STUDIES ON MECHANISM OF SEX DIFFERENCE IN DRUG- OXIDIZING ACTIVITY OF LIVER MICROSOMES*

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The main pathway of drug inactivation occurs in liver microsomes by mixed function oxygenation in the presence of NADPH and oxygen (1).

The participation in these reactions of a carbon monoxide-binding hemoprotein called P-450 as the oxygen activating component has been established (1-6). One of characteristics of the drug-oxidizing enzymes of liver microsomes is to have marked sex difference in the activity of rats (7-12).

In previous paper, we reported that the magnitude of the sex difference in the drug-oxidizing activity in rats was markedly different according to the substrate used (11, 12). On the other hand, it has been observed that there are no clear sex difference in the activities of microsomal drug-oxidizing enzymes of other species of animals (8, 13). More recently, it was observed in our laboratory that the activity of microsomal NADPH-linked electron transport system, such as, the activity of NADPH oxidase, NADPH-cytochrome c reductase and NADPH-neotetrazolium reductase and the content of P-450 were higher in male rats than in female rats (14, 15).

Therefore, it is of interest to establish the relationship between the activities of microsomal NADPH-linked electron transport system and drug-oxidizing enzymes in connection with the sex difference and to investigate whether the such sex difference in the activity of microsomal NADPH- or NADH-linked electron transport system is observed in other species of animals.

METHODS

Male and female rats of Wistar strain, weighing about 200 g and 160 g, respectively, and male and female rabbits, weighing about 2.2 kg and 2.0 kg, respectively, and male and female mice of *dd* strain, weighing about 22 g and 20 g, respectively, were used.

Preparation of microsomes: The animals were decapitated and the liver were removed, chopped into small pieces, washed well, and homogenized with 3 volumes of 1.15% (isotonic) KCl solution in a Teflon-glass homogenizer.

The homogenates were centrifuged at $9,000 \times g$ for 20 minutes. The supernatant

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solutions were then centrifuged at $105,000\times g$ for one hour and the microsome were suspended in 1.15% KCl solution.

Assays of drug-metabolizing enzymes: The incubation mixtures consisted of $9,000\times g$ supernatants equivalent to 500 mg of liver, 20 μ moles of glucose-6-phosphate, 0.6 μ moles of NADP, 50 μ moles of nicotinamide, 50 μ moles of $MgCl_2$, 1.4 ml of 0.2 M sodium phosphate buffer (pH 7.4), various substrates (hexobarbital, 3.0 μ moles; aminopyrine, 5.0 μ moles; aniline, 5.0 μ moles), and water to a final volume of 5.0 ml.

The mixtures were incubated at 37°C for 30 minutes under air. The hydroxylation of hexobarbital was determined by measuring the disappearance of the substrate according to the method of Cooper and Brodie (16). The N-demethylation of aminopyrine was determined by measuring the formation of 4-aminoantipyrine according to the method of La Du *et al.* (17). The hydroxylation of aniline was determined by measuring the formation of *p*-aminophenol according to the method described in a previous paper (11).

Assays of components of electron transport systems in liver microsomes:

NADPH oxidase was assayed spectrophotometrically according to the method of Gillette *et al.* (18). NADPH-cytochrome c reductase was determined according to the method of Williams and Kamin (19). NADPH-neotetrazolium reductase was determined according to the method described in a previous paper (20).

NADH oxidase, NADH-cytochrome c reductase and NADH-neotetrazolium reductase were determined according to the methods similar to those of NADPH oxidase, NADPH-cytochrome c reductase and NADPH-neotetrazolium reductase, respectively.

The content of cytochrome P-450 and b_5 was determined as described in previous paper (15). The difference spectrum of a microsomal preparation was measured by a Hitachi autorecording spectrophotometer with cuvettes of 1 cm optical pathway.

Determination of microsomal protein: Microsomal protein was measured according to the method of Lowry *et al.* (21).

RESULTS

1. The relative activity of NADPH-linked electron transport system and drug-metabolizing enzymes of female rats

NADPH-linked electron transport system and mechanism of drug-oxidation are represented schematically as given in Fig. 1. The activity of NADPH-linked electron transport system in female rats was much higher than that of drug-metabolizing enzymes (Table 1). For example, the activity of NADPH oxidase, which may represent the turn-over rate of the reduction and oxidation circle of cytochrome P-450, was about 90 times higher than that of aminopyrine N-demethylation and the activity of NADPH-cytochrome c reductase was about 970 times higher. Since the activity of these NADPH-linked electron transport system was determined at room temperature (20–23°C), the differences should be much higher at body temperature. Since the content of cytochrome P-450 was about 0.88 $m\mu$ mole/mg microsomal protein, the turn-over rate of the reduction and oxidation

FIG. 1. Schema for the NADPH-linked electron transport systems and drug-oxidizing system of liver microsomes and possible site of the action of androgen.

fp : Flavoprotein X : Unknown factor RH : Substrate

TABLE 1. The relative activity of NADPH-linked electron transport system and drug-metabolizing enzymes of female rats.

	Activity* (10^{-10} mole/mg protein/min)	Relative activity (Aminopyrine=1)
Aminopyrine N-demethylation	1.5 ± 0.1 (24)	
Hexobarbital hydroxylation	11.8 ± 0.7 (24)	7.9
Aniline hydroxylation	5.5 ± 0.2 (23)	3.7
NADPH oxidase	134 ± 6 (24)	89
NADPH-cytochrome c reductase	1455 ± 58 (24)	970
NADPH-NT reductase	569 ± 31 (24)	379
Cytochrome P-450	8.8 ± 0.5 (24)	5.9

* Cytochrome P-450 was expressed as content (10^{-10} mole/mg protein).

All the results are given as average $\pm$ standard error.

The figures in the brackets indicate number of the determination.

TABLE 2. Sex difference in the N-demethylation of aminopyrine and hydroxylation of hexobarbital by liver microsomes of rats, mice and rabbits.

Species	Sex	Aminopyrine N-demethylation (μ mole/g/min)	Differ. (%)	Hexobarbital hydroxylation (μ mole/g/min)	Differ. (%)
Rats	Female	28.4 ± 1.5 (18)		4.1 ± 0.3 (18)	
	Male	113.3 ± 8.1 (18)	+ 298*	18.5 ± 1.1 (17)	+ 351*
Mice	Female	41.4 ± 2.1 (15)		7.2 ± 0.5 (15)	
	Male	40.8 ± 1.5 (16)	- 2	8.4 ± 0.4 (16)	+ 17
Rabbits	Female	65.3 ± 3.1 (10)		10.9 ± 0.8 (11)	
	Male	66.5 ± 4.0 (11)	0	11.5 ± 1.0 (11)	+ 6

The results are expressed by the substrate metabolized by g wet weight of liver and given as average $\pm$ standard error.

The figures in the brackets indicate number of the determination.

The stars indicate the significant difference ($p < 0.05$).

circle of cytochrome P-450 under the absence of exogenous substrate is about 15 times per minute.

These results indicated that one of the rate limiting step for the oxidation of drugs may present in the oxygen transfer mechanism between oxygenated cytochrome P-450 and substrate.

2. *Sex difference in the N-demethylation of aminopyrine and hydroxylation of hexobarbital by liver microsomes of rats, mice and rabbits*

There was clear sex difference in the activities of N-demethylation of aminopyrine and hydroxylation of hexobarbital in liver microsomes of rats (Table 2). In contrast to these results, there was no sex difference in liver microsomes of rabbits and mice.

3. *Sex difference in the hydroxylation of aniline by liver microsomes of rats, mice and rabbits*

In contrast to the activities of aminopyrine N-demethylation and hexobarbital hydroxylation, there was a small sex difference in the activity of aniline hydroxylation in liver microsomes of rats (Table 3). Moreover, there was no sex difference in the activity of aniline hydroxylation in liver microsomes of rabbits and mice.

TABLE 3. Sex difference in the hydroxylation of aniline by liver microsomes of rats, mice and rabbits.

Species	Sex	Aniline hydroxylation ($\mu\text{mole/g/min}$)	Difference (%)
Rats	Female	14.1 ± 0.5 (18)	
	Male	18.7 ± 0.8 (17)	+33*
Mice	Female	35.1 ± 1.2 (14)	
	Male	39.0 ± 2.1 (16)	+11
Rabbits	Female	19.5 ± 1.5 (11)	
	Male	18.3 ± 1.8 (10)	-6

See the legend for Table 2.

4. *Sex difference in the activity of NADPH oxidase, NADPH-cytochrome c reductase, NADPH-dichlorophenolindophenol diaphorase and NADPH-neotetrazolium reductase and the content of cytochrome P-450 in liver microsomes of rats, mice and rabbits*

The activities of NADPH oxidase and NADPH-cytochrome c reductase were slightly

TABLE 4. Sex difference in the activities of NADPH oxidase and NADPH-cytochrome c reductase in liver microsomes of rats, mice and rabbits.

Species	Sex	NADPH oxidase ($\mu\text{mole/g/min}$)	Differ. (%)	NADPH-cyt c reductase ($\mu\text{mole/g/min}$)	Differ. (%)
Rats	Female	287 ± 10 (17)		4110 ± 158 (18)	
	Male	395 ± 17 (18)	+38*	5620 ± 297 (18)	+37*
Mice	Female	345 ± 21 (16)		3310 ± 130 (16)	
	Male	321 ± 27 (16)	-7	3130 ± 155 (16)	-5
Rabbits	Female	141 ± 20 (11)		3020 ± 121 (11)	
	Male	132 ± 21 (10)	-7	2850 ± 138 (11)	-6

See the legend for Table 2.

higher in male rats than in female rats. In contrast to these results, there was no sex difference in rabbits and mice (Table 4). Similar results were observed when dichlorophenolindophenol was used as electron acceptor in stead of cytochrome c. The activity of NADPH-neotetrazolium reductase in liver microsomes isolated from male rats was higher than that of female rats. However, there was no sex difference in the activity of rabbits and mice liver microsomes (Table 5). The content of cytochrome P-450 was slightly higher in male rats than in female rats. However, there was no sex difference in the content of cytochrome P-450 of rabbit and mouse liver microsomes (Table 5).

TABLE 5. Sex difference in the activity of NADPH-neotetrazolium (NT) reductase and the content of cytochrome P-450 in liver microsomes of rats, mice and rabbits.

Species	Sex	NADPH-NT reductase (m μ mole/g/min)	Differ. (%)	Cyt P-450 (m μ mole/g)	Differ. (%)
Rats	Female	1230 $\pm$ 49 (17)		20.5 $\pm$ 1.5 (18)	
	Male	2510 $\pm$ 158 (17)	+104*	27.2 $\pm$ 1.3 (17)	+33*
Mice	Female	1380 $\pm$ 58 (16)		26.9 $\pm$ 1.8 (15)	
	Male	1410 $\pm$ 80 (15)	+ 2	27.5 $\pm$ 1.2 (15)	+10
Rabbits	Female	1093 $\pm$ 122 (11)		42.8 $\pm$ 2.9 (11)	
	Male	1145 $\pm$ 143 (11)	+ 5	39.0 $\pm$ 3.4 (10)	- 9

See the legend for Table 2.

5. Sex difference in the activities of NADH oxidase, NADH-cytochrome c reductase, NADH-dichlorophenolindophenol diaphorase and NADH-neotetrazolium reductase and the content of cytochrome b₅ in liver microsomes of rats, mice and rabbits

In contrast to the activity of NADPH-linked electron transport system, there was no sex difference in the activities of NADH oxidase, NADH-cytochrome c reductase, NADH-dichlorophenolindophenol diaphorase and NADH-neotetrazolium reductase of rat liver microsomes (Tables 6 and 7).

It was of interest that there was no sex difference in the content of cytochrome b₅ in rat liver microsomes which is considered as a component of NADH-linked electron transport system (Table 7). Moreover, there is no sex difference in the activity of NADH-linked electron transport system in liver microsomes of rabbits and mice.

TABLE 6. Sex difference in the activities of NADH oxidase and NADH-cytochrome c reductase in liver microsomes of rats, mice and rabbits.

Species	Sex	NADH oxidase (m μ mole/g/min)	Differ. (%)	NADH-cyt c reductase (μ mole/g/min)	Differ. (%)
Rats	Female	161 $\pm$ 7 (18)		60 $\pm$ 2.5 (18)	
	Male	153 $\pm$ 11 (18)	-5	54 $\pm$ 3.1 (18)	-10
Mice	Female	219 $\pm$ 8 (15)		31 $\pm$ 3.1 (16)	
	Male	213 $\pm$ 13 (16)	-2	28 $\pm$ 1.8 (16)	-11
Rabbits	Female	185 $\pm$ 13 (7)		25 $\pm$ 1.3 (7)	
	Male	171 $\pm$ 14 (8)	-7	24 $\pm$ 2.9 (8)	- 4

See the legend for Table 2.

TABLE 7. Sex difference in the activity of NADH-neotetrazolium (NT) reductase and the content of cytochrome b_5 in liver microsomes of rats, mice and rabbits.

Species	Sex	NADH-NT reductase (m μ mole/g/min)	Differ. (%)	Cyt b_5 (m μ mole/g)	Differ. (%)
Rats	Female	521 $\pm$ 21 (18)		16.4 $\pm$ 1.3 (18)	
	Male	549 $\pm$ 33 (17)	+ 5	16.3 $\pm$ 0.6 (18)	0
Mice	Female	440 $\pm$ 19 (16)		11.5 $\pm$ 0.5 (15)	
	Male	398 $\pm$ 25 (16)	-10	10.7 $\pm$ 0.4 (16)	-7
Rabbits	Female	—		19.0 $\pm$ 1.5 (8)	
	Male	—		20.4 $\pm$ 1.1 (8)	+7

See the legend for Table 2.

DISCUSSION

In the present investigation, it was demonstrated that there were slight sex differences in the activities of aniline hydroxylation and NADPH-linked electron transport system and cytochrome P-450 content of rat liver microsomes, but there were no sex difference in the activity of NADH-linked electron transport system and cytochrome b_5 content of rat liver microsomes. Moreover, there were no sex difference in the activities of drug oxidation and NADPH- and NADH-linked electron transport system and the contents of cytochrome P-450 and b_5 of rabbits and mice liver microsomes.

Therefore, it might be supposed that the slight sex difference in the activity of aniline hydroxylation in rats liver microsomes is likely correlated to the difference in the activity of NADPH-linked electron transport system and the content of cytochrome P-450.

Moreover, it is likely that the clear sex difference in the activities of aminopyrine N-demethylation and hexobarbital hydroxylation is not only due to the difference in the activity of NADPH-linked electron transport system, but also to the difference in the step of the terminal oxidation (Fig. 1).

Since the activity of drug-oxidation in liver micorsomes is regulated by anabolic action of male sex hormone (9, 10, 22, 23), the site of action of androgen on the activities of aminopyrine N-demethylation and hexobarbital hydroxyaltion are likely connected with the NADPH-linked electron transport system, cytochrome P-450 and the terminal oxidase. From the results presented in this paper it is clear that the action of male sex hormone on the terminal oxidase is likely major factor for the stimulation of the activities of aminopyrine N-demethylation and hexobarbital hydroxylation, but the action of male sex hormone is likely negligible for the activity of aniline hydroxylation (Fig. 1).

These views are in accordance with the recent observation of Schenkman *et al.* (24). Moreover, they observed that the affinities of hexobarbital and aminopyrine to the terminal oxidase were much greater in male rats than in female rats. Furthermore, Castro and Gillette observed that the higher affinity of N-ethylmorphine to the terminal oxidase is only observed in rats (25).

The mechanism of androgen to increase the activities of NADPH-linked electron trans-

port system and the terminal oxidase are not clear, but the following possibility could be speculated.

1) Androgen may stimulate the biosynthesis and/or prevent the degradation of each the terminal oxidase.

2) Androgen may stimulate the biosynthesis of a specific protein or phospholipids and then may modified the ultra-fine structure of endoplasmic reticulum and results in an increase in the accessibility of some substrates to the terminal oxidase.

3) Androgen may stimulate the biosynthesis of phospholipids and then may modified the state of elcetron charge on microsomes and results in an increase in the affinity for the formation of the substrate-enzyme complex and active oxygen transfer.

The first one is likely most probable speculation and the third speculation is more attractive, but it is apparently difficult to consider with numerous data from our laboratory (11, 12, 14, 15, 26).

However, it is of interest to recall that the biosynthesis of phospholipid by rats liver microsomes is influenced by androgen (27). Since the sex differences in NADPH-linked electron transport system and drug-oxidizing activity of liver microsomes were observed only in rats, but not in the other animals, it is probable that androgen stimulates the activities of NADPH-linked electron transport system and the terminal oxidase mediated through a common factor and this common factor is genetically absent in the other animals (Fig. 1).

Thus, androgen may act as an exogenous regulator by inactivating an endogenous repressor according to the operon theory of Monad and Jacob (28).

Further studies on molecular level will be necessary to elucidate these problems.

SUMMARY

1. The marked sex difference was observed in the N-demethylation of aminopyrine and hydroxylation of hexobarbital, on the other hand, only slight sex difference was observed in the hydroxylation of aniline in rats liver microsomes.

2. There was no sex difference in the N-demethylation of aminopyrine and hydroxylation of hexobarbital and aniline in mice and rabbits.

3. The activities of microsomal NADPH oxidase, NADPH-cytochrome c reductase and the content of cytochrome P-450 were about 30–45% higher in male rats than in female rats and much greater sex difference was observed in the activity of NADPH-neotetrazolium reductase.

4. There was no sex difference in the activity of NADPH-linked electron transport system in liver microsomes of mice and rabbits.

5. There was no sex difference in the activities of NADH oxidase, NADH-cytochrome c reductase and NADH-neotetrazolium reductase and the content of cytochrome b_5 in liver microsomes of rats, mice and rabbits.

6. These results indicate that the slight sex difference in the hydroxylation of aniline likely related to the slight sex difference in the activity of microsomal NADPH-linked

electron transport system, on the other hand, the marked sex difference in the N-demethylation of aminopyrine and hydroxylation of hexobarbital is mainly due to the difference in the activity of the terminal oxidase.

7. Thus, there are following two sites for the action of androgen in rat liver microsomes. The first site of the action is at the level of NADPH-linked electron transport system and the second site is at the level of the terminal oxidase.

Possible mechanism of the androgen to stimulate the activity of microsomal drug-oxidation was discussed.

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