Biochemical Pharmacology, Vol. 38, No. 22, pp. 3925–3931, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

EVALUATION OF THE INVOLVEMENT OF A MALE SPECIFIC CYTOCHROME P-450 ISOZYME IN SENESCENCE-ASSOCIATED DECLINE OF HEPATIC DRUG METABOLISM IN MALE RATS

SHOICHI FUJITA,* REIKO MORIMOTO, MASATO CHIBA, KENICHI KITANI† and TOKUJI SUZUKI

Department of Biopharmaceutics, Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoicho, Chiba, Japan 260, and † First Laboratory of Clinical Physiology, Tokyo Metropolitan Institute of Gerontology, 35-2, Sakaecho, Itabashiku, Tokyo, Japan

(*Received* 3 *January* 1989; *accepted* 30 *May* 1989)

Abstract—The major "male specific" species of cytochrome P-450 (P-450m1) was purified and an antibody against it used to evaluate the involvement of this isozyme in alterations of drug metabolism in senescence. P-450m1 exhibited strikingly high imipramine (IM) *N*-demethylase activity while it showed no IM 2-hydroxylation, which is an alternate pathway of IM metabolism in rat liver microsomes. The antibody to P-450m1 inhibited 80% of imipramine N-demethylation in young male rats. In old male rats, which have been shown to have lower IM N-demethylase activity, a 60% inhibition was observed. The inhibitable portion of this activity in old male rats is about one third of that in young rats, but the remaining portion not inhibited by this antibody is almost identical in young and old rats. IM 2-hydroxylation on the other hand was not inhibited by this antibody at all. It also inhibited about 30% of diazepam(DZ) N-demethylation in young rats but showed no inhibition in old rats, resulting in the loss of the age difference in the remaining portion. DZ 3-hydroxylation was not inhibited by this antibody, in spite of the fact that it showed a markedly higher activity in young male than in young female rats with a subsequent reduction in old age in male rats. This study provides the first direct evidence that differences in the amount of the major male specific P-450 isozyme (P-450m1) are responsible for the age- and sex-associated differences in some of the drug metabolizing activities. It also became apparent that P-450m1 may not be the only isozyme responsible for these differences.

Alteration of drug metabolism with senescence is of clinical importance. While investigating age-associated alterations in drug metabolizing enzyme activities in rat liver microsomes, we have recognized that only activities which show large sex difference (male > female) in young animals decrease markedly in senescent male rats, while no marked alteration in enzyme activity is observed in female rats [1–5]. In fact the sex difference in drug metabolism completely disappears in two-year-old rats due to the marked decrease in activity in male rats.

Sex difference in metabolism of xenobiotics has been well documented [6]. The presence of at least two male specific‡ forms of cytochrome P-450 was first recognized by Fujita *et al.* using EPR spectroscopy [7]. They proposed that the difference in relative abundance of multiple species of cytochrome P-450 in liver microsomes from male and female rats may be responsible for the sex difference in drug metabolism. Subsequently, several laboratories have purified sex specific forms of cytochrome P-450 [8–15]. The male specific form has been shown to possess higher steroid and xenobiotic metabolizing activities in reconstituted system than the female specific form [10, 16]. The difference in the quantity of the male specific form of P-450 in male and female rat liver microsomes has been invoked the cause of sex differences in metabolism of xenobiotics in rat liver microsomes. The direct evidence for this hypothesis has been obtained by the use of monospecific antibody for the sex difference of testosterone 16α hydroxylase activity in rat liver microsomes [12]. As for the sex difference in metabolism of xenobiotics, however, only circumstantial evidence such as the close correlation between the quantity of the male specific P-450 isozyme in liver microsomes and the magnitude of metabolic activity of the corresponding microsomes has been shown, but no direct evidence has been reported [16].

Since the senescence-associated sharp decline of drug metabolism was observed only in male rats, Fujita *et al.* have proposed that this may be due to the decrease in male specific form(s) of cytochrome P-450 [1, 4]. They have demonstrated that the relative abundance of P-450 isozymes in old male rat liver is almost identical to that of females of any age, but different from that of young male rats by the pattern analysis of HPLC elution profiles of solubilized liver microsomes [17]. Kamataki *et al.* [18] in the same year demonstrated the parallel decrease of their male specific P-450 isozyme (P-450 male) with the age-associated decrease in drug metabolism.

These findings strongly support the above hypothesis, but the crucial evidence that the male specific P-450 isozyme is indeed engaging in the drug metabolism tested in liver microsomes has been lacking. This has been a subject of criticism from the inves-

* To whom reprint requests should be addressed.

‡ "Male specific" or "female specific" species of cytochrome P-450 refer to cytochrome P-450 isozymes present in male or female rat liver microsomes, respectively, in exceedingly larger quantity than in liver microsomes from rats of the other gender.

BP 38:22-C

tigators who support other hypotheses of the mechanism of the age-associated alteration of drug metabolism. The decreased functional cytochrome P-450 reductase [19] resulting in the decrease in activities of P-450 mediated reactions or the altered microsomal membrane fluidity [20, 21] affecting activities of microsomal enzymes have been proposed as the alternative cause for alterations of drug metabolism with old age. Unless evidence indicating that the metabolic activities observed in liver microsomes are carried out by the male specific cytochrome P-450, is provided, the parallelism between the decrease in the activities and the decrease in the content of the P-450 isozyme does not necessarily mean that the latter phenomenon is the cause of the former.

The present investigation was undertaken to prepare an antibody to the major male specific P-450 isozyme which is capable of inhibiting drug metabolism and with the use of this antibody, to confirm and to evaluate the involvement of this P-450 isozyme in sex- and age-associated difference in drug metabolism.

MATERIALS AND METHODS

Chemicals. NADPH, glucose-6-phosphate (G6P), and G6P-dehydrogenase were purchased from Oriental Yeast Co. (Tokyo). Dilauroylphosphatidylcholine, amitriptyline HCl and imipramine HCl were obtained from Sigma (St Louis, MO). Desipramine and 2-hydroxyimipramine were gifts from Ciba Geigy (Basel). Diazepam, temazepam (3-hydroxydiazepam), nordiazepam (*N*-desmethyldiazepam) and nitrazepam were gifts from Japan Rosh Co. Ltd (Tokyo). Nortriptyline HCl (Dainippon Pharmaceutical Co., Tokyo), *cis*- and *trans*-10-hydroxyamitriptyline HCl (Lundbeck, Copenhagen) were also gifts. Other chemicals used were all of analytical grade.

Animals. Male Sprague–Dawley rats were raised under SPF condition. Young and old (3- and 24-month-old) rats were used to prepare liver microsomes [22]. A group of young rats was treated with i.p. injection of phenobarbital (100 mg/kg/day) for 4 days. Another group of young rats were treated with i.p. injection of 3-methylcholanthrene dissolved in corn oil (40 mg/kg/day) for 4 days. Microsomes prepared from treated and untreated young rats were used to prepare purified cytochromes P-450.

Purification of cytochromes P-450. P-450b and P-450c, the major species of P-450 induced by phenobarbital and 3-methylcholanthrene, respectively, were purified according to previously described methods [23, 24]. The specific P-450 contents of the final samples were 12 and 14 nmol/mg protein, respectively.

P-450m1 was purified from uninduced 3-month-old male rat liver microsomes by cholate-solubilization (3 mg/mg protein), polyethylene glycol 6000 (8–18%) precipitation, followed by the standard P-450 male purification procedure described by Kamataki *et al.* [10] with modifications. After *w*-amino-*n*-octyl-Sepharose 4B column chromatography, P-450m1 was eluted from the DE52 column isocratically with 3 mM phosphate buffer containing 20% glycerol, 0.2% Emulgen 911, 1 mM dithiothreitol, 0.5% sodium cholate, and 0.1 mM EDTA. The P-450m1 containing fraction was rechromatographed through the DE52 column with the same buffer, and a third time with the same buffer except that it contained 0.2% (instead of 0.5%) sodium cholate. P-450m1 contained in this fraction at this stage was electrophoretically homogeneous. After the P-450m1 containing fraction was eluted from the column, the sodium cholate concentration in the elution buffer was raised to 0.5% to elute the remaining P-450 species which showed different molecular weight (M_r = 47,000) from P-450m1. The P-450m1 containing fraction was then subjected to hydroxyapatite column chromatography to concentrate the sample and for further purification followed by another such column to remove Emulgen. The specific content of the final P-450m1 preparation was 14.4 nmol/mg, and the apparent M_r = 51,000. Judging from the *N*-terminal sequence, the apparent M_r, chromatographic behavior and spectral characteristics, P-450m1 appears to be identical to the major male specific isozyme designated as RLM5 [8], P-450 UT-A [9], P-450 male [10], P-450h [11], P-450 2c [12], P-450 16α [13], P-450M1 [14] and P-450 UT-2 [15]. The female specific species of cytochrome P-450, P-450f1 was purified from liver microsomes from 3-month-old female rats by essentially the same procedure as above. The apparent M_r was 49,000. SDS–polyacrylamide gel electrophoresis [25] was used for molecular weight determination. Protein concentration was determined by the method of Lowry *et al.* [26].

Preparation of anti-P-450m1 antibody. Antibody against P-450m1 was raised in a male Japan White rabbit according to the method of Noshiro and Omura [27]. The IgG was purified from antiserum with ammonium sulfate (20–35%) precipitation followed by DE52 column chromatography according to Noshiro and Omura [27] and Kamataki *et al.* [28]. This antibody did not cross react with cytochrome P-450c, P-450b, or P-450f1, the female specific P-450 isozyme in Ouchtelony double diffusion plates. It formed a precipitation line with male rat liver microsomes and with cytochrome P-450m1.

Assay procedures for catalytic activities of liver microsomes and of the reconstituted monooxygenase system with purified cytochromes P-450. Amitriptyrine N-demethylation and *trans*- and *cis*-10 hydroxylation were assayed after 1 min incubation of amitriptyline with liver microsomes (1 mg protein/ml) or the reconstituted system with a purified P-450 in 0.1 M sodium phosphate buffer (pH 7.4) containing NADPH generating system. An HPLC procedure capable of simultaneously detecting all three primary metabolites of amitriptyline as well as *cis*- and *trans*-10 hydroxy nortriptyline, the secondary metabolites, was used to quantitate the metabolites. After termination of the reaction, which was linear for 5 min, with 1 M borate buffer (pH 10), amitriptyline and its metabolites were extracted with ethylacetate, evaporated to dryness, and dissolved in an HPLC mobile phase (H_2O:CH_3CN:MeOH = 53:48:50 plus 2% butylamine). The sample was applied to reversed-phase ODS column (Inertsil ODS, Gasukuro Kogyo Ltd, Tokyo). The substrate and its metab-

olites formed were detected at a wavelength of 239 nm. The HPLC system consisted of a Shimadzu LC 3A pump, injector and Shimadzu SPD-2A spectrophotometric detector.

Assay procedures for aminopyrine N-demethylation, *p*-nitroanisole O-demethylation, hexobarbital oxidation, diazepam 3-hydroxylation and N-demethylation and imipramine N-demethylation and 2-hydroxylation in liver microsomes were described previously [1, 3, 29]. Assay methods for these activities with a reconstituted P-450 monooxygenase system were essentially the same as those employed for microsomal assays. Reconstituted P-450 monooxygenase system generally consisted of P-450 (0.1 nmol/ml), cytochrome *c* reductase (0.4 unit/ml), dilauroylphosphatidylcholine (5 μg/ml), sodium cholate (0.1 mg/ml), G6P (20 mM), G6P-dehydrogenase (1.5 units/ml), $MgCl_2$ (3 mM) and substrates. In *p*-nitroanisole *O*-demethylase system, dilauroylphosphatidylcholine concentration was 0.75 μg/ml. Substrate concentrations used were, aminopyrine, 1.0, mM; *p*-nitroanisole, 1.0 mM; hexobarbital, 1.0 mM; imipramine, 5 and 700 μM; diazepam, 100 μM; and amitriptyline, 125 μM.

Catalytic assays in the presence of anti-P-450m1 antibody. Hepatic microsomal imipramine N-demethylation and 2-hydroxylation, and diazepam N-demethylation and 3-hydroxylation in liver microsomes from 3-month-old rats were assayed in the presence of various concentrations of anti-P450m1 antibody (1–8 mg/mg microsomal protein). Substrate concentration used was 100 μM for both reactions. For aging study, 5 mg of anti-P-450m1 antibody was added to 1 ml incubation mixture containing 0.5 mg microsomal protein (i.e. 10 mg antibody/mg microsomal protein) to ascertain the maximal inhibition. Other assay procedures were the same as described above.

RESULTS AND DISCUSSION

The major species of cytochrome P-450 in male rat liver microsomes (P-450m1) was purified to electrophoretic homogeneity. Table 1 summarizes the activities of P-450m1 in comparison with P-450b and P-450c in the reconstituted monooxygenase system and of liver microsomes from male and female Sprague–Dawley rats. Aminopyrine *N*-demethylase activity of P-450m1 corresponds to that of P-450 male reported by Kamataki *et al.* [10]. Hexobarbital oxidase activity of P-450m1 was of the same magnitude as that of P-450h reported by Ryan *et al.* [11]. P-450b showed a higher turnover rate for this reaction.

Exceptionally high activities were observed with P-450m1 for N-demethylations of imipramine (IM) and amitriptyline (AT), which showed large sex differences in young rats (Table 1). The P-450 turnover number for these reactions at high substrate concentrations showed the highest values ever reported for a P-450 activity. However, those activities in male rat liver microsomes (< 10 nmol/min/nmol P-450) were not as high as can be expected from P-450 turnover numbers obtained from the reconstituted system with P-450m1. Differences in the NADPH-cytochrome P-450 reductase to P-450 ratio between that in the reconstituted system and that in the microsomal assay system or the unfavorable topology of cytochrome P-450m1 in liver microsomal membrane for catalytic activity may have caused this discrepancy. IM 2-hydroxylation showing only a small sex difference in rat liver microsomes [5, 29, Table 1], on the other hand, was not detected at all in the reconstituted system with P-450m1. Cytochromes P-450b and P-450c did not show such a strong *regio* selectivity in IM metabolism.

Only low AT 10-hydroxylase activity was observed in the reconstituted system with this male specific form of P-450. Cytochrome P-450b showed high stereo-selectivity in 10-hydroxylation of AT. *Trans*-10-hydroxylase activity was about 20 times as high as *cis*-10-hydroxylase activity.

In the reconstituted monooxygenase system with P-450m1, DZ *N*-demethylase activity was almost 3 times as high as DZ 3-hydroxylase activity. In contrast, in male rat liver microsomes, the former (*N*-demethylase activity) was less than a half of the latter (3-hydroxylase activity) (Table 1). This discrepancy between activity of P-450m1 and of male rat liver microsomes is of importance especially in view of the fact that DZ 3-hydroxylase activity showed larger sex difference than DZ *N*-demethylase activity. It is suggested that the contribution of P-450m1 to the sex difference in DZ 3-hydroxylation in rat liver microsomes is smaller than to that in N-demethylation.

The effect of varying concentrations of anti-P-450m1 antibody on IM N-demethylation and 2-hydroxylation as well as DZ N-demethylation and 3-hydroxylation in microsomes from 3-month-old male Sprague–Dawley rats was tested to see the extent of involvement of P-450m1 in these reactions (Fig. 1). A marked decrease in IM *N*-demethylase activity was observed by the addition of the antibody, while no effect was observed in IM 2-hydroxylase activity even after the antibody concentration was raised to the level which caused a maximum (80%) inhibition of N-demethylation (Fig. 1). This is consistent with the result of the reconstitution study above, where extremely high IM *N*-demethylase activity and no 2-hydroxylase activity were observed (Table 1). The same antibody did not inhibit IM N-demethylation or 2-hydroxylation in female rat liver microsomes. It is clear from these observations that about 80% of IM *N*-demethylase activity and none of 2-hydroxylase activity is catalysed by P-450m1 in liver microsomes from 3-month-old male rats. This provides a biochemical base for our previous observation that while IM *N*-demethylase activity is markedly higher in male rat liver than in female rat liver microsomes, such sex difference is absent in IM 2-hydroxylation [5, 29, Table 1].

A significant sex difference in DZ 3-hydroxylase activity was also observed (Table 1). The average value for DZ *N*-demethylase activity in male rat liver microsomes was higher than that in female rat liver microsomes, but the difference was not statistically significant ($P = 0.052$). The anti-P-450m1 antibody, however, inhibited DZ N-demethylation only up to 35% and did not inhibit 3-hydroxylation (Fig. 1), although the latter showed larger sex difference than the former. This suggests the possibility that another male specific species of P-450 is involved in DZ 3-hydroxylation. It should be noted that several other

Table 1. Catalytic activity of cytochrome P-450m1 as compared with activities of cytochromes P-450b and P-450c and of liver microsomes from 3-month-old male and female rats

Substrate	(mM)	Reaction	Activities				
			P-450m1 (nmol/min/nmol P-450)[a]	P-450b	P-450c	Liver microsomes male (nmol/min/mg protein)[b]	female
Aminopyrine	(1.0 mM)	N-Demethylation	9.84	12.9	6.30	4.21 ± 0.26	2.14 ± 0.14**
p-Nitroanisole	(1.0 mM)	O-Demethylation	0.96	1.57	10.9	0.51 ± 0.23	0.58 ± 0.15
Hexobarbital	(1.0 mM)	Oxidation	23.0	30.9	2.03	1.29 ± 0.11	0.36 ± 0.05**
Amitriptyline	(125 μM)	N-Demethylation	381	38.0	30.1	6.05 ± 1.21	1.37 ± 0.39**
		t-10-Hydroxylation	7.14	15.3	6.78	0.97 ± 0.08	0.73 ± 0.15*
		c-10-Hydroxylation	4.93	0.88	3.54	0.14 ± 0.02	0.09 ± 0.03*
Diazepam	(100 μM)	N-Demethylation	8.96	—	—	0.38 ± 0.11	0.27 ± 0.05
		3-Hydroxylation	3.19	—	—	0.88 ± 0.18	0.44 ± 0.05**
Imipramine	(5.0 μM)	N-Demethylation	13.2	—	—	0.43 ± 0.07	0.17 ± 0.02**
	(100 μM)		242	—	—	2.55 ± 0.37	0.77 ± 0.07**
	(700 μM)		389	9.84	19.9	3.88 ± 0.63	2.21 ± 0.17**
	(5.0 μM)	2-Hydroxylation	ND	—	—	0.77 ± 0.14	0.81 ± 0.17
	(100 μM)		ND	—	—	1.14 ± 0.13	0.91 ± 0.14
	(700 μM)		ND	5.14	11.3	1.28 ± 0.15	0.87 ± 0.05**

[a] Averages of duplicate or triplicate determinations (variabilities were < 20%).
[b] Activities are expressed as mean ± SD (N = 4).
*, ** Significantly different from the corresponding monooxygenase activities in male rat liver microsomes (* $P < 0.05$, ** $P < 0.01$).
ND: not detectable.

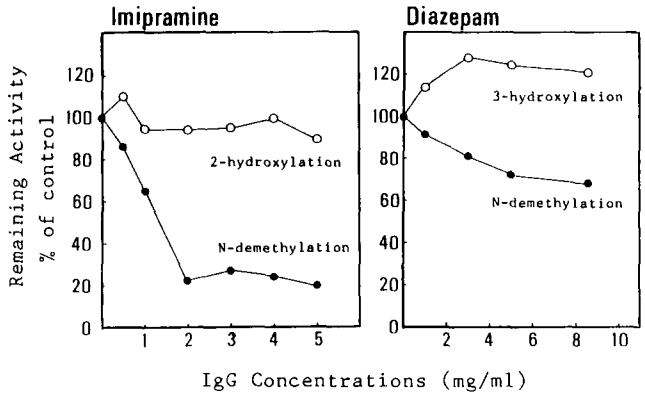

Fig. 1. Effects of anti-P-450m1 antibody on imipramine and diazepam metabolism in rat liver microsomes. Varying concentrations of anti-P-450m1 IgG were added to the microsomal assay mixture (final volume 1 ml) containing 1 mg protein/ml equivalent of microsomes obtained from three 3-month-old male rats, NADPH generating system, and 100 μM imipramine (left) or desipramine (right) as substrates. Typical results obtained using the same preparation of microsomes for both imipramine and diazepam metabolism are shown. The activity was expressed as the percentage of control activity which was determined under the same conditions as above except in the presence of preimmune IgG in the place of anti-P-450m1 IgG. Preimmune IgG did not exert any inhibitory effect to these reactions. Control activities for imipramine N-demethylation were 1.85 nmol/min/mg in the absence and 1.88 nmol/min/mg in the presence of 5 mg/ml preimmune IgG, and for imipramine 2-hydroxylation were 0.650 nmol/min/mg in the absence and 0.654 nmol/min/mg in the presence of 5 mg/ml preimmune IgG. Those for diazepam N-demethylation were 0.37 nmol/min/mg in the absence and 0.39 in the presence of 5 mg/ml preimmune IgG, and for diazepam 3-hydroxylation were 0.93 in the absence and 0.88 in the presence of 5 mg/ml preimmune IgG. Open circles indicate hydroxylation activities and filled circles, demethylation activities.

male specific forms of P-450 (P-450M2, M3 [14], P-450RLM2 [30], P-450g [31], P-450 2a/PCN-E [32]) has been reported. It awaits further investigations to elucidate the cause of age-associated decrease of DZ 3-hydroxylation.

Since senescence-associated decline in drug metabolizing enzyme activities is limited to those which show markedly higher activity in young male than in young female rats [1–5], the decrease in P-450m1 may be responsible for this phenomenon. To test this possibility, anti-P-450 antibody was added to the IM or DZ metabolic reaction mixture with microsomes from young and old male rats. In the absence of the antibody, the IM *N*-demethylase activity in old rats is less than a half of young rats. About 80% of activity in young rats and 60% of

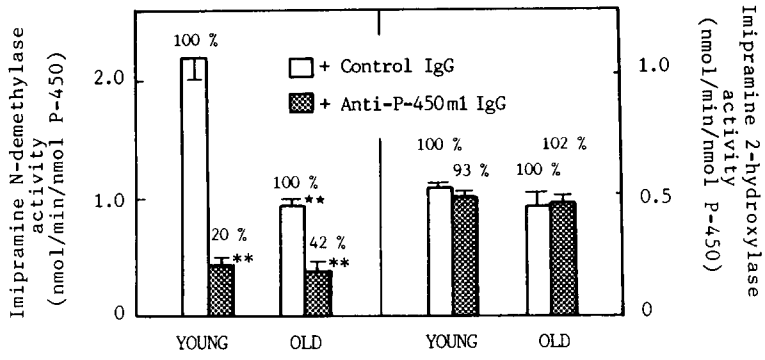

Fig. 2. Effect of anti-P-450m1 antibody on imipramine metabolism in liver microsomes from young and old male rats. Anti-P-450m1 IgG in a concentration (5 mg/ml) which can give maximum inhibition of imipramine *N*-demethylase activity was added to assay mixtures for imipramine metabolism using young and old male rat liver microsomes. Activities in the presence of 5 mg/ml preimmune IgG are shown by open bars and those in the presence of anti-P-450m1 IgG are shown by shaded bars. The error bars represent the standard deviations from the mean of 4 determinations using liver microsomes from different rats. The remaining activities (in percentage of controls) of IM *N*-demethylase and 2-hydroxylase in liver microsomes from young and old rats in the presence of this antibody were indicated in the figure. *, ** Significantly different from control activities in the presence of preimmune IgG ($P < 0.05$, $P < 0.01$, respectively). ★, ★★ Significantly different from the activity of liver microsomes from 3-month-old male rats ($P < 0.05$, $P < 0.01$, respectively).

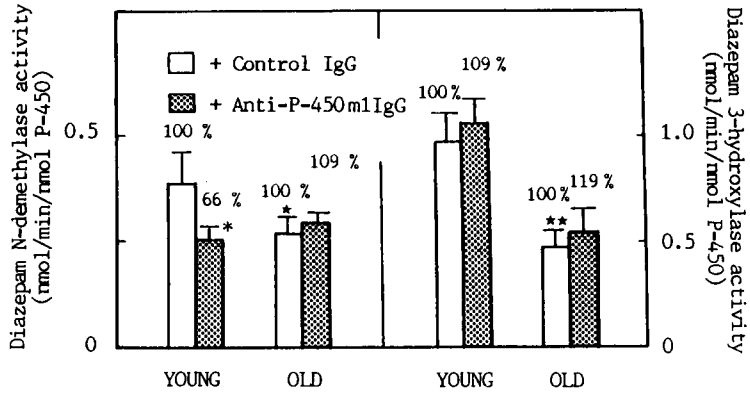

Fig. 3. Effect of anti-P-450m1 antibody on diazepam metabolism in liver microsomes from young and old male rats. Anti-P-450m1 IgG in a concentration (5 mg/ml) which can give maximum inhibition of diazepam *N*-demethylase activity was added to assay mixtures for diazepam metabolism using young and old male rat liver microsomes. Activities in the presence of 5 mg/ml preimmune IgG are shown by open bars and those in the presence of anti-P-450m1 IgG are shown by shaded bars. The error bars represent the standard deviations from the mean of 4 determinations using liver microsomes from different rats. The remaining activities of DZ *N*-demethylase and 3-hydroxylase in liver microsomes from young and old rats in the presence of this antibody were indicated in the figure. *, ** Significantly different from control activities in the presence of preimmune IgG ($P < 0.05$, $P < 0.01$, respectively). ★, ★★ Significantly different from the activity of liver microsomes from 3-month-old male rats ($P < 0.05$, $P < 0.01$, respectively).

activity in old male rats were inhibited by this antibody (Fig. 2). The absolute value of antibody-inhibited portion in old rats is about one third of that in young rats, indicating a marked decrease in P-450m1 mediated IM N-demethylation in old age. The remaining portion not inhibited by this antibody, and therefore, presumably not catalysed by P-450m1 did not alter with age. IM 2-hydroxylase activity was not inhibited by the antibody in either young or old rats. These results clearly show that the age-associated alteration in imipramine metabolism is due to the decrease in P-450m1 level with age.

In the absence of anti-P-450m1 antibody, DZ *N*-demethylase activity was about 30% higher in young than in old rats, while DZ 3-hydroxylase activity was about 2-fold higher in young than in old rats (Fig. 3). In the presence of the antibody, 30% of the activity in young rats was inhibited, but in old rats, no inhibition was observed. This resulted in the disappearance of the age difference in the uninhibited portion of *N*-demethylase activity, indicating that the age-associated alteration in DZ *N*-demethylase activity was due to the decrease in P-450m1 with age. DZ 3-hydroxylase activity was not inhibited

by this antibody at all in either young or old rats, indicating that DZ 3-hydroxylase activity is not catalysed by P-450m1 and that age-associated alteration in DZ 3-hydroxylase activity is not due to the decrease in P-450m1 content. Therefore, in this case, the criticism raised against the assumption that the parallel decrease in a P-450 isozyme content with activities of drug metabolism represents the cause and the effect relationship was correct. Another male specific P-450 isozyme [14, 30–32] may be involved in DZ 3-hydroxylation and in its alteration with age.

The present observations provide a conclusive answer to the long debated cause for the senescence-associated alteration of drug metabolism. We were able to obtain direct evidence that the senescence-associated decrease in the content of P-450m1 is responsible for the senescence-associated decrease in enzyme activities involved in the metabolism of some drugs.

These results are consistent with our hypothesis that the senescence-associated alteration in drug metabolism is caused by senescence-associated alterations in the relative abundance of multiple species of P-450 in liver microsomes into female type composition [1–5]. This would result in the functional feminization of male rat liver. This agrees well with our previous observations: drug metabolizing enzyme activities which are higher in young male than in young female rats decrease with age while androstenedione 5α-reductase activity which is higher in young female than in young male rats increases with age in male rats, resulting in the disappearance of the sex difference in these activities [3]. It appears that neonatal androgenic imprinting of male rat liver is reversed in old male rats, contrary to the current notion that it is irreversible [33].

Acknowledgements—This work was supported in part by grants from the Japan Research Foundation For Clinical Pharmacology to T. S. and The Research Foundation For Pharmaceutical Sciences to S.F.

REFERENCES

1. Fujita S, Uesugi T, Kitagawa H, Chiba M, Suzuki T, Ohta M and Kitani K, Hepatic microsomal monooxygenase and azoreductase activity in aging Fischer-344 rats. Importance of sex difference for aging studies. In: *Liver and Aging — 1982* (Ed. Kitani K), pp. 55–74. Elsevier Biomedical Press, Amsterdam, 1982.
2. Fujita S, Tatsuno J, Kawai R, Kitagawa H, Suzuki T and Kitani K, Age-associated alteration of lidocaine metabolism is position selective. *Biochem Biophys Res Commun* **126**: 117–122, 1985.
3. Fujita S, Chiba M, Suzuki T and Kitani K, Effect of senescence on the hepatic metabolism of drugs affecting the central nervous system in rats and mice. In: *Liver and Aging — 1986* (Ed. Kitani K), pp. 103–112. Elsevier Science Publishers, Amsterdam, 1986.
4. Kitagawa H, Fujita S, Suzuki T and Kitani K, Disappearance of sex difference in rat liver drug metabolism in old age. *Biochem Pharmacol* **34**: 579–581, 1985.
5. Chiba M, Fujita S, Kitani K and Suzuki T, Age-associated alteration in imipramine metabolism is position selective. *Biochem Pharmacol* **35**: 2628–2630, 1986.
6. Kato R, Sex-related differences in drug metabolism. *Drug Metabol Rev* **3**: 1–32, 1974.
7. Fujita S, Chevion M and Peisach J, Influence of sex hormones on relative quantities of multiple species of cytochrome P-450 in rat liver microsomes: a possible answer to the sexual differences in metabolic activities of rat liver microsomes. *Isr J Chem* **21**: 87–90, 1981.
8. Cheng K-C and Schenkman, JB, Purification and characterization of two forms of rat liver microsomal cytochrome P-450. *J Biol Chem* **257**: 2378–2385, 1982.
9. Guengerich FD, Dannan GA, Wright ST, Martin MV and Kaminsky LS, Purification and characterization of liver microsomal cytochrome P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**: 6019–6030, 1982.
10. Kamataki K, Maeda K, Yamazoe Y, Nagai T and Kato R, Sex difference of cytochrome P-450 in the rat: purification, characterization and quantitation of constitutive forms of cytochrome P-450 from liver microsomes of male and female rats. *Arch Biochem Biophys* **225**: 758–770, 1983.
11. Ryan DE, Iida S, Wood AW, Thomas RE, Lieber CS and Levin W, Characterization of three highly purified cytochrome P-450 from hepatic microsomes of adult male rats. *J Biol Chem* **259**: 1239–1250, 1984
12. Waxman DJ, Rat hepatic cytochrome P-450 isozymes 2c. *J Biol Chem* **259**: 15481–15490, 1984.
13. Morgan TE, MacGeoch C and Gustafsson JA, Sexual differentiation of cytochrome P-450 in rat liver. Evidence for constitutive isozyme as the male-specific 16α hydroxylase. *Mol Pharmacol* **27**: 471–479, 1985.
14. Matsumoto T, Emi Y, Kawabata S and Omura T, Purification and characterization of three male-specific and female-specific forms of cytochrome P-450 from rat liver microsomes. *J Biochem* **100**: 1359–1371, 1986.
15. Funae Y and Imaoka S, Simultaneous purification of multiple forms of rat liver microsomal cytochrome P-450 by high-performance liquid chromatography. *Biochim Biophys Acta* **842**: 119–132, 1985.
16. Yamazoe Y, Shimada M, Kamataki T and Kato R, Effects of hypohysectomy and growth hormone treatment on sex specific forms by cytochrome P-450 in relation to drug and steroid metabolism in rat liver microsomes. *Jap J Pharmacol* **42**: 371–382, 1986.
17. Fujita S, Kitagawa H, Chiba M, Suzuki T, Ohta M and Kitani K, Age and sex associated differences in the relative abundance of multiple species of cytochrome P-450 in rat liver microsomes. A separation by HPLC of hepatic microsomal cytochrome P-450 species. *Biochem Pharmacol* **34**: 1861–1864, 1985a.
18. Kamataki T, Maeda K, Shimada M, Kitani K, Nagai T and Kato R, Age-related alteration in activities of drug-metabolizing enzymes and contents of sex-specific forms of cytochrome P-450 in liver microsomes from male and female rats. *J Pharmacol Exp Ther* **233**: 222–228, 1985.
19. Schmucher DL, Wang RK and Kwong P, Age-dependent alterations in rat liver microsomal NADPH cytochrome c (P-450) reductase. In: *Liver and Aging — 1982* (Ed. Kitani K), pp. 75–97. Elsevier Biomedical Press, Amsterdam, 1982.
20. Armbrecht HJ, Birnbaum LS, Zenser TV and Davis BB, Changes in hepatic microsomal membrane fluidity with age. *Exp Gerontol* **17**: 41–48, 1982.
21. Stier A, Finch SAE, Greinert R, Hohne M and Muller R, Membrane structure and function of the hepatic microsomal cytochrome P-450 system. In: *Liver and Aging — 1982* (Ed. Kitani K), pp. 3–14. Elsevier Biomedical, Amsterdam, 1982.
22. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes — I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
23. Harada N and Omura T, Selective induction of two different molecular species of cytochrome P-450 by

phenobarbital and 3-methylcholanthrene. *J Biochem* **89**: 237–248, 1981.
24. Kuwahara S, Harada N, Yoshioka H, Miyata T and Omura T, Purification and characterization of four forms of cytochrome P-450 from liver microsomes of phenobarbital-treated and 3-methylcholanthrene-treated rats. *J Biochem* **95**: 703–714, 1984.
25. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
26. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
27. Noshiro M and Omura T, Immunochemical study on the electron pathway from NADH to cytochrome P-450 of liver microsomes. *J Biochem* **83**: 61–73, 1978.
28. Kamataki T, Belcher OH and Neal RA, Studies of the metabolism of diethyl *p*-nitrophenyl phosphorothionate (parathion) and benzphetamine using an apparently homogeneous preparation of rat liver cytochrome P-450: effect of cytochrome P-450 antibody preparation. *Mol Pharmacol* **12**: 921–932, 1976.
29. Chiba M, Nishihara E, Fujita S and Suzuki T, Position selective sex difference in imipramine metabolism in rat liver microsomes. *Biochem Pharmacol* **34**: 898–900, 1985.
30. Jansson I, Mole J and Schenkman JB, Purification and characterization of a new form (RLM2) of liver microsomal cytochrome P-450 from untreated rat. *J Biol Chem* **260**: 7084–7093, 1985.
31. Dannan GA, Guengerich FP, Waxman DJ, Hormonal regulation of rat liver microsomal enzymes. Role of gonadal steroids in programming, maintenance, and suppression of d4-steroid 5α-reductase, flavin-containing monooxygenase, and sex-specific cytochrome P-450. *J Biol Chem* **261**: 10728–10735, 1986.
32. Rampersaud A, Bandiera S, Ryan ED, Levin W, Thomas PE and Waltz FG Jr, A regulatory polymorphism for rat hepatic cytochrome P-450g. *Arch Biochem Biophys* **252**: 145–151, 1987.
33. Gustafsson JA and Stenberg A, Irreversible androgenic programming at birth of microsomal and soluble rat liver enzymes active on 4-androstene-3,17-dione and 5α-androstane-3α, 17β-diol. *J Biol Chem* **249**: 711–718, 1974.