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RENAL CYTOCHROME P-450-DEPENDENT METABOLISM OF ARACHIDONIC ACID IN SPONTANEOUSLY HYPERTENSIVE RATS

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Abstract—Renal cytochrome P-450-dependent monooxygenases metabolize arachidonic acid to products some of which affect vascular tone and (Na^+,K^+)ATPase activity. We measured these metabolites in spontaneously hypertensive (SHR) and control normotensive Wister–Kyoto (WKY) rats. Systolic tail blood pressure in SHR increased from 112 to 202 mm Hg and in WKY from 97 to 136 mm Hg at 5 and 20 weeks respectively. Renal cortical and outer medullary microsomes were incubated with [^{14}C]arachidonic acid; metabolites formed via the cytochrome P-450 pathway were defined as those dependent on NADPH, inhibited by SKF-525A, and unaffected by indomethacin. The P-450-dependent metabolites were higher in SHR vs WKY at 5, 7 and 11 weeks in the cortex and at 7 and 11 weeks in the outer medulla. In the outer medulla, the formation of these metabolites peaked at 7 weeks. Using reverse-phase HPLC, the cytochrome P-450-dependent metabolites were separated into three radioactive peaks: peak I had a retention time of 17.5 min and comigrated as 11,12-dihydroxyeicosatrienoic acid standard. Peak II had a retention time of 19 min and comigrated with ω-hydroxylation compounds. Peak III had a retention time of 27 min and comigrated with 11,12-epoxyeicosatrienoic acid. In the renal cortex, peak I was higher in SHR vs WKY at 5, 7, and 9 weeks and peak III at 5, 7, 9 and 11 weeks. In the outer medulla, peak I was higher in SHR at 5 and 7 weeks, and peaks II and III at 7 weeks. Cytochrome P-450 content in the renal cortex was always higher in SHR vs WKY. We conclude that renal cytochrome P-450-dependent metabolites of arachidonic acid may participate in the circulatory changes of SHR, particularly during the developmental stage.

Metabolism of arachidonic acid (AA) involves three pathways: (a) cyclooxygenase, leading to the formation of prostaglandins (PGs), thromboxane A_2 (TxA_2), and prostacyclin (PGI_2); (b) lipoxygenases, leading to the formation of hydroxy- and dihydroxy-eicosatetraenoic acids (HETEs and diHETEs) and leukotrienes; and (c) cytochrome P-450-dependent monooxygenase system which metabolizes AA to a variety of oxygenated products such as HETEs, epoxyeicosatrienoic acids (EETs) and ω- and ω-1 hydroxylated acids by an NADPH-dependent mechanism [1–4]. The pattern of AA metabolism in the kidney is distinct and presumably participates in integrated renal function.

Cytochrome P-450 exists in multiple forms that differ in substrate specificity, positional specificity, and stereospecificity [5]. The renal content of the components of the cytochrome P-450-dependent mixed-function oxidase system has been measured, and it was found to be much less than that of the liver [6]. The highest level of cytochrome P-450 and the components of this system was found in the renal cortex [7–10]. The renal cortical cytochrome P-450 system has been shown to metabolize AA to the ω- and ω-1 hydroxylation products, the EETs, which can undergo hydrolysis by epoxide hydrolase to form the corresponding dihydroxyeicosatrienoic acids (DHTs), and trihydroxyeicosatrienoic acids [1]. In epithelial cells isolated from the thick ascending limb of Henle's loop (TALH) of the rabbit kidney, AA is specifically metabolized by a cytochrome P-450-dependent pathway to products which affect (Na^+,K^+)ATPase activity and vascular tone [11, 12]. Production of these AA metabolites is increased in TALH cells isolated from kidneys of rabbits made hypertensive by aortic coarctation [13].

Altered renal AA metabolism has been described in spontaneously hypertensive rats (SHR) [14–19] which have been studied extensively as an animal model of human essential hypertension [20, 21]. No data are available on renal AA metabolism through the cytochrome P-450-dependent pathway in SHR and normotensive Wistar–Kyoto rats (WKY). In view of the possible role of cytochrome P-450-dependent AA metabolites in the control of renal function [12, 22], and of the pivotal role of the kidney in the regulation of blood pressure, this study was undertaken.

METHODS

Materials. Bovine serum albumin (Fraction V), glucose-6-phosphate, NADPH, NADP, benzo[*a*]pyrene, and 7-ethoxycoumarin were

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obtained from the Sigma Chemical Co., St. Louis, MO. Glucose-6-phosphate dehydrogenase was purchased from Boehringer Biochemicals, Indianapolis, IN. [1-^{14}C]AA (56.5 mCi/mmol) was purchased from Amersham, Arlington Heights, IL. All other reagents were obtained from Fisher Certified Reagents, Springfield, NJ.

Animals. Male SHR and WKY were purchased from Charles River, Wilmington, MA, and fed and housed under identical conditions. Blood pressure from the tail was measured every day for 4 consecutive days, without anesthesia, using a plethysmograph.

Tissue preparation. Rats were anesthetized with sodium-pentobarbital (100 mg/kg, i.p.), and the abdominal cavity was opened. The aorta was tied above the renal arteries, and 0.9% ice-cold saline was flushed through the aorta into the kidneys. The renal cortex was dissected free from the medulla, and the outer medulla from the papilla. The cortex and outer medulla were homogenized separately in Tris–HCl sucrose buffer, pH 7.6. The homogenate was centrifuged at 2,000 *g* for 10 min. The supernatant fraction was centrifuged at 10,000 *g* for 15 min. Microsomes were obtained by centrifugation of the 10,000 *g* supernatant fraction at 100,000 *g* for 60 min and resuspended in 0.1 M potassium phosphate buffer, pH 7.6.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* [23], using bovine albumin as the standard.

AA metabolism. Microsomal suspensions (0.3 mg protein) from renal cortex and outer medulla were incubated with [^{14}C]AA (0.2 to 0.4 μCi), with or without indomethacin (10 μM), NADPH-generating system (0.1 mM glucose-6-phosphate, 0.4 mM NADP, and 1 unit of glucose-6-phosphate dehydrogenase), and SKF-525A (200 μM) in a total volume of 1 ml for 30 min at 37°. The reaction was terminated by acidification to pH 3.5–4.0 with citric acid, and the AA metabolites were extracted with ethyl acetate and subjected to thin-layer chromatography (TLC) for separation of AA and oxygenated metabolites using the upper phase of ethyl acetate–isooctane–acetic acid–water (110:50:20:100). The TLC plates were evaluated on a computerized Berthold 512LB radiochromatogram scanner. Radioactive zones were also visualized by autoradiography, cut, and counted in a Searle model 6880 Mark III liquid scintillation counter. High performance liquid chromatography (HPLC) was used to separate metabolites of AA. Reverse-phase HPLC was performed on a C18 microsorb column (250 × 4.6 mm, Rainin Instrument Co., Woburn, MA) using a linear gradient of 1.25%/min from acetonitrile–water–acetic acid (50:50:0.1) to acetonitrile–acetic acid (100:0.1) at a flow rate of 1 ml/min. Radioactivity was monitored by a flow detector (Radiomatic Instrument & Chemical Co., Tampa, FL). Samples were also collected every 0.5 min and counted in the liquid scintillation counter.

Cytochrome P-450 content. Cytochrome P-450 content was measured from the reduced carbon monoxide difference spectrum by using sodium dithionite as the reducing agent [24]. The absorbance difference between 450 and 490 nm was used to calculate the cytochrome P-450 content, using a molar extinction coefficient of 91 mM^{-1} cm^{-1}.

Aryl hydrocarbon hydroxylase (AHH) activity. The AHH activity was determined by using benzo[*a*]pyrene as the substrate according to the method of Nebert and Gelboin [25] as modified by Abraham *et al.* [26]. The fluorescence was quantitated by comparison with a standard curve obtained with various dilutions of 3-OH benzo[*a*]pyrene (a gift of Dr. Croci Tiziano of the American Health Foundation, Valhalla, NY).

7-Ethoxycoumarin O-deethylase (7-EC) activity. The activity of 7-EC was measured by determining the formation of 7-hydroxycoumarin according to the method of Aitio [27]. Microsomal preparations (0.5 mg protein) were added to the incubation mixture which consisted of 7-ethoxycoumarin (10 μM), an NADPH-generating system (1 mM NADP, 1 mM glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase), 0.1 mM $MnCl_2$, 0.1 M $MgCl_2$, and 0.1 mM Tris–HCl, pH 7.4. The reaction was terminated with 0.5 ml trichloroacetic acid (TCA, 0.31 M), and the pH of the mixture was brought to about 10 by adding 4 ml of 1.6 M NaOH–glycine buffer, pH 10.3. The protein precipitate was spun down, and the amount of 7-hydroxycoumarin was measured using a Perkin–Elmer fluorometer with excitation of 390 nm and emission of 440 nm. The instrument was standardized with authentic 7-hydroxycoumarin and calibrated with quinine sulfate in sulfuric acid. The readings were corrected by measuring fluorescence in a similarly treated complete incubation mixture with zero incubation time.

Statistical analysis. Results are expressed as means ± SEM. Statistical analysis employed analysis of variance (ANOVA) designed to identify differences between SHR and WKY. When a significant difference was observed, each time point was then analyzed by an unpaired *t*-test. The difference was considered to be significant when $P < 0.05$.

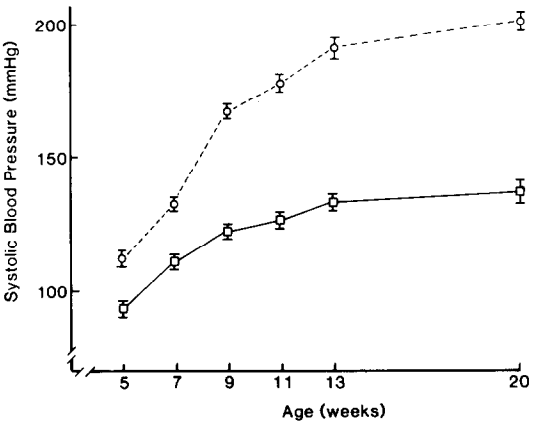

Fig. 1. Systolic blood pressure in SHR and WKY at different ages. Blood pressure was measured by tail cuff plethysmography in SHR (○) and WKY (□) at 5 (N = 6), 7 (N = 11), 9 (N = 9), 11 (N = 11), 13 (N = 3) and 20 (N = 6) weeks. Blood pressure was significantly higher in SHR vs WKY ($P < 0.001$) for each period.

RESULTS

Blood pressure increased in SHR from 112 to 202 mm Hg between the ages of 5 and 20 weeks (Fig. 1); the major increase in blood pressure occurred between 5 and 13 weeks. In contrast, the increase in blood pressure in WKY for the same period was moderate, reaching 136 mm Hg at 20 weeks (Fig. 1).

Metabolism of AA was determined in microsomes from cortex and outer medulla of kidneys from 5-, 7-, 9-, 11-, 13-, and 20-week-old SHR and WKY. In the absence of an NADPH-generating system, AA was converted primarily to cyclooxygenase products. The percent conversion of [^{14}C]AA to PGs by cortical and outer medullary microsomes was low and not significantly different in SHR and WKY.

Figure 2 shows a TLC separation of AA metabolites formed by cortical microsomes of 7-week-old SHR. The cytochrome P-450-dependent AA metabolites are defined as those metabolites whose formation is absolutely dependent on NADPH addition, inhibited by SKF-525A, an inhibitor of cytochrome P-450-dependent enzymes via a type I binding mechanism [28], and unaffected by indomethacin. Addition of an NADPH-generating system to the incubation medium increased the conversion of [^{14}C]AA by several-fold in both cortical and outer medullary microsomes from SHR and WKY kidneys, yielding several radioactive peaks (Fig. 2, third lane) that were inhibited by more than 50% with SKF-525A (200 μM) (Fig. 2, fourth lane). The conversion of AA to the cytochrome P-450-dependent metabolites was significantly higher in SHR vs WKY at 5, 7, and 11 weeks in the cortex and at 7 and 11 weeks in the outer medulla (Fig. 3). In the outer medulla, the formation of cytochrome P-450-dependent metabolites peaked at 7 weeks in the SHR (Fig. 3B). The cytochrome P-450-dependent metabolites were separated by reverse-phase HPLC into three radioactive peaks: peak I had a retention time of 17.5 min and comigrated with 11,12-DHT standard, peak II of 19 min and comigrated with ω-hydroxylation compounds, and peak III of 27 min and comigrated with 11,12-EET (Fig. 4). Product formation was dependent on the presence of NADPH (Fig. 4). In the renal cortex, peak I was higher in SHR vs WKY at 5, 7 and 9 weeks and peak III at 5, 7, 9 and 11 weeks, (Fig. 5). In the outer medulla, peak I was higher in SHR at 5 and 7 weeks, and peaks II and III at 7 weeks (Fig. 6).

Cytochrome P-450 content in the renal cortex was always higher in SHR vs WKY (Fig. 7). No difference was found in AHH activity, a cytochrome P-450-dependent monooxygenase, at any age, while the

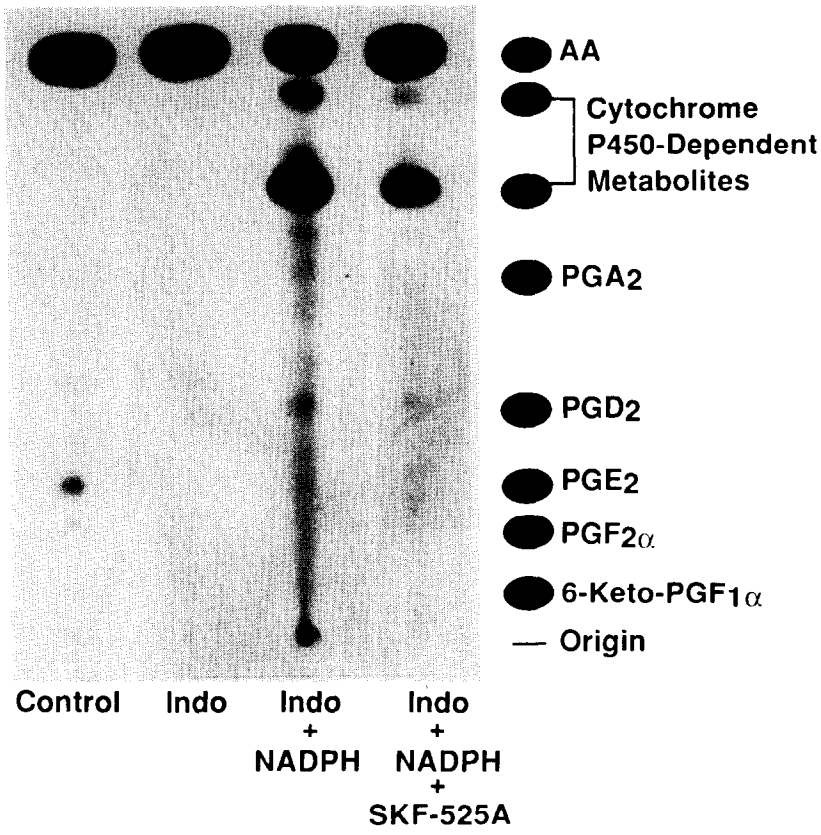

Fig. 2. Autoradiography of TLC separation of AA metabolites. Microsomes (0.3 mg protein) from the renal cortex of 7-week-old SHR were incubated with [^{14}C]AA (0.2 μCi) for 30 min as described in Methods, with or without indomethacin, SKF-525A and NADPH.

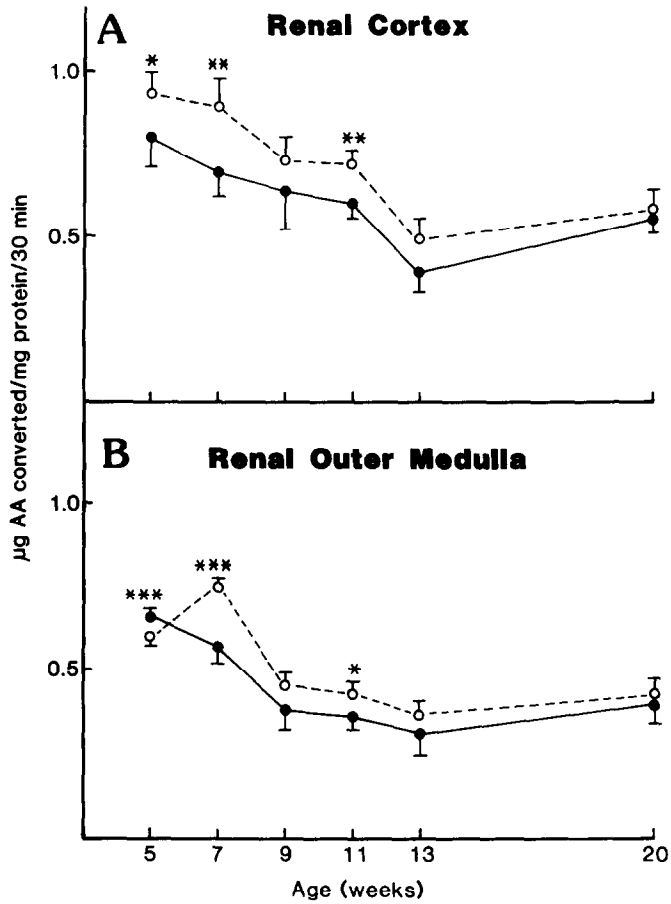

Fig. 3. Renal cortical and outer medullary cytochrome P-450-dependent AA metabolites in SHR and WKY. Microsomes from renal cortex (A) and outer medulla (B) of SHR (○) and WKY (●) of 5 (N = 6), 7 (N = 11), 9 (N = 9), 11 (N = 11), 13 (N = 3) and 20 (N = 6) weeks were incubated with [^{14}C]AA (0.2 μCi), indomethacin and NADPH for 30 min as described in Methods. Metabolites were separated by TLC as shown in Fig. 2. Radioactive zones were visualized by autoradiography and counted in a liquid scintillation counter. Key: (*)$P < 0.05$; (**)$P < 0.02$; and (***)$P < 0.005$.

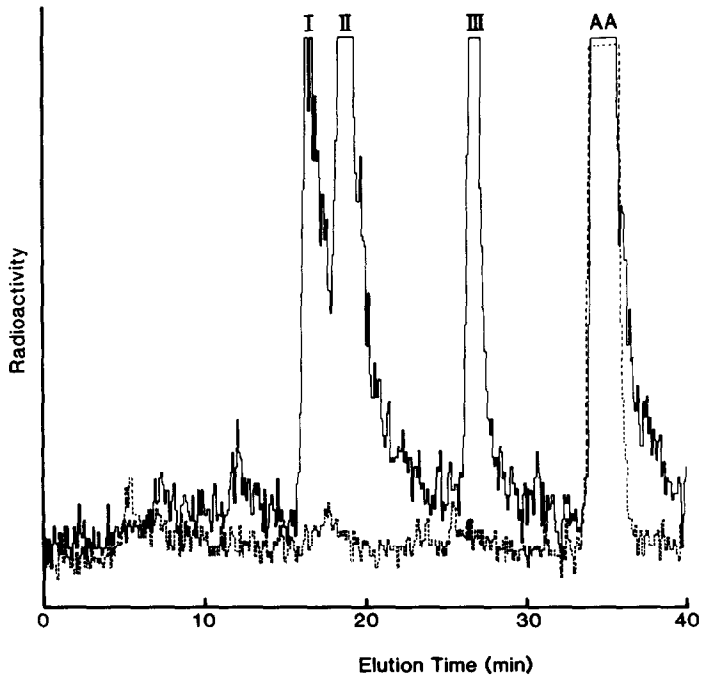

Fig. 4. Separation by reverse-phase HPLC of cytochrome P-450-dependent AA metabolites. Microsomes (0.3 mg protein) from the renal cortex of 7-week-old SHR were incubated with [^{14}C]AA (0.4 μCi), indomethacin, and NADPH, and reverse-phase HPLC was performed as described in Methods. Cytochrome P-450-dependent metabolites of AA (——) were not formed in the absence of NADPH (---). Peak I comigrated with 11,12-DHT standard, peak II with ω-hydroxylation compounds, and peak III with 11,12-EET.

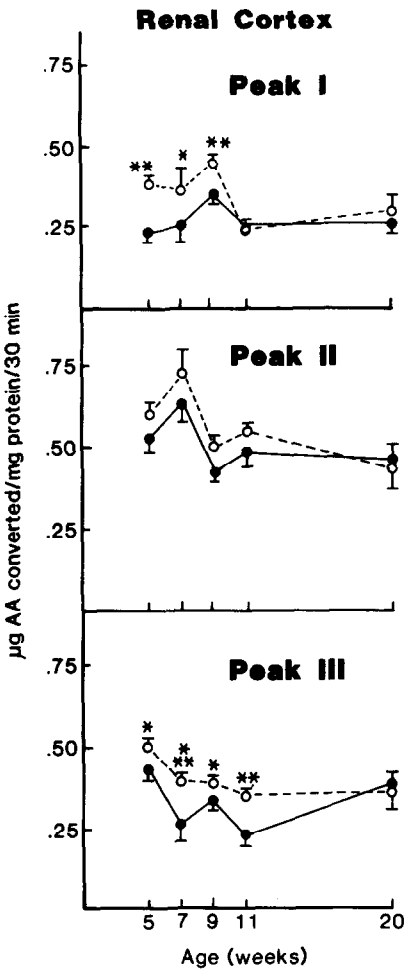

Fig. 5. Renal cortical cytochrome P-450-dependent metabolites in SHR and WKY. Microsomes (0.3 mg/protein) from the renal cortex of SHR (○) and WKY (●) were incubated with [^{14}C]AA (0.4 μCi), indomethacin and NADPH, and reverse-phase HPLC was performed as described in Methods. Radioactivity corresponding to peaks I, II and III (Fig. 4) was measured in a liquid scintillation counter at 5 (N = 3), 7 (N = 9), 9 (N = 6), 11 (N = 6) and 20 (N = 6) weeks. Key: (*)P < 0.05; (**)P < 0.02; and (***)P < 0.005.

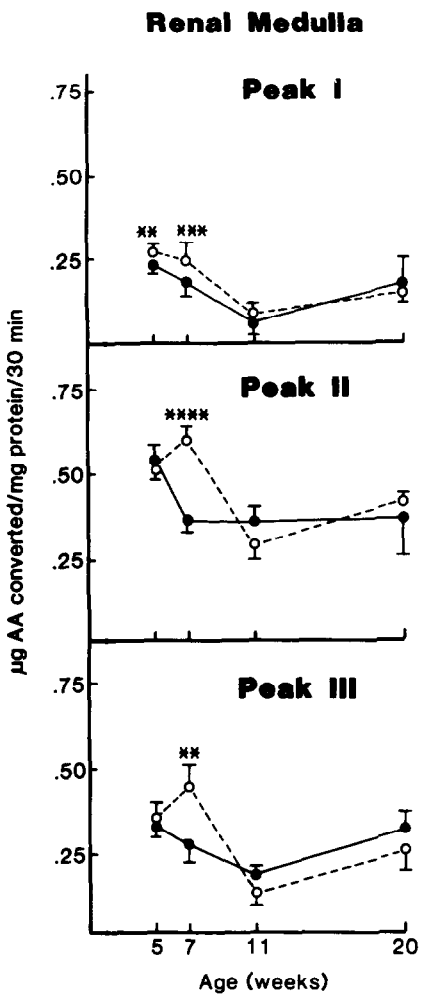

Fig. 6. Renal outer medullary cytochrome P-450-dependent metabolites in SHR (○) and WKY (●) of 5 (N = 6), 7 (N = 6), 11 (N = 3) and 20 (N = 3) weeks. Key: (**)P < 0.02; (***)P < 0.005; and (****)P < 0.001.

activity of 7-EC, a different cytochrome P-450-dependent monooxygenase, was higher in WKY vs SHR at 9 and 20 weeks (Fig. 7).

DISCUSSION

In this study, we demonstrated fluctuation of the renal cytochrome P-450 system and its oxygenase activities in the SHR as related to the increase of blood pressure when compared to the WKY. Our results showed that both SHR and WKY cortical and outer medullary microsomes metabolized AA through the cytochrome P-450-dependent pathway. The cytochrome P-450-dependent metabolites of AA, those whose formation was dependent on NADPH, unaffected by indomethacin and inhibited by SKF-525A, showed variations with age in their levels and types of products between the SHR and WKY. The conversion of AA through the cyclooxygenase pathway was low both in the cortex and outer medulla. The conversion of AA by cortical and outer medullary microsomes via the cytochrome P-450-dependent pathway was significantly higher in the SHR as compared to the WKY at 5, 7, 11 and 7, 11 weeks, respectively, whereas no difference was present in 20-week-old rats. The increase of AA conversion to the cytochrome P-450-dependent metabolites was coincident with the phase of greatest increase in blood pressure. Once the blood pressure was established by the 20th week of age, there were no differences between SHR and WKY in cytochrome P-450-dependent metabolism of AA. It should be noted that the renal functional disturbances described in the SHR, such as decreased renal blood flow and glomerular filtration rate [29] and salt and water retention [30], are evident only in the developmental phase of hypertension and disappear with the established phase, sometime after

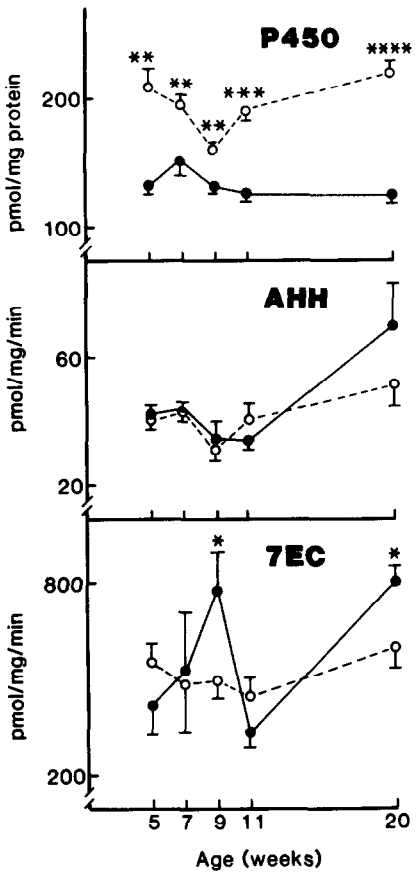

Fig. 7. Renal cortical cytochrome P-450 content and AHH and 7-EC activities in SHR and WKY. Cytochrome P-450 content (P-450), AHH and 7EC were determined as described in Methods in SHR (○) and WKY (●) of 5 (N = 3), 7 (N = 3), 9 (N = 6), 11 (N = 6), and 20 (N = 6) weeks. Key: (*)$P < 0.05$; (**)$P < 0.02$; (***)$P < 0.01$; and (****)$P < 0.001$.

12 weeks of age. These temporal relationships are consistent with, but do not provide direct evidence for, involvement of renal cytochrome P-450-dependent AA metabolites in elevation of blood pressure, particularly as some of these metabolites have been found to affect (Na^+,K^+)ATPase activity and vascular tone [12]. More direct evidence has been obtained recently by depleting cytochrome P-450 with stannous chloride, an inducer of heme oxygenase which degrades cytochrome P-450 and other hemoproteins [31]. Treatment of the young SHR with stannous chloride prevents elevation of blood pressure [31].

The increase in the cytochrome P-450-dependent AA metabolites in both cortical and outer medullary microsomes of the SHR is attributed to three major metabolites: peaks I, II and III. Peak III of the HPLC separation comigrated with 11,12-EET and peak I comigrated with its hydrolysis products, 11,12-DHT. Epoxide metabolites of AA have been reported to stimulate hormonal secretion [32, 33], to affect ion transport [22] and to modify vascular tone [12]. Peak II of the HPLC separation comigrated with 19- and 20-HETE. As the biological activities of the cytochrome P-450-dependent AA metabolites are only partially described, it would be premature to assign any one of these products a role in the development of hypertension in the SHR.

We have also measured renal cytochrome P-450 content. This is the first demonstration of elevation of renal cytochrome P-450 levels in the SHR when compared to the WKY. However, there were no parallel increases of two cytochrome P-450-dependent enzymes, AHH and 7-EC. These differences between enzyme activities and cytochrome P-450 content suggest that the increase in cytochrome P-450-dependent AA metabolism is due to elevated activity of a specific isozyme of cytochrome P-450, particularly an isozyme that prefers AA as a substrate, as previously reported for the outer medulla of the rabbit kidney [34].

In conclusion, we have shown increased renal cytochrome P-450-dependent metabolism of AA during the developmental stage of hypertension in the SHR which subsides when hypertension becomes established.

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