Invited Paper 4

DISTRIBUTION AND SOME CHARACTERISTICS OF CYTOCHROME P-450 IN THE KIDNEY

Hitoshi ENDOU

Department of Pharmacology, Faculty of Medicine
University of Tokyo, 7-3-1 Hongo, Bunkyo-ku,
Tokyo 113. Japan

Abstract.....Intrarenal distribution of cytochrome P-450 (P-450) was investigated with different segments of isolated single nephrons from rabbits and rats by using a new ultra-micro method of P-450 determination. In both animals, P-450 was localized only in the proximal tubule. Other segments such as the glomerulus, the loop of Henle, the distal tubule, and the collecting tubule possessed no P-450 at all. Within the proximal tubule, the straight portion (S_2 and/or S_3 segments) revealed a higher specific content of P-450 than the convoluted one. An inducer of P-450, 3, 4-benzo(a)pyrene increased the P-450 of a definite portion (second 3 mm from the glomerulus) almost doubly in rabbits. In rats, both 3-methylcholanthrene (3MC) and 48 hr starvation (Fast) induced P-450, but only the later increased laurate- ω -hydroxylation. P-450 in the proximal convoluted and straight tubules was induced separately by Fast and 3MC, respectively. These results indicate that renal mixed function oxidase should be confined to the proximal tubule and that, like the liver, multiple forms of renal P-450 should exist in the different proximal segments.

Key words: Cytochrome P-450, isolated nephron segments, sodium phenobarbital, 3-methyl cholomthrene, 3, 4-benzo(a)pyrene, starvation.

INTRODUCTION

Numerous papers have reported on the hepatic P-450 in the past 20 years. On the contrary, extrahepatic P-450s have been studied especially in comparison with the

This work was supported by Grant-in-Aid for special Project Research. Fundamental research on toxicological action.

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hepatic P-450 not only in their functions but also in their multiplicity (Ellin and Orrenius, 1975; Serabjit-Singh et al., 1979; Wolf et al., 1980; Franklin et al., 1980; Liem et al., 1980; Okita et al., 1981; Robertson et al., 1981; Dees et al., 1982; Hasumura et al., 1983). Recently, Guengerich's group (Guengerich and Mason, 1979; Kaminsky et al., 1979) has reported that the antibodies against P-450s purified from phenobarbital (PB) and 3-methylcholanthrene (3MC) treated rat liver could cross-react with PB and 3MC induced P-450s from kidney microsomes, demonstrating that there should be common P-450 subunit(s) between liver and kidney microsomes. More recently, we could identify that the molecular subunit(s) of rat kidney P-450 were apparently more simple than those of the liver by one dimensional continuous gradient polyacrylamide gel electrophoresis (Hasumura et al., 1983).

Although mixed function oxidase activity in the kidney with the exception of fatty acid ω -hydroxylation (Anders, 1980) is generally much lower than that of the liver, renal P-450 should be confined to a specific population of the kidney. Since anatomical structure of the kidney is very complicated and the basic morphological and functional units of the kidney are nephrons, microdissection of a single nephron and P-450 determination in each segment would shed light on the clear-cut investigation of renal P-450.

The purpose of this study is to demonstrate P-450 localization within a single nephron in rabbits and rats, and to show existence of P-450 multiple forms induced by the treatment with several compounds. A part of this study has been reported elsewhere (Endou et al., 1982a; Endou, 1983).

MATERIALS AND METHODS

Biochemical Materials : Collagenase (type I), β -nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), 3, 3', 5, 5', -tetramethylbenzidine (TMBZ) and 3,4-benzo(a)pyrene (BP) were obtained from Sigma and 3MC was from Spectrum Chemical MFG. Corp. Sodium phenobarbital was kindly donated by Fujinaga Pharmaceutical Co. Ltd. Radioactive lauric acid, [$^{12-14}\text{C}$]-laurate was supplied by Commissariat a L'Energie Atomique. Silica gel thin layer, Kieselgel 60, was obtained from Merck. All other reagents were of the highest grade commercially available.

Treatment of animals : Male New Zealand white rabbits weighing between 1.8 and 2.2 kg were used. The rabbits were given a single intraperitoneal injection of BP (20 mg/kg) dissolved in corn oil; the controls received an equivalent volume of the corn oil. After 24 hr, the animals were sacrificed.

Male Sprague-Dawley rats weighing between 160 and 220 g were treated as described: 12 h fast; 48 hr fast; daily intraperitoneal injection of BP (20 mg/kg), 3MC (30 mg/kg) dissolved in corn oil for 3 days, and PB (80 mg/kg) in saline for 3 days; and control (i. p. injection of corn oil or saline).

Preparation of microsomes : Microsomes were prepared from perfused kidneys. Homogenates in 0.25 M sucrose were centrifuged at $10,000\times g$ for 10 min. The supernatants were recentrifuged at $105,000\times g$ for 60 min. The pellets (microsomes) were

washed twice with 0.15 M KCl and suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol.

Microdissection of nephron segments : Each nephron segment was dissected from perfused rabbit kidneys with 0.1% collagenase and rat kidneys with 0.1% collagenase and 0.1% hyaluronidase by the modified method (Endou et al., 1983) of Morel et al. (1978). Since P-450 activities of the microdissected rat nephron segments decreased more rapidly than those of the rabbit, each segment from rats was used for P-450 assay within 2 hrs after the enzyme treatment. Ten glomeruli and each 3 to 4 mm long nephron segment were used for P-450 analysis and for protein measurements.

Analytical Procedures : Protein was measured according to Lowry et al. (1951).

The amount of P-450 was measured as described by Omura and Sato (1964) using a Hitachi 340 Dual Beam Recording Spectrophotometer.

Estimation of laurate- ω -hydroxylation with renal microsomes prepared from treated rats was made by a modified method of Ichihara et al. (1969). Metabolites of lauric acid were separated on a thin-layer of silica gel 60 with ether-benzene-ethanol-formic acid (250 : 250 : 10 : 1, by volume) as the solvent. The radioactivities of ω -hydroxylated metabolites, identified with standards of authentic metabolites and laurate, were measured by liquid scintillation counting.

Micro-assay of P-450 (Endou, 1983) was based upon minimizing continuous gradient polyacrylamide gels (Ruechel et al., 1973) in which peroxidase activity of P-420 (Hrycay and O'Brien, 1971), a spectrally modified form of P-450 with sodium dodecyl sulfate (SDS), was stained with TMBZ and hydrogen peroxide (Thomas et al., 1976). Densitograms of stained gels in 7% acetic acid were made with a Joyce-Loebl MK III CS microdensitometer. Quantitative estimation of P-450 was carried out by measuring the peak height on the chart and comparing it with a standard line of peroxidase stained peak heights using partially purified P-450 prepared from rat kidney microsomes by the method of Imai and Sato (1974).

RESULTS

I. Rabbit Experiments.

Distribution of P-450 in kidney slices and effect of BP : Since renal P-450 was distributed not only in the microsomes (Kato, 1966) but also in the mitochondria (Ghazarian et al., 1974), specific contents of P-450 in the subcellular fractions from different slices were determined. As shown in Figure 1, renal microsomal P-450 was distributed in the cortex as well as in the outer medulla. Although no result is depicted in Figure 1, P-450 was not detected in the inner medulla (also named papilla). It may be noteworthy that considerable amounts of P-450 were measured in the mitochondrial fractions ($10,000\times g$ pellets) from both the cortex and the outer medulla. By the treatment with BP, only microsomal P-450 from the cortex was induced almost doubly, but not that from the outer medulla, suggesting that a BP-inducible P-450 subunit may be enriched in certain nephron segment(s) of the cortex.

Fig. 1. Distribution of P-450 content in mitochondrial and microsomal fractions from control and BP treated rabbit slices. Column height and bars represent the mean $\pm$ S. D., $n=4$. The white column indicates the control and the grey one, BP treatment. Since the papilla possessed no P-450 in both fractions, data are not depicted.

Intranephron distribution of P-450 : Peroxidase activities of hemeproteins containing P-420, a modified form of P-450 by SDS treatment, were stained after SDS gel electrophoresis. Under this condition only hemeproteins could be visualized, since the peroxidase activity of non-hemeproteins was inactivated in the presence of SDS. Figure 2 shows the intranephron distribution of P-450 in rabbits. It is striking to observe that only the proximal tubule contained P-450 and that other segments such as the glomerulus, the distal tubule, and the cortical collecting tubule possessed no P-450. Although results are not shown, no P-450 could be detected in the loop of Henle. Within the proximal tubule, the S_2 segment, which consists of the late convoluted and early straight portions, revealed the highest specific contents of P-450 per mm length as well as per μ g protein.

Increasing effect of BP on the intraproximal P-450 : According to the results depicted in Figure 1, it can be speculated that the P-450 of certain nephron segment(s) within the cortex should be specifically induced. Since renal P-450 was distributed only in the proximal tubule (Figure 2), P-450 contents along the single proximal tubule were estimated serially. As clearly demonstrated in Table 1, P-450 of a definite portion, the second 3 mm segment, was increased significantly after the treatment with BP, indicating that the molecular subunit of P-450 induced by BP may be enriched in this portion.

Effect of BP on densitometric profiles of P-450 : Figure 3 represents typical densitograms of hemeproteins from control and BP treated S_2 segments of the proximal

Cytochrome P-450 in the kidney

Fig. 2. Distribution of P-450 along a single nephron in rabbits. Each column and corresponding bar represent the mean $\pm$ S. D. Numbers in parenthesis indicate number of samples assayed separately. Hatched column indicates specific content, fmole/ μ g protein, which was calculated from P-450 content per mm length (value of white column) and protein amount of each nephron segment. Abbreviations of the nephron segments are ; the glomerulus (Glm), segment 1 to 3 of the proximal tubule (S_1 - S_3), the distal tubule (DT), and the cortical collecting tubule (CCT).

tubules. At least 2 peaks of P-450 could be identified ; the main peak in the control and the predominant peak in the BP treated tubule. By the treatment with BP, a subpeak located at the anodic site in the control increased and became a predominant peak as clearly shown at the lower chart of Figure 3. Since 4 to 40% continuous gradient polyacrylamide gels were used in this study, the molecular weight of the BP inducible peak should be larger than the main peak in the control.

II. Rat experiments.

Effects of several P-450 inducers on laurate- ω -hydroxylation : Figure 4 represents specific P-450 contents and laurate- ω -hydroxylation activities of rat kidney microsomes after fast for 12 hrs and 48 hrs, and after the treatment with BP, 3MC, and PB. Fast induced a time-dependent increase of P-450 contents and laurate- ω -hydroxylation. On the other hand, BP and 3MC induced P-450 but not laurate- ω -hydroxylation. PB changed neither P-450 nor laurate- ω -hydroxylation. These results indicate that there should be at least two different P-450 molecules in rat kidney microsomes : with and without increasing activity of laurate- ω -hydroxylation.

Distribution of P-450 along a single nephron of rats : As can be observed in Figure 5, P-450 was localized only in the proximal tubule also in rats. Within the proximal

Table 1. P-450 content in various segments of the proximal tubule from control and BP treated rabbits.

Segment	P-450, fmoles/ μ g protein		P value
	Control	BP	
First 3 mm from glomerulus	50.9*	67.1	NS
Second 3 mm from glomerulus	49.0	103.9	<0.01
Third 3 mm from glomerulus	93.0	135.5	NS
Second 3 mm from terminal portion	174.2	176.2	NS
First 3 mm from terminal portion	142.6	107.4	NS

* All values are the means of two to four determinations.
NS represents no statistical significance.

EFFECT OF 3,4-BENZPYRENE ON HEMEPROTEIN PATTERN
OF STRAIGHT PROXIMAL TUBULE IN RABBIT

Fig. 3. Densitometric profiles (620 nm) of SDS-polyacrylamide gels stained with TMBZ and hydrogen dioxide after electrophoresis of the S_2 segments. In the control segment, a prominent peak with a slightly visible shoulder on the right side (higher molecular side) can be observed. After treatment with BP, the peak corresponding to the shoulder increased and became a dominant peak as is clearly shown at the bottom.

Cytochrome P-450 in the kidney

Fig. 4. Effects of inducers on microsomal P-450 and laurate ω -hydroxylation in rats. White and hatched columns and bars represent the means $\pm$ S. D. ; P-450, nmol/mg ; laurate ω -hydroxylation, nmol/mg/10 min.

Fig. 5. Distribution of P-450 along a single nephron in rats. Each column and corresponding bar represent the mean $\pm$ S. D. The abbreviations are the same as stated in Fig. 2, except the proximal convoluted tubule (PCT), the proximal straight tubule (PST), the thick ascending limb of Henle's loop (TAL), and the distal convoluted tubule (DCT).

Fig. 6. Effects of 48-hour fast and 3MC on distributional patterns of P-450 in PCT(a) and PST(b).

tubule, the straight tubule (PST) contained a higher specific P-450 content than the convoluted one (PCT). Although classification of nephron segments used in rat experiments is not the same as that in rabbits, these results (Figure 5) are fully consistent with those in rabbits (Figure 2), suggesting that there is no qualitative species difference in intranephron distribution of P-450 between rats and rabbits.

Distributional heterogeneity of P-450 multiple forms within the proximal tubule :

Since an apparent discrepancy could be observed between P-450 contents and laurate- ω -hydroxylation activities induced by fast and chemicals such as BP and 3MC (Figure 4), changes of P-450 contents in PCT and PST were determined by 48-hour fast and 3MC. Figure 6a shows changes in P-450 contents of PCT from rats of control, 48 hr fast, and 3MC treatment. Fast for 48 hrs significantly increased P-450 contents of PCT per mm length and per μ g protein, whereas 3MC did not change P-450 content in PCT. On the other hand, effects of fast and 3MC on P-450 contents of PST were the reverse (Figure 6b). Treatment of rats with 3MC caused P-450 in PST to increase significantly. Fast for 48 hrs, however, did not affect P-450 contents in PST. These results suggest that two forms of P-450 may be distributed differently from PCT to PST in rats.

DISCUSSION

Intrarenal distribution of P-450: In 1966, Kato first reported the presence of P-450 in rat kidney microsomes. Because of a lower specific content of P-450 in the kidney than the liver, less attention might have been previously paid to renal P-450. A cortical-papillary gradient (highest in the cortex lowest in the papilla) for mixed function oxidase activities and P-450 contents has been reported by Zenser et al. (1978). Figure

1 in this paper confirms this evidence. From the fact that rats treated with 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), an inducer of microsomal P-450, have increased renal P-450 and proliferation of the smooth endoplasmic reticulum in the S₃ cells of the proximal tubule (Fowler et al., 1977), intranephron distribution of P-450 could be speculated in the S₃ segment of the proximal tubule. More direct evidence, in which an antibody against purified microsomal P-450 from pig kidney cortex could bind to the proximal tubule cells (Masters et al., 1981), suggests that renal P-450 may be distributed in the proximal tubule. This qualitative finding is supported by the quantitative assays of P-450 with microdissected nephron segments in this paper (Figure 2 and 5).

Relationship between P-450 distribution and nephron functions : Renal P-450 is distributed not only in the microsomes (Kato, 1966), but also in the mitochondria (Ghazarian et al., 1974). Each P-450 plays important roles in drug metabolism (Anders, 1980), ω -hydroxylation of medium-chain fatty acids (Ellin and Orrenius, 1975), and 25(OH)vitamin D₃-1 α -hydroxylation (Ghazarian et al., 1974). In the kidney, mitochondrial P-450 is distributed in a fashion similar to the microsomal one within different slices (Figure 1) and proximal tubules (Endou, 1983). It may be interesting, therefore, to compare P-450 distribution in nephron segments (Figure 2 and 5) with intranephron distribution of 25(OH)vitamin D₃-1 α -hydroxylase activity. In rachitic chickens, 1 α -hydroxylase activity could be detected in PCT and the thick loop cortex (Brunette et al., 1978). Although the later portion is difficult to compare with the mammalian nephron because of differences in anatomical structure of avian nephrons, a part of the nephron, PCT in chickens, is consistent with the P-450 containing segment in this study (Figure 2 and 4). In rabbit fetal kidney, we could demonstrate that 1 α -hydroxylase activity has been found only in the proximal tubule, with PST showing higher activity (Akiba et al., 1980). This finding is quite consistent with the present results of P-450 localization in a single nephron. In vitamin D-deficient rats, PCT has been found to produce 1 α , 25(OH)₂ vitamin D₃ more effectively than PST (Kawashima et al., 1981a). The discrepancy between higher 1 α -hydroxylase activity with lower amount of P-450 in PCT in rats, and lower hydroxylase with higher P-450 in PST has been explained by high blood level of calcitonin in the fetus which selectively stimulates 1 α -hydroxylase in PST (Kawashima et al., 1981b).

Multiple forms of renal P-450: The mitochondrial P-450 of the kidney has been shown to be distinct from the microsomal one (Ghazarian et al., 1974). As studied intensively with liver microsomal P-450(s) (Lu and West, 1980), multiple forms of microsomal P-450 in rabbit kidneys have been reported (Armbrecht et al., 1979; Liem et al., 1980; Kusunose et al., 1981; Ogita et al., 1982; Ogita et al., 1983). Two forms of P-450, of which molecular weights are 54,500 and 58,900 (Armbrecht et al., 1970), were detected. Similar results were found by Liem et al. (1980) and Ogita et al. (1982). Figure 3 in this paper also supports these findings. The result shown in Figure 3, in which treatment of rabbits with BP increased the higher molecular P-450 from the S₂ segment, is consistent with the clear evidence of P-448 enabling BP to catalyze (Ogita et al., 1982). Multiple forms of rat kidney microsomal P-450 could be purified, because the results in

this study (Figure 4 and 6) suggest this possibility.

It may be more interesting to know that individual segments along the proximal tubule may contain different form(s) of P-450 and specify distinct metabolic functions in each segment. Further study should be carried out on drug metabolism and ω - and/or (ω -1)-hydroxylation of endogenous active compounds such as prostaglandins and hormones using microdissected nephron segments and isolated kidney cells. We recently reported a trial for sorting isolated kidney cells in pure populations by a flow cytometer (Endou et al., 1982b).

ACKNOWLEDGEMENTS

The author thanks Miss N. Todoroki for her expert secretarial assistance. This study was supported in part by grants from the Japanese Ministry of Education, Science and Culture (No. 56122018 and 57570069), the Foundation for the Promotion of Research on Medicinal Resources, and Mitsukoshi Prize of Medicine 1982.

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