CYTOCHROME P-450 MONOOXYGENASE SYSTEM IN THE RABBIT KIDNEY: ITS INTRANEPHRON LOCALIZATION AND ITS INDUCTION*

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Abstract—Two components of the renal cytochrome P-450 (P-450) monooxygenase system, P-450 and NADPH-cytochrome c (cyt. c) reductase, were estimated using rabbit kidney slices and isolated nephron segments. Renal P-450 was distributed in the mitochondrial and microsomal fractions of both slices and nephron segments. P-450 (mitochondrial plus microsomal) was localized exclusively in the proximal tubule, with the highest activity in the S2 portion (18.14±5.47 fmoles/mm, 134.4 fmoles/μg protein). Intraperitoneal injection of 3,4-benzo(a)pyrene (BP) induced a 2-fold increase of only the microsomal P-450. The intraproximal site of BP action was localized in a definite portion, a segment 3 to 6 mm distant from the glomerulus (49.0±7.7 to 103.9±3.5 fmoles/μg protein), suggesting that inducible P-450 molecules may be enriched in this portion. Multiplicity of renal P-450 could be demonstrated electrophoretically. Comparison of the pattern from BP treated rabbits with control hemeprotein indicated that BP induced a higher molecular weight P-450. The highest concentrations of NADPH-cyt. c reductase were distributed in the cortical microsomes, although it was also detectable in the papilla. The reductase was distributed along the entire single nephron, with highest concentrations in the S2 portion of the proximal tubule. In conclusion, the P-450 monooxygenase system is localized in the proximal tubule and in the control condition, the highest activity is found in the S2 portion.

The cytochrome P-450 (P-450) monooxygenase system, which consists of P-450, a flavoprotein (NADPH-cytochrome P-450 reductase), phospholipids, and the NADPH generating system, has been reported to be widely distributed in most animal tissues and organelles and in plants and microorganisms (1, 2). It is believed that this system functions generally to monooxygenate lipophilic substances such as fatty acids, prostaglandins, and steroids as well as a host of foreign compounds including drugs, petroleum products, anesthetics, insecticides, and carcinogens (2).

Although hepatic microsomal P-450 has been intensively investigated (2, 3), renal P-450 has also been established to have unique metabolic functions. Renal P-450 is distributed in both mitochondrial (4) and microsomal (1, 5) fractions. A well known function of renal mitochondrial P-450 is the \( \alpha \)-hydroxylation of 25-hydroxy vitamin D\(_3\) (4). On the other hand, renal microsomal P-450 is involved in metabolism of some drugs (6, 7) and \( \alpha \) - and (\( \alpha \)-1)-hydroxylation of fatty acids (8-10) and prostaglandins (11).

In 1966, Kato first reported the presence of P-450 in kidney microsomes (5). Because the kidney cortex microsomal P-450 monooxygenase system catalyzes both fatty acid hydroxylation in the \( \alpha \) - and (\( \alpha \)-1)-positions (9, 10) and aryl hydrocarbon hydroxylation

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the intrarenal localization of P-450 and NADPH-cyt. c reductase was examined with rabbits in this study. In previous papers, we reported that P-450 is exclusively localized in the proximal tubule (12) and in isolated cells originating from the proximal tubule (13) in rats. Since the proximal tubule has been classified into 3 segments (S1, S2 and S3) in rats (14) and rabbits (15) mainly according to the morphological differences, special care was taken for dissecting the proximal tubule in this study.

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 (Saint Louis, U.S.A.). Cytochrome c from Candida krusei was purchased from the Sankyo Co., Ltd. (Tokyo, Japan). All other chemicals were the analytical grade of commercial sources.

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 body weight) dissolved in corn oil; the controls received an equivalent volume of the corn oil. After 24 hr, the animals were sacrificed.

Isolation and classification of nephron segments: Single nephron segments from control and BP treated rabbits were isolated according to Morel et al. (16) with a slight modification. The following segments were collected for assaying P-450, NADPH-cyt. c reductase and protein: the glomerulus (Glm), the early portion of the proximal tubule (S1) within 3 mm from the Glm, the straight portion of the middle proximal tubule (S2), the straight portion of the proximal tubule (S3) within 1 mm from the beginning of the thin descending limb of Henle's loop, the distal tubule (DT) including bright and granular portions, and the light portion of the cortical collecting tubule (CCT). In order to demonstrate the intraproximal distribution of P-450 activity continuously within a single nephron, each 4 mm long tubule fragment from an entire proximal tubule was used, except for the terminal portion in which four 1 mm long terminal segments of the tubule were pooled. For clarification of the BP effect on P-450 induction using nephron segments, each 3 mm long proximal tubule fragment from glomerular and terminal sides was specifically used.

Estimation of the protein content in nephron segments: To determine enzyme activity both per mm tubule length and μg protein, the protein content of the individual nephron segments was determined by the method of Lowry et al. (17) in a 65 μl final volume, using a microcuvette. Table 1 shows the mean values of protein from 6 different nephron segments.

Preparation of mitochondrial and microsomal fractions from slices and isolated nephron segments: Perfused kidney slices of cortex, outer medulla (medulla), and inner medulla (papilla) were homogenized with nine parts of 0.25 M sucrose in a glass-teflon homogenizer. The homogenates were centrifuged at 600 g for 10 min. The supernatant was then centrifuged at 10,000 g for 10 min, and the sediment was collected (mitochondrial fraction). The resultant supernatant was centrifuged at 100,000 g for 60 min. The pellet thus obtained (microsomal fraction) and the mitochondrial fraction were washed once with 1.15% KCl and resuspended in 0.1 M phosphate buffer (pH 7.5) to a protein concentration of approximately 10 mg per ml. Each individual nephron segment containing 2 μg protein was microdissected from the nephron and homogenized with 10 μl of 0.25 M sucrose in a glass capillary tube.
Table 1. Protein content of the isolated nephron segments

| Nephron segment                  | Protein content (ng/glomerulus or mm, mean±S.D.) |
|---------------------------------|-------------------------------------------------|
| Glomerulus                      | 64.8± 5.1                                       |
| S1 portion of the proximal tubule| 185.6±23.6                                      |
| S2 portion of the proximal tubule| 135.0±12.2                                      |
| S3 portion of the proximal tubule| 124.6±15.1                                      |
| Distal tubule                   | 87.4±12.9                                       |
| Cortical collecting tubule      | 82.7±14.3                                       |

Each nephron segment from the control and BP-treated rabbit kidneys was microdissected under a stereomicroscope and photographed. Protein content of each single nephron segment was measured by the method of Lowry et al. (17) in a final assay volume of 65 μl. Since no significant difference in each protein amount from the control and BP-treated rabbits was observed, all values in the two conditions were combined. The values are the means of 10 to 15 determinations from 6 rabbits.

Microdetermination of P-450 estimated as peroxidase activity of P-420 in the nephron segments: The principle of the P-450 microassay is based on the fact that high spin P-420, a form of P-450 modified with a detergent such as Lubrol WX, shows an increase of peroxidase activity (18), which can be stained in polyacrylamide gels after SDS (sodium dodecyl sulfate)-electrophoresis with TMBZ and hydrogen peroxide (19). A 4 to 40% continuous gradient polyacrylamide gel prepared in a 10 μl glass capillary tubing (inner diameter; 0.63 mm, Brand, FRG) has been reported to be useful especially for quantitative analysis of minute amounts of electrophoretically separated enzyme (20). To identify the peak corresponding to P-450, microsomal P-450 from control rabbit kidney cortex was partially purified by the method of Imai and Sato (21). By mixing solubilized microsomal protein with the purified P-450, the resulting P-450 band(s) could be identified. To obtain a standard curve of P-450 activities, a portion of cortical microsomes was used for the determination of P-450 activity according to Omura and Sato (22) and protein measurement (17). One hundred to six hundred nanograms of the microsomes solubilized with 0.25% (W/V) SDS were electrophoresed in the presence of 0.1% (W/V) SDS in the electrode buffer at 60 V for 30 min at room temperature. After electrophoresis, the gels were pushed out from glass tubes with a steel wire, and peroxidase activity was stained at 25°C by the method of Thomas et al. (19). Densitograms of the stained gels were made with a Joyce-Loebl microdensitometer, MK III CS. The peak heights of P-420 peroxidase activities on the densitograms are linear from 0 to 160 femoles of P-450 as shown in Fig. 1. Each nephron segment was microdissected within 4 hr after the treatment with collagenase, photographed for tubular length estimation, solubilized with 0.25% (W/V) SDS in 20% (V/V) glycerol in a siliconized glass pipette and applied immediately onto the microgels. P-450 contents in the nephron segments were calculated from the standard curve (Fig. 1).

Determination of NADPH-cytochrome c reductase activity: The NADPH-cyt. c reductase activities of nephron segments
Fig. 1. Linearity between kidney microsomal P-450 content and densitometric peak heights. Kidney microsomes were prepared from control rabbits and P-450 activity as well as protein concentration were measured. One hundred to six hundred nanograms of the microsomes solubilized with 0.25% SDS were separated electrophoretically and stained for peroxidase activity. Densitograms were performed using wedge D with a Joyce-Loeble microdensitometer.

The millimolar extinction difference (cm⁻¹ mM⁻¹) between reduced and oxidized electron acceptors was 21.1 at 550 nm at 25 °C for cyt. c.

Results

Distribution of P-450 and effect of BP on P-450 from subcellular fractions in kidney slices: As shown in Fig. 2, in control rabbits, mitochondrial P-450 was distributed in both the cortex and the medulla, but no P-450 was detected in the papilla. P-450 content in the medulla was higher than in the cortex. Microsomal P-450 was also present in the cortex and the medulla, but not in the papilla. The specific activity in the cortex was significantly higher than that in the medulla. A single intraperitoneal injection of 20 mg BP/kg body wt. resulted in a two-fold increase in specific P-450 activity only in cortical microsomes (Fig. 2).

Intranephron localization of P-450: Hemeproteins of individual microdissected nephron segments were stained after SDS-electrophoresis since the peroxidase activity of non-hemeproteins was inactivated in the presence of SDS during gel electrophoresis. As clearly demonstrated in Fig. 3, the peak corresponding to P-450 was observed only in the proximal tubule. No visible P-450 band was found in the glomerulus, the distal tubule, or the cortical collecting tubule. In contrast to P-450, a prominent band of cyt. c identified by the co-electrophoresis with purified cyt. c was observed in all nephron segments tested.

Intraproximal distribution of P-450: Since renal P-450 was localized exclusively in the proximal tubule (Fig. 3), intraproximal heterogeneity of P-450 distribution was examined. Each 4 mm long tubule fragment was cut from a single entire proximal tubule and analyzed for P-450 detection. Figure 4 shows a typical series of distributional patterns of hemeproteins including P-450.
Fig. 3. Localization of P-450 along a single nephron of rabbits. Each nephron segment was microdissected and stained for peroxidase activity with TMBZ and H2O2 after SDS-gel electrophoresis. Bands that corresponded to P-450 and cyt. c were identified by mixing their purified standard with each nephron segment. A clear P-450 band can be observed only in the proximal tubule.

Interestingly, the P-450 peak is increased gradually according to the distance from the glomerulus and finally decreased at the terminal portion. On the other hand, the band of cyt. c can be observed to decrease continuously from the first to the last segments of the proximal tubule, indicating that the intracellular contents of mitochondria should be different in these segments.

Figure 5 represents the intranephron distribution of P-450 per mm length and per μg protein calculated with the values shown in Table 1. The S2 segment shows the highest content of P-450, and no P-450 could be observed in the glomerulus, the distal tubule, or the cortical collecting tubule. Although the result is not shown here, the thick ascending limb of Henle’s loop did not contain P-450.

P-450 distribution in subcellular fractions from various portions of microdissected nephrons: Since renal P-450 has been

Fig. 4. Intraproximal distribution of P-450 in rabbits. Each 4 mm long segment from a single nephron except the last portion was assayed for peroxidase activities. Four 1 mm long segments from 4 terminal portions of the proximal tubule were pooled and analyzed as the last segment.

Fig. 5. Distribution of P-450 along a single nephron. Each column and corresponding bar represent the mean±S.D. Numbers in parentheses indicate number of samples assayed separately. Hatched column indicates specific enzyme activity, fmoles/μg protein, which was calculated from activity per mm length (value of white column) and protein amount of each nephron segment (presented in Table 1). Abbreviations of the nephron segments are: the glomerulus (Glm), segment 1 to 3 of the proximal tubule (S1–S3), the distal tubule (DT), and the cortical collecting tubule (CCT).
reported to exist not only in mitochondria but also in microsomes, subcellular fractionation of nephron segments was necessary to explain the real P-450 localization. Figure 6a shows densitograms of mitochondrial hemeproteins for which peroxidase activity was stained following SDS gel electrophoresis. Proximal tubules from S1, S2, and S3 possessed P-450, with the S2 segment revealing the highest content. As can be easily speculated from the results in Fig. 3, no mitochondrial P-450 was observed in the distal and collecting tubules, in which a prominent peak of cyt. c could be seen.

Similarly to the distribution pattern of mitochondrial P-450, microsomal P-450 was detected in S1, S2, and S3 portions of the proximal tubule (Fig. 6b). Comparing the peak heights of cyt. c from the lower nephron (DT and CCT) found in Fig. 6a with those in Fig. 6b, a small amount of mitochondrial proteins contaminated the microsomal fractions. Nevertheless, it can be concluded that both fractions, mitochondria and microsomes from the proximal tubules, contain P-450.

**Effect of BP on P-450 content and existence of renal P-450 multiplicity:** Because a single intraperitoneal injection of BP increased microsomal P-450 from the cortex (Fig. 2), the effect of BP on P-450 content in the proximal tubule was examined. As shown in Table 2, BP caused a significant increase in the P-450 activity of a very limited portion of the proximal tubule, the segment 3 to 6 mm distant from the glomerulus, indicating that one type of the P-450 molecules induced by BP may be enriched in this portion.

Densitograms of hemeproteins from the early portion of the proximal straight tubule shown in Fig. 7 suggest that there should

| Table 2. Cytochrome P-450 content in various segments of the proximal tubule from control and 3,4-benzo(a)pyrene treated rabbits |
|---------------------------------------------------------------|
| Segment                                      | Cytochrome P-450, fmoles/µg protein | P value |
| Control                              | 3,4-benzo(a)pyrene                  |       |
| First 3 mm from glomerulus             | 50.9*                               | NS     |
| Second 3 mm from glomerulus            | 49.0                                | <0.01  |
| Third 3 mm from glomerulus             | 93.0                                | NS     |
| Second 3 mm from terminal portion      | 174.2                               | NS     |
| First 3 mm from terminal portion       | 142.6                               | NS     |

*All values are the means of two to four determinations. NS represents no statistical significance.
Fig. 7. Densitometric scans (620 nm) of SDS-polyacrylamide gels stained with TMBZ and H₂O₂ after electrophoresis of the proximal straight tubules from control (above) and BP-treated (below) rabbits. In the control segment, a prominent peak with a slightly visible shoulder on the right side (higher molecular side) can be seen. After treatment with BP, the peak corresponding to the shoulder increased and became a dominant peak as is clearly shown at the bottom, indicating the existence of at least two forms of P-450.

Fig. 8. Distribution of NADPH-cyt. c reductase activity in microsomes from rabbit kidney slices. Enzyme activity was measured with cyt. c (from Candida krusei) as a substrate at 25°C. Values are the means of 3 independent experiments with control rabbits.

Fig. 9. Distribution of NADPH-cyt. c reductase activity along a single nephron in control rabbits. The assay condition was the same as described in Fig. 8, except for the use of a microcuvette with a final volume of 55 µl. The abbreviations of the nephron segments are the same as stated in Fig. 5.

be at least two molecules of P-450, a main peak in the control and the dominant peak induced by BP. By SDS-electrophoresis with a continuous gradient polyacrylamide gel, protein bands can be separated by molecular weight. The molecular weight of the P-450 inducible by BP, therefore, can be speculated to be higher than that of the main peak found in control P-450.

Distribution of NADPH-cyt. c reductase in slices and nephron segments: Figure 8 illustrates the distribution of NADPH-cyt. c reductase activity in microsomes from the cortex, the medulla, and the papilla. The highest activity was found in the cortical microsomes. Unlike the intrarenal distribution of P-450 (Fig. 2), the papillary microsomes showed approximately half the activity of those in the cortex.

As shown in Fig. 9, all cortical nephron segments possessed NADPH-cyt. c reductase activity. Among the 6 segments, the S2 and S3 portions showed the highest activity. From the results depicted in Figs. 5 and 9, it can be concluded that the P-450 monooxygenase system is localized only in the proximal tubule in the rabbit kidney.
Discussion

The present findings of intranephron localization of P-450 and NADPH-cyt. c reductase in microdissected nephron segments from rabbit kidney cortex indicate that the P-450 monooxygenase system in microsomes as well as in mitochondria exists only in the proximal tubule. In order to determine P-450 content in nephron segments, a new micromethod of assaying P-450 was necessary because the micromethods reported previously (24, 25) require more amounts of sample proteins (mg order) than those contained in microdissected nephron segments (μg order). Combining a sensitive and stable method of staining hemeprotein including P-450 (19) with microgel electrophoresis (26) resulted in the ultramicro assay of P-450 used in this study. In general, gel electrophoresis has been used for qualitative analysis of protein samples. A continuous gradient polyacrylamide gel, however, is a useful tool for kinetic studies of isoenzymes (20) and quantitative analysis of the separated protein (27) because protein bands electrophoresed in this gel are packed so tightly that diffusion of bands can not easily occur, especially during incubation for enzymatic staining. Moreover, multiple forms of the P-450 molecules can be observed with this assay system. If rabbit liver microsomes are analyzed with the continuous gradient polyacrylamide gels, five to six multiple bands corresponding to P-450 could be observed (to be published). In cases of kidney microsomes as well as solubilized nephron segments, more simple bands of P-450 were recognized, as shown in Fig. 3, Fig. 4, Fig. 6a, Fig. 6b and Fig. 7.

Although 5 to 6 forms of P-450 have been reported to exist in rabbit liver microsomes (28), little is known concerning P-450 multiplicity in the kidney. Recently, Kusunose et al. (29) have reported multiple forms of P-450 in rabbit kidney cortex separated with high performance liquid chromatography using an anion exchange resin. Their results are consistent with the present study because only one P-450 molecule was induced by the treatment with 3-methylcholanthrene, an inducer similar to the BP used in this paper.

Since 1α-hydroxylase of 25(OH)vitamin D₃ is localized in kidney mitochondria (30) and mitochondrial P-450 is indispensable to the 1α-hydroxylation (4), it may be interesting to compare P-450 localization in nephron segments (Figs. 5 and 6a) with intranephron distribution of 1α-hydroxylase activity. In rachitic chickens, the proximal convoluted tubule and the thick loop cortex have been reported to reveal 1α-hydroxylase activity. In cases of kidney microsomes as well as solubilized nephron segments, more simple bands of P-450 were recognized, as shown in Fig. 3, Fig. 4, Fig. 6a, Fig. 6b and Fig. 7.

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strating that P-450 was distributed in the proximal tubule. Although they could not estimate P-450 contents quantitatively, their results in the pig kidney are qualitatively consistent with the present study in the rabbit kidney. In view of Fig. 7 in this paper and the study by Kusunose et al. (29) that there should be at least 2 forms of microsomal P-450 in rabbit kidneys, intraproximal distribution of individual forms of P-450 and induction of each molecule by drugs and/or fatty acids should be further studied.

Unlike the existence of P-450 multiplicity, no multiple forms of NADPH-cyt. c reductase have been reported up to the present time. Immunofluorescence of NADPH-cyt. c reductase in rat and minipig kidneys stained with specific antibody against the reductase has been found to appear in the proximal tubule, but not in the distal and the collecting tubules (37). Thus, the present result (Fig. 9) is inconsistent with the results in rats and minipigs. In order to explain this difference, further investigation of NADPH-cyt. c reductase in different animal kidneys needs to be done using various conditions and assay methods.

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