SPECIES AND SEX DIFFERENCES IN THE SUBSTRATE-INDUCED SPECTRAL CHANGE OF P-450 IN RELATION TO THE ACTIVITY OF DRUG OXIDATION IN LIVER MICROSOMES

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A number of foreign compounds of high lipid-solubility are oxidatively metabolized to more water soluble compounds by liver microsomal hydroxylase system, called drug-metabolizing enzymes, in the presence of NADPH and oxygen (1, 2).

There are marked species differences among various animals in the activity of drug-metabolizing enzymes, and these species differences are assumed to be responsible factors for the species differences in the effect and toxicity of a variety of drugs (2–5).

Recent studies have established that a hemoprotein called P-450 (6) is involved in the monooxygenase reactions in liver microsomes as the oxygen-activating component (7–9). More recently, Imai and Sato (10), and Schenkman et al. (11) have reported that a number of drugs, substrates of hepatic microsomal monooxygenases, react with the microsomal cytochrome to give two characteristic types of spectral change. These results have suggested that the spectral changes observed are indicative of substrate interaction for enzymic hydroxylation (11) and that the magnitude of the substrate binding with cytochrome P-450 is one of important factors for the rate of the over-all hydroxylation of drugs by liver microsomes. It was therefore of interest to investigate whether the magnitude of the substrate binding with P-450 is related to the activity of hydroxylations of drugs by liver microsomes of various species of animals. In the present communication, the magnitude of the substrate binding with P-450 has been investigated in both sexes of rats, mice and rabbits.

MATERIALS AND METHODS

Male and female rats of Wistar strain, weighing about 180 and 160 g, respectively, male and female mice of the dd strain, weighing about 25 and 22 g, respectively, and male and female rabbits, weighing about 2.1 and 1.9 kg, respectively, were used.

Preparation of microsomes

The animals were killed by bleeding or decapitation and the livers were removed immediately, chopped into small pieces, washed thoroughly and homogenized with 3 volumes of 1.15% KCl solution in a Teflon-glass homogenizer. The homogenate was

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centrifuged at 10,000 x g for 20 minutes. The supernatant fraction was then centrifuged at 105,000 x g for 1 hour, and the microsomes were suspended in 1.15% KCl solution.

Assays of hexobarbital and aniline hydroxylation

The incubation mixture consisted of 1 ml of 10,000 x g supernatant equivalent to 250 mg liver, 10 μmoles of glucose 6-phosphate, 0.8 μmole of NADP, 25 μmoles of nicotinamide, 25 μmoles of MgCl₂, 0.7 ml of 0.2 M sodium phosphate buffer (pH 7.4), hexobarbital (2 μmoles) or aniline (5 μmoles), and water to a final volume of 2.5 ml. The mixtures were incubated at 37°C for 30 minutes under air. The hydroxylation of hexobarbital was determined by the disappearance of the substrate according to Cooper and Brodie and the hydroxylation of aniline was determined by the formation of p-aminophenol according to the method described by Kato and Gillette as reported in a previous paper (5).

Determination of substrate-induced difference spectra

The spectral changes induced by hexobarbital and aniline were determined as described in a previous paper (12) according to Schenkman et al. (11). The difference spectra were recorded at room temperature with a Hitachi EPS-3T recording spectrophotometer with an integral sphere attachment.

Determinations of microsomal protein and cytochrome P-450

The content of microsomal protein was measured according to the method of Lowry et al. (13). The content of cytochrome P-450 was determined by the difference spectrum of the carbon monoxide complex as described in a previous paper (12) and the results are expressed as μmole per mg microsomal protein according to Omura and Sato (6).

RESULTS

1. Spectral change of P-450 induced by hexobarbital in liver microsomes from rats, mice and rabbits

The content of cytochrome P-450 was higher in male rats than in female, but there was no clear sex difference in mice and rabbits (Table 1). The content of P-450 in rabbits was considerably higher than those in mice and rats.

In agreement with the observation of Schenkman et al. (14), the magnitude of spectral

| Species  | Sex   | Microsomal protein (mg/g wet weight) | Cytochrome P-450 (μmole/mg protein) |
|----------|-------|-------------------------------------|------------------------------------|
| Rats     | Male  | 28.1 ± 0.04 (12)                    | 0.96 ± 0.06 (12)                   |
| Rats     | Female| 27.9 ± 0.03 (12)                    | 0.72 ± 0.04 (12)                   |
| Mice     | Male  | 29.3 ± 0.05 (9)                     | 1.08 ± 0.05 (9)                    |
| Mice     | Female| 29.4 ± 0.07 (9)                     | 1.04 ± 0.07 (9)                    |
| Rabbits  | Male  | 27.3 ± 0.06 (8)                     | 1.63 ± 0.12 (8)                    |
| Rabbits  | Female| 27.6 ± 0.04 (8)                     | 1.58 ± 0.10 (8)                    |

The results are expressed as averages ± S.E. The figures in the parentheses indicate number of the determination. Pooled liver from 3-4 mice were used for each determination.
change induced by hexobarbital in liver microsomes was markedly greater in male than in female rats, but there was no clear sex difference in mice and rabbits (Table 2).

The magnitude of the spectral change was considerably greater in male and female rabbits than in male and female mice and in female rats. The magnitude of hexobarbital-induced spectral change per P-450, indicating the binding capacity of P-450 with hexobarbital, was higher in male rats than in female. It is of interest to note that the binding capacity of P-450 with hexobarbital was apparently similar in all microsomes isolated from both sexes of rabbits and mice, and female rats.

2. Spectral change of P-450 induced by aniline in liver microsomes from rats, mice and rabbits

The magnitude of spectral change induced by aniline was slightly greater in male rats than in female rats, but there was no sex difference in mice and rabbits (Table 3).

The greatest magnitude of the spectral change was observed in rabbits and the smallest was observed in female rats. The magnitude of the spectral change in rabbits was about twice greater than in female rats.

It is of interest to note that the magnitude of aniline induced spectral change per P-

| Species | Sex | Hexobarbital-induced spectral change | Aniline-induced spectral change |
|---------|-----|-------------------------------------|---------------------------------|
|         |     | (ΔOD×10^3/mg protein)               | (ΔOD×10^3/mg/mole P-450)       |
| Rats    | Male| 17.9±0.7 (12)                       | 11.8±0.7 (12)                  |
| Rats    | Female| 7.0±0.5 (12)                       | 8.9±0.5 (12)                  |
| Mice    | Male| 8.1±0.4 (9)                         | 13.7±0.8 (9)                  |
| Mice    | Female| 8.4±0.3 (9)                         | 13.9±0.7 (9)                  |
| Rabbits| Male| 15.2±0.9 (8)                        | 19.2±1.0 (8)                  |
| Rabbits| Female| 15.0±1.2 (8)                        | 18.2±1.3 (8)                  |

The results are expressed by the difference in the optical density between 421 mμ and 500 mμ and given as averages ±S.E. The figures in the parentheses indicate number of the determination. The concentration of hexobarbital was 1.6 mm.

The results are expressed by the difference in the optical density between 421 mμ and 500 mμ and given as averages ±S.E. The figures in the parentheses indicate number of the determination. The concentration of aniline was 2.0 mm.
450, indicating the binding capacity of P-450 with aniline, was apparently similar in all microsomes isolated from rats, mice and rabbits.

These results with aniline and hexobarbital indicate that there is no species difference in microsomes of rats, mice and rabbits for the binding capacity of P-450 with aniline and hexobarbital except for male rats in which the binding capacity is increased by the action of androgen (15).

3. The activity of hexobarbital hydroxylation in relation to the content of P-450 and the magnitude of spectral change in liver microsomes from rats, mice and rabbits

As shown in Table 4, there is considerable species difference in the hydroxylation activity of hexobarbital per mg protein, but the species difference in the hydroxylation activity per P-450 was relatively small except for male rats in which the activity is stimulated by androgen.

However, it is of interest to note that the hydroxylation activity per the magnitude of the spectral change was identical in both sexes of rats, mice and rabbits. These results suggest that the species and sex difference in the hydroxylation activity of hexobarbital is clearly related to the amount of the bound substrate with P-450 in the hydroxylation reaction.

4. The activity of aniline hydroxylation in relation to the content of P-450 and the magnitude of spectral change in liver microsomes from rats, mice and rabbits

There is considerable species difference in the hydroxylation activity of aniline per mg protein, and the activity of microsomes from mice was twice higher than that from rats (Table 5). The hydroxylation activity per P-450 was still higher in mice than in rats and rabbits.

In contrast to the results obtained with hexobarbital, there was clear species difference for the hydroxylation activity of aniline per the spectral change. As shown in Table 5, the hydroxylation activity was highest in mice and lowest in rabbits. The activity in rabbits was about 57% of those in mice. This result suggests that the species difference

| Species  | Sex   | Hexobarbital hydroxylation (mumole/30 min) |
|----------|-------|------------------------------------------|
|          |       | per mg protein | per mumole P-450 | per ΔOD×10^3 |
| Rats     | Male  | 108.7±6.5(12) | 103.2±6.9(12) | 6.03±0.58(12) |
| Rats     | Female| 38.5±2.1(11)  | 53.4±3.1(11)  | 5.42±0.43(11) |
| Mice     | Male  | 45.3±2.9 (9)  | 41.9±2.8 (9)  | 5.59±0.49 (9) |
| Mice     | Female| 46.0±2.1 (9)  | 44.2±2.5 (9)  | 5.48±0.61 (9) |
| Rabbits  | Male  | 84.5±5.5 (8)  | 51.8±3.7 (8)  | 5.57±0.03 (8) |
| Rabbits  | Female| 82.4±6.7 (8)  | 52.2±4.2 (8)  | 5.49±0.70 (8) |

The results are expressed by the hydroxylation per mg protein, mumole P-450 or ΔOD×10^3 and given as averages±S.E. The figures in the parentheses indicate number of the determination. The concentration of hexobarbital was 1.6 mM.
TALE 5. The activity of aniline hydroxylation in relation to the content of P-450 and the magnitude of spectral change in liver microsomes from rats, mice and rabbits.

The results are expressed by the hydroxylation per mg protein, mpmole P-450 or AOD x 10³ and given as averages±S.E. The figures in the parentheses indicate number of the determination. The concentration of aniline was 2.0 mm.

in the hydroxylating activity of aniline is not only related to the amount of the bound substrate with P-450, but also other factor, presumably the rate of reduction of P-450-aniline complex by NADPH-cytochrome P-450 reductase, may be involved in the species difference in the hydroxylation of aniline.

DISCUSSION

The importance of the interaction of substrates of microsomal hydroxylase with P-450 has been recognized by several lines of evidence. For example, the mechanism of the action of androgen or estrogen on the activity of hexobarbital hydroxylation is assumed to be related to the increase or decrease in the binding capacity of P-450 with hexobarbital (15). Moreover, the effects of phenobarbital and methylcholanthrene on the activities of hexobarbital and aniline hydroxylations seem to be related to the effects on the magnitudes of the substrate interaction with P-450 (12).

In the present studies, it has been demonstrated that there is no sex difference in the magnitude of spectral change induced by hexobarbital and in the binding capacity of P-450 with hexobarbital in agreement with the lack of sex difference in the activity of hexobarbital hydroxylation in mice and rabbits (16). Moreover, it has been demonstrated that although there is marked species difference in the activity of hexobarbital hydroxylation and the content of cytochrome P-450, there is no clear species difference in the binding capacity of P-450 with hexobarbital, except for male rats in which the binding capacity is stimulated by androgen (15). Similarly, there is no clear species difference in the binding capacity of P-450 with aniline.

It is of interest to note that the hydroxylating activity of hexobarbital per the magnitude of the spectral change in microsomes isolated from rats, mice and rabbits is essentially same, whereas the hydroxylating activity of aniline per the magnitude of the spectral change in mice is about 74 and 50%, respectively, higher than that in rabbits and rats.

These results suggest that the sex difference in the amount of the bound substrate with P-450 may be a responsible factor for the species difference in the activity of hexobar-
bital hydroxylation, whereas other factors may be involved in the species difference in the hydroxylation of aniline. The species difference in the rate of the reduction of P-450-aniline complex by NADPH-cytochrome P-450 reductase might be assumed to be a responsible factor (17).

It has been known that hexobarbital induced type I spectral change, whereas aniline induced type II spectral change and this difference seems to be related to the difference in the binding site of the substrates (10, 11). Gigon et al. (17) recently reported that the addition of the substrates which induce type I spectral change, such as hexobarbital, accelerates the rate of reduction of P-450-substrate complex, whereas the addition of the substrates which induce type II spectral change, such as aniline, decelerates the rate of reduction.

Therefore, the observed difference for the species difference between the hydroxylating activity of hexobarbital and aniline per the spectral change may be related to the difference in the binding site of P-450. However, further detailed studies with a variety of substrates will be required for better understanding of the mechanism of species difference in the hydroxylation of drugs by liver microsomes.

SUMMARY

1. The magnitudes of spectral changes of cytochrome P-450 induced by hexobarbital and aniline in liver microsomes were investigated in rats, mice and rabbits in order to determine whether the microsomal hydroxylating activities are related to the magnitudes of the spectral changes.

2. There are no sex differences in the magnitude of spectral change induced by hexobarbital and in the binding capacity of P-450 with hexobarbital in mice and rabbits in agreement with the lack of sex difference in the activity of hexobarbital hydroxylation.

3. Although there is marked species difference in the activity of hexobarbital hydroxylation and the content of P-450, there is no clear species difference in the binding capacity of P-450 with hexobarbital, except for male rats in which the binding capacity is stimulated by androgen. Similarly, there is no clear species difference in the binding capacity of P-450 with aniline.

4. The hydroxylating activity of hexobarbital per the magnitude of the spectral change in microsomes isolated from rats, mice and rabbits is essentially the same, whereas the hydroxylating activity of aniline per the magnitude of the spectral change in mice is about 74 and 50%, respectively, higher than that in rabbits and rats.

5. These results suggest that the sex difference in the amount of the bound substrate with P-450 may be responsible factor for the species difference in the activity of hexobarbital hydroxylation, whereas other factor(s) may be involved in the species difference in the hydroxylation of aniline. The rate of the reduction of P-450-aniline complex is assumed to be a responsible factor.
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