Vol. 103, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 16, 1981 Pages 1-7

PARTIAL PURIFICATION AND CHARACTERIZATION OF CYTOCHROME P-450 RESPONSIBLE FOR THE OCCURRENCE OF SEX DIFFERENCE IN DRUG METABOLISM IN THE RAT

Tetsuya Kamataki, Kaori Maeda*, Yasushi Yamazoe,

Tsuneji Nagai* and Ryuichi Kato

Department of Pharmacology, School of Medicine,
Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160
and *Department of Pharmaceutics, Hoshi Institute of
Pharmaceutical Sciences, 2-4-41 Ebara, Shinagawa-ku
Tokyo 142, Japan

Received September 9,1981

SUMMARY: Cytochrome P-450 in cholate-solubilized microsomes from untreated male and female rats was divided into two fractions (I and II) by ω-amino-n-octyl Sepharose 4B columns. A marked sex difference in the O-depropylation of 7-propoxycoumarin was seen between the I fractions from male and female rats in which male rat fraction I exhibited higher activity than that of female rats. Addition of cytochrome b_5 resulted in about 2-fold increase in the O-depropylation activities of the I fractions from male and female rats but not in those of the II fractions. Based on these and other results, we propose that at least one of multiple forms of cytochrome P-450 responsible for the occurrence of the sex difference in drug metabolism requires cytochrome b_5 for maximal activity.

There are marked sex differences in the activities of hepatic drug metabolizing enzymes as well as in the magnitude of drug responses in the rat. The occurrence of the sex difference is dependent on the drugs used and the hormonal status of the animals. Viewing these lines of evidence, Kato and his co-workers proposed

---

Abbreviations used are: EDTA, ethylene diamine tetraacetic acid; SDS, sodium dodecyl sulfate; HEPES, sodium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate; Buffer A, 10 mM potassium phosphate buffer (pH 7.4) containing 0.5 % sodium cholate, 0.2 % Emulgen 911, 0.1 mM EDTA, 1.0 mM dithiothreitol and 20 % glycerol; Buffer B, 200 mM potassium phosphate buffer (pH 7.4) containing 0.5 % sodium cholate, 0.5 % Emulgen 911, 0.1 mM EDTA, 1.0 mM dithiothreitol and 20 % glycerol; Buffer C, 100 mM potassium phosphate buffer (pH 7.25) containing 0.5 % sodium cholate and 20 % glycerol.

0006-291X/81/210001-07$01.00/0

that in male rats one or more of multiple drug metabolizing enzymes were synthesized under androgen-sensitive gene control (1).

It has been widely accepted that cytochrome P-450 in liver microsomes plays a central role as a drug metabolizing enzyme. The presence of multiple forms of cytochrome P-450 has also been confirmed (2-4). Thus, it seemed most reasonable to assume that one or more forms of cytochrome P-450 existing to a greater extent in male rats than in female rats were responsible for the occurrence of sex differences (5,6).

In the present communication, we report that a form(s) of cytochrome P-450 present in male rats but in lesser amounts in female rats requires cytochrome b_5 for maximal activity expressed as the O-depropylation of 7-propoxycoumarin.

MATERIALS AND METHODS

Separation of multiple forms of cytochrome P-450 from microsomes of male and female rats Microsomes(1.5-2.0 g) from untreated male and female rats of Sprague-Dawley strain (8-11 week old) were solubilized with sodium cholate and applied to an ω-amino-n-octyl Sepharose 4B column (3.5 x 40 cm) by the method of Imai and Sato(7,8) with modifications(9). The columns were washed with 500 ml of 10 mM potassium phosphate (pH 7.4) containing 0.5 % sodium cholate, 0.1 mM EDTA, 1.0 mM dithiothreitol and 20 % glycerol. Cytochrome P-450 fractions (I and II) were eluted with Buffers A and B. A portion of pooled I or II fraction (25-30 nmoles of cytochrome P-450) was applied to a hydroxylapatite column (2.5 x 10 cm) equilibrated with 10 mM potassium phosphate (pH 7.25) containing 20 % glycerol. Before application to this column, the II fractions were dialyzed overnight against 2000 ml of Buffer A, and diluted with the same amount of 20 % glycerol. The hydroxylapatite column was washed with 30 mM potassium phosphate (pH 7.25) containing 20 % glycerol to remove excess detergents. Cytochrome P-450 was eluted with 300 mM potassium phosphate (pH 7.25) containing 20 % glycerol and the eluates were dialyzed overnight against 2000 ml of 10 mM potassium phosphate (pH 7.25) containing 20 % glycerol.

Purification of cytochrome b_5 We have developed a simple method to purify rat liver cytochrome b_5 with buffers commonly used for the purification of cytochrome P-450. Liver microsomes (5-7 g) from untreated male rats were solubilized with Buffer A, which contained cholate and Emulgen 911, at a concentration of about 10 mg protein per ml for 2 hr with stirring. Solubilization was carried out at 0-4°. The detergent-treated microsomes were

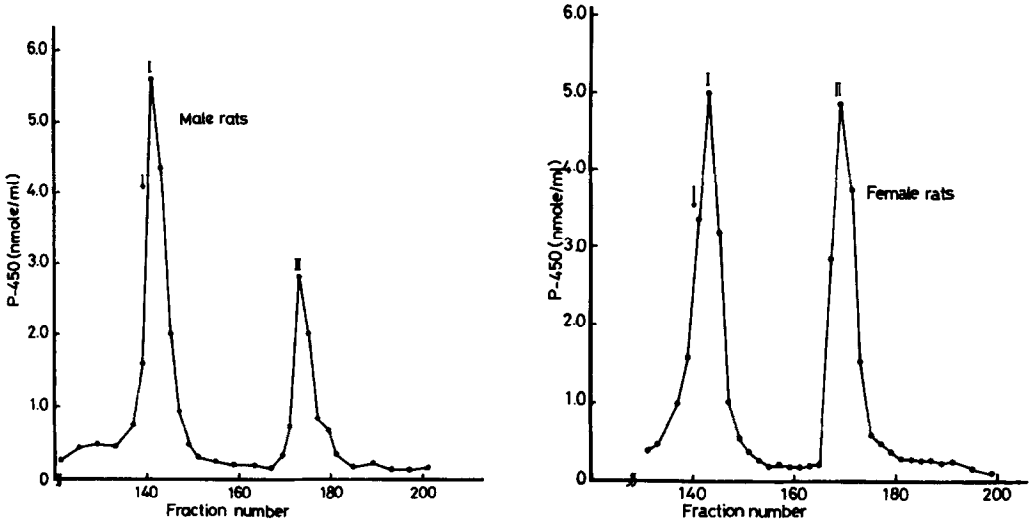

Fig. 1. Elution profiles from ω-amino-n-octyl Sepharose 4B columns of cytochrome P-450 in microsomes of male and female rats. The I and II fractions were eluted with Buffers A and B. At the points indicated by arrows in the charts, the buffer was changed to Buffer B.

centrifuged at 105,000 x g for 1 hr, and then the supernatant fraction was applied to a Whatman DE-52 column (5 x 10 cm) equilibrated with Buffer A. The column was washed with 600 ml of Buffer A containing 100 mM sodium chloride. The red band on the DE-52 column was cut after removal by introduction of nitrogen gas from the bottom of the column. Cytochrome b_5 was extracted with 50 mM Tris-HCl (pH 7.7) containing 280 mM potassium chloride, 0.2 % Emulgen 913 and 20 % glycerol. The extract diluted with the same amount of 50 mM Tris-HCl (pH 7.7) containing 0.2 % Emulgen 913 and 20 % glycerol was then applied to a DEAE-Sephadex A-50 (3.5 x 11 cm) column equilibrated with 50 mM Tris-HCl (pH 7.7) containing 140 mM potassium chloride, 0.2 % Emulgen 913 and 20 % glycerol. The column was washed with 900 ml of the same buffer. Cytochrome b_5 was eluted broadly. The fractions containing cytochrome b_5 were pooled and applied to a ω-amino-n-octyl Sepharose 4B column (2.5 x 12 cm) previously equilibrated with the same buffer used for the DEAE-Sephadex A-50 column. The column was washed with 2 times the column volume of Buffer C. Cytochrome b_5 was eluted with Buffer C containing 0.3 % sodium deoxycholate, and the eluate was dialyzed twice against 1000 ml of 10 mM potassium phosphate (pH 7.25) containing 20 % glycerol. The specific content of cytochrome b_5 in the final preparation ranged from about 38 to 47 nmoles per mg protein with the recovery of 17-20 %.

Purification of NADPH-cytochrome P-450 reductase NADPH-cytochrome P-450 reductase was purified to specific activities greater than 50 units per mg of protein by the method of Yasukochi and Masters (10) with some minor modifications. The activity of the reductase was defined as a unit which reduced one μmole of cytochrome c per min.

Table I. The absorption maxima in the carbon monoxide difference spectra, and the recoveries and specific contents of cytochrome P-450 in the I and II fractions eluted ω-amino-n-octyl Sepharose 4B columns

Sex	Fraction	Peak in CO-diff.(nm)	Rec.(%)*	S.C.(nmole/mg)**
Male	I	450.5	23.8-27.5	2.56-3.78
	II	449.0	15.4-19.3	1.40-2.42
Female	I	449.0	19.3-31.5	2.00-3.12
	II	448.0	14.0-25.2	0.98-2.22

* Recovery of cytochrome P-450 from microsomes
** Specific content

Assay methods Cytochrome P-450 was determined by the method of Omura and Sato (11) and the content was calculated according to the equation described by Imai and Sato (12). NADPH-cytochrome P-450 reductase was measured by the method of Phillips and Langdon (13). Cytochrome b_5 was determined from reduced minus oxidized difference spectra using NADH or sodium dithionite as a reducing agent (14). Protein was determined by the method reported by Lowry et al (15).
Materials ω-Amino-n-octyl-Sepharose 4B was synthesized by the method of Cuatrecasas (16). Emulgen 911 and 913 were generous gifts from Kao-Atlas Co., Japan. 7-Propoxycoumarin was synthesized by the method previously described (6).

RESULTS AND DISCUSSION

The elution profiles of cytochrome P-450 from ω-amino-n-octyl-Sepharose 4B columns are shown in Fig. 1. Cytochrome P-450 was divided into two peaks (I, II) by washing the columns successively with Buffers A and B. The absorption maxima in the reduced carbon monoxide difference spectra, and the recovery and specific contents of cytochrome P-450 in the fractions of I and II are shown in Table I. Cytochrome P-450 in the II fractions from male and female rats had an absorption maximum at shorter wave length than did cytochrome P-450 from corresponding I fractions. In addition, both I and II fractions of female rats showed absorption maxima at a shorter wave length than those of male fractions, probably suggesting that female microsomes contain larger amounts of cytochrome P-448 than do the male microsomes. This is in accordance with our previous result that 7-propoxycoumarin O-de-

Table II. Comparison of 7-propoxycoumarin O-depropylation activities of cytochrome P-450 in the I and II fractions of male and female rats

	Activity (nmole/nmoleP-450/min)			
	Male I	Male II	Female I	Female II
Minus cytochrome b_5	0.313	0.512	0.055	0.791
Plus cytochrome b_5	0.748	0.534	0.090	0.779

The incubation mixture consisted of 0.1 nmole of cytochrome P-450, 0.5 unit of NADPH-cytochrome P-450 reductase, 15 μg of dilauroyl-L-3-phosphatidylcholine, 50 μg of sodium deoxycholate, 50 mM HEPES (pH 7.4), 0.1 mM EDTA, an NADPH-generating system (0.5 mM NADP, 5 mM glucose 6-phosphate, 0.5 unit of glucose 6-phosphate dehydrogenase and 5 mM magnesium chloride) and 0.5 mM 7-propoxycoumarin in a final volume of 0.5 ml. When necessary, cytochrome b_5 was added. Incubations were carried out at 37° for 15 min. 7-Hydroxycoumarin formed during incubations was determined by the method of Aitio (17).
Activities presented in the Table are means of duplicate determinations.

propylation activity of female microsomes is more sensitive to inhibition by 7,8-benzoflavone than that of male microsomes (6).

Comparison of 7-propoxycoumarin O-depropylase activities and the effect of cytochrome b_5 on the activities of cytochrome P-450 in the I and II fractions of male and female rats are shown in Table II. Both I fractions from male and female microsomes showed an appreciable sex difference while no such sex difference was seen between the II fractions. Addition of purified cytochrome b_5 resulted in the enhancement of the O-depropylation activities in the I fractions but not in the II fractions of male and female rats. Based on these results, we confirm that a form(s) of cytochrome P-450 responsible for the occurrence of the sex difference in 7-propoxycoumarin O-depropylation is present in the I fraction and this cytochrome P-450 requires cytochrome b_5 for maximal activity. The latter idea probably accounts for the sex difference in the NADH-synergism of NADPH-dependent drug oxidations. Correia and Mannering (18) reported that the NADH-synergism of NADPH-dependent ethylmorphine N-demethylase activity was seen in male rats to a larger extent than in female rats. In

accordance with these results, we also found that male microsomes showed the NADH-synergism in 7-propoxycoumarin O-depropylation (31 %) and benzphetamine N-demethylation (29 %). A similar NADH-synergism was not seen in female microsomes. Based on these data, it is suggested that microsomes from male and female rats contain different forms of cytochrome P-450, thus resulting in the sex differences in the NADH-synergism of the drug metabolizing activities.

Our preliminary experiments have shown that the cytochrome P-450 from fraction I of male and female microsomes shows marked sex differences with respect to hexobarbital hydroxylation, 7-propoxycoumarin O-depropylation and protein bands on SDS-polyacrylamide gel electrophoresis. The molecular weight of a protein band in male rats was 52,000 while those of two protein bands in female rats were 50,000 and 47,500. These results and the data presented in this paper suggest that a major cytochrome P-450 fraction obtained by application of the I fractions from male and female rats to Whatman DE-52 columns contains cytochrome P-450 responsible for the sex differences in drug metabolizing activities.

ACKNOWLEDGEMENT

This research was supported by the Grant-in-Aid from the Ministry of Education, Science and Culture of Japan. The authors are indebted to Miss Mieko Soshino for her skillful technical assistance. The authors are grateful to Dr. Patrick W. Beatty, Shell Development Company, for his kind suggestion in the preparation of this manuscript.

REFERENCES

1. Kato, R. (1974) Drug Metab. Rev., 3, 1-32
2. Coon, M. J., Vermilion, J. L., Vatsis, K. P., French, J. S., Dean, W. L., and Haugen, D. A. (1977) Drug Metabolism Concept (D. M. Jerina, ed), pp46-71, Amer. Chem. Soc., Washington
3. Guengerich, F. P. (1979) Pharmac. Ther., 6, 99-121
4. Lu, A. Y. H., and West, S. B. (1980) Pharmac. Rev., 31, 277-295

5. Kahl, R., Buecker, N., and Netter, K. J. (1977) Microsomes and Drug Oxidations (V. Ullrich ed), pp177-183, Pergamon Press, New York
6. Kamataki, T., Ando, M., Yamazoe, Y., Ishii, K., and Kato, R. (1980) Biochem. Pharmac., 29, 1015-1022
7. Imai, Y., and Sato, R. (1974) J. Biochem. 75, 689-697
8. Imai, Y., and Sato, R. (1974) Biochem. Biophys. Res. Commun., 60, 8-14
9. Kamataki, T., Belcher, D. H., and Neal, R. A. (1976) Mol. Pharmac. 12, 921-932
10. Yasukochi, Y., and Masters, B. S. S. (1976) J. biol. Chem., 251, 5337-5344
11. Omura, T., and Sato, R. (1964) J. biol. Chem. 239, 2379-2385
12. Imai, Y., and Sato, R. (1967) Europ. J. Biochem. 1, 419-426
13. Phillips, A. H., and Langdon, R. G. (1962) J. biol. Chem. 237, 2652-2660
14. Omura, T., and Takesue, S. (1970) J. Biochem. 67, 249-257
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. biol. Chem. 193, 265-275
16. Cuatrecasas, P. (1970) J. biol. Chem. 245, 3059-3065
17. Aitio, A. (1978) Anal. Biochem. 85, 488-491
18. Correia, M. A., and Mannering, G. J. (1973) Mol. Pharmac. 9, 470-485

11. Omura, T., and Sato, R. (1964) J. biol. Chem. 239, 2379-2385
12. Imai, Y., and Sato, R. (1967) Europ. J. Biochem. 1, 419-426
13. Phillips, A. H., and Langdon, R. G. (1962) J. biol. Chem. 237, 2652-2660
14. Omura, T., and Takesue, S. (1970) J. Biochem. 67, 249-257
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. biol. Chem. 193, 265-275
16. Cuatrecasas, P. (1970) J. biol. Chem. 245, 3059-3065
17. Aitio, A. (1978) Anal. Biochem. 85, 488-491
18. Correia, M. A., and Mannering, G. J. (1973) Mol. Pharmac. 9, 470-485