18:2) and arachidonic acid (20:4) (*P* < 0.01) and a lower proportion of stearic acid (18:0) (*P* < 0.001)). Analysis of the fatty acid content of microsomal phospholipids derived from diabetic male rats also gave changes from the control male. The acute diabetic gave a reduced content of arachidonate (20:4) (*P* < 0.01), whereas the chronic diabetic gave a reduced content of palmitoleate (16:1) (*P* < 0.05) and an increased content of stearate (18:0), oleate (18:1) and linoleate (18:2) (all *P* < 0.01), as well as a very marked reduction in arachidonate (20:4) (*P* < 0.001). It should be noted that the differences between the male- and female-derived phospholipids were not the same as those caused by diabetes (either acute or chronic). The percentage of unsaturated fatty acids was somewhat lower in the female- than male-derived phospholipids (37 com-

pared to 48%) and the value for the diabetic state was intermediate between the two control values, and did not differ between acute and chronic diabetes (42%).

Effect of reconstitution with phosphatidylcholine species with differing fatty acyl chains on lignocaine metabolism

Delipidated microsomal proteins from male rats were reconstituted by the addition of synthetic phosphatidylcholine with known and differing fatty acyl side chains (ranging from dicaproyl (6:0) to diarachidonyl (20:4)). The results obtained are shown in Table 4. It is seen that the N-deethylation of lignocaine by these preparations was markedly affected by the species of PC used; diarachidonyl-PC (20:4) was the most effective in the reconstitution system

Table 4 The effect of reconstitution with phosphatidylcholines (PCs) of differing acyl chains on the metabolism of lignocaine by a delipidated male microsomal protein preparation

Lipid used	3-Hydroxylation	N-deethylation
DAPC (A)	0.43 ± 0.03	1.49 ± 0.06
DOPC (B)	0.43 ± 0.02	1.24 ± 0.04
DBPC (C)	0.44 ± 0.01	1.03 ± 0.03
DSPC (D)	0.43 ± 0.02	0.97 ± 0.03
DLPC (E)	0.45 ± 0.01	0.97 ± 0.03
DCPC (F)	0.35 ± 0.01	0.79 ± 0.06
	<u>A B C D E F</u>	<u>A B C D E F</u>

Results are expressed as nmol product formed min⁻¹ nmol⁻¹ P-450 and are means ± s.d. of 6 independent samples. Statistical analysis was by Duncan's multiple range test — those group letters underlined indicate that the groups were not significantly different (*P* > 0.05). DAPC = diarachidonyl-PC; DOPC = dioleoyl-PC; DBPC = dibehenoyl-PC; DSPC = distearoyl-PC; DLPC = dilauroyl-PC; DCPC = dicaproyl-PC.

whereas dilauryl- (12:0), distearyl- (18:0) and dibehenoyl-PC (22:0) all gave a lower but constant activity. Dioleoyl-PC (18:1) gave a significantly higher activity than distearyl-PC (18:0) ($P < 0.05$) and dicaproyl-PC (6:0) gave the lowest activity. There were no differences in the effects of the PC species on 3-hydroxylation, except for dicaproyl-PC which resulted in a significantly lower activity than any of the other PCs.

Discussion

Lignocaine is a clinically important drug whose metabolism has been shown to be sex differentiated in the rat liver (Nyberg *et al.*, 1977; Skett *et al.*, 1980) with the N-deethylation (thought to be catalysed by cytochrome P-450IIC11) being markedly greater in male than female rats, whereas the 3-hydroxylation (thought to be catalysed by cytochrome P-450IIA1) shows no sex difference. Although there is no reported sex difference in the metabolism of lignocaine in man, there are sex differences in the metabolism of other drugs (for review, see Skett, 1988), and any effects seen on the metabolism of lignocaine in the rat may be relevant to the metabolism of these drugs in man.

There is evidence to suggest that this sex difference may be related to the lipids in which the enzymes are embedded in the endoplasmic reticulum of the liver cell. Early work in this laboratory (Barr & Skett, 1984) indicated that male- and female-derived microsomal lipids had different effects on lignocaine metabolism (in line with the sex differences seen in metabolism). Extension of this work using delipidated and reconstituted microsomal proteins (Meftah & Skett, 1987a), showed that the sex difference in lignocaine N-deethylation could be explained by the presence of sex-specific lipids with female-derived lipids able to reduce the activity of male-derived enzymes to the level seen in the female. Such correlations between differences in drug metabolism and microsomal lipids have been postulated previously (Belina *et al.*, 1975; Turcan *et al.*, 1981).

The data in this study (Table 1) confirm the role of sex-dependent microsomal lipids in the control of lignocaine N-deethylation and also show that lipids derived from diabetic male rats can affect this enzyme. The lipids derived from the chronic (21 days) diabetic animals appeared to be more effective than that from the acute (3 day) diabetic animals. In this case, it is specifically the phospholipids which have been used, indicating that the sex-dependent effects of the microsomal lipids reside in this fraction. The diabetic state is known to decrease male-specific drug metabolism in the rat liver (Skett & Joels, 1985) and, indeed, has been shown to have a specific reductive effect on lignocaine N-deethylation in the

male rat. The lower N-deethylase activity of the male-derived microsomal proteins in the presence of diabetic-derived lipids is, therefore, not unexpected and further enhances the idea of lipid control of sex-dependent drug metabolism. It is seen, therefore, that both female- and diabetes-derived microsomal phospholipids can decrease lignocaine N-deethylase activity.

In order to correlate these effects to phospholipid changes it was necessary to analyse the different phospholipid fractions. We analysed the phospholipids in terms of head group and fatty acid content. It was noted that female-derived phospholipids contained less total phospholipid (expressed g^{-1} liver tissue) than male-derived lipids, which was reflected in a lower content of PC and phosphatidylethanolamine (PE). The PC/PE ratio was markedly higher in females than in males. This confirms the findings of Belina *et al.* (1975). This was mimicked by the chronic diabetes-derived phospholipids except for the effect on the PC/PE ratio which was unaffected by the diabetic state. The phospholipids derived from the acute diabetic animals showed a pattern of lipids intermediate between the control male and control female. The actual amount of phospholipid per g liver is unimportant to this study as the same quantity of lipid was used in each reconstitution. What may be important, however, is the relative proportions of the different phospholipids. It has previously been noted that increasing the proportion of PE can enhance some drug metabolising enzyme activities (Ingelman-Sundberg & Glaumann, 1980) and, indeed, for the substrate used in this study, we have previously shown that N-deethylase activity of delipidated male-derived proteins is increased in the presence of PE (Meftah & Skett, 1987a). This is also seen for the activity of a purified, sex-specific cytochrome P-450 isozyme (cytochrome P-450 2c) indicating that we are examining a direct interaction between the lipid and the terminal oxidase enzyme (Meftah & Skett, 1987b). There is, however, no change in PC/PE ratio in the diabetes-derived phospholipids compared to control males, although this lipid mixture has a profound effect on N-deethylase activity (Table 1). It cannot simply be the PC/PE ratio that is causing the effects seen.

Analysis of the fatty acid content of the phospholipids (Table 3) showed that there were marked sex differences also in this parameter. There was a lowered linoleic (18:2) and arachidonic (20:4) acid and increased stearic (18:0) acid content in the female-derived phospholipids and, thus, the percentage of unsaturated fatty acids dropped from 48% to 37%. These differences are similar to those obtained by Feuer *et al.* (1980). Could any of these differences correlate to the effect on lignocaine N-deethylase activity? If we examine the fatty acid content of the

diabetes-derived phospholipids (which exert the same effect on N-deethylase activity), we see that the only effect of acute diabetes is a significant decrease in arachidonic acid ($P < 0.01$), whereas the chronic diabetic-derived lipids show a decrease in arachidonic and palmitoleic (16:1) acids and an increase in stearic, oleic (18:1) and linoleic (18:2) acids. In both cases the percentage unsaturated was intermediate between the control male and control female. These data are similar to those of Faas & Carter (1983) and Mercuri *et al.* (1967), except that the former group saw an increase in total phospholipid in diabetic animals. Faas & Carter (1980, 1983) suggested that the changes in fatty acid content were a consequence of the altered activity of the fatty acid desaturase enzymes. These differences between the female- and diabetic-derived phospholipids would seem to rule out linoleic acid as a candidate for the effector and, indeed, the overall percentage of unsaturated fatty acids in the phospholipids. We are, thus left with stearic and arachidonic acids as the possible factors affecting lignocaine N-deethylase activity. Attempts to correlate the activity of the lignocaine N-deethylase to all of the parameters so far measured showed that there was a weak correlation with PC and PE content (correlation coefficient of 0.859 and 0.886 respectively), although this was negated by the use of the same amount of lipid from each sample in the reconstitution, and a strong correlation (correlation coefficient of 0.997) with the arachidonic acid content of the phospholipid. There were no other significant correlations seen. It seemed most likely, therefore, that the phospholipid content of arachidonic acid was the factor controlling N-deethylase activity.

In order to test this hypothesis further, delipidated male-derived proteins were reconstituted with synthetic PC species of known fatty acid content and the activity of the N-deethylase measured. Diarachidonyl-PC was by far the most efficient in reconstituting N-deethylase activity being 50%

better than the PC containing saturated fatty acids (lauric, stearic and behenic (22:0) acids). Dioleoyl-PC (18:1) was significantly better than its saturated homologue (distearoyl-PC) ($P < 0.05$), whereas dicaproyl-PC (6:0) showed the lowest activity probably due to the formation of micelles by this PC rather than vesicles. (Dencher, 1986). This non-specific effect of dicaproyl-PC is further shown by the fact that it decreases 3-hydroxylase activity as well (an effect not noted for any of the other PC species). Thus, chain length above C-12 has little effect on N-deethylase activity but degree of unsaturation seems to be important and arachidonic acid, as noted above, seems to be the best side-chain in PC to reconstitute lignocaine N-deethylase activity. This would fit with the evidence of Marshall & McLean (1971) and Rowe & Wills (1976) that diets deficient in unsaturated fatty acids led to decreased drug metabolism and that unsaturated fatty acids can alter the spin-state of cytochrome P-450 and, thus, its efficiency as a terminal oxidase (Gibson & Tamburini, 1984).

All of the evidence, therefore, points heavily towards the phospholipid fraction of the endoplasmic reticulum membrane being important in the regulation of sex-dependent drug metabolism and that the arachidonic acid content of the phospholipid is, perhaps, the important factor in this control. This may relate to the control of sex dependent metabolism of other drugs in man but, due to the lesser importance of this phenomenon in this species, may be more related to a general phenomenon of regulation of drug metabolism by the microsomal membrane composition as well as the enzyme complement of the membrane.

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