Downregulation of Renal CYP-Derived Eicosanoid Synthesis in Rats With Diet-Induced Hypertension

Mong-Heng Wang, Anita Smith, Yiqiang Zhou, Hsin-Hsin Chang, Songbai Lin, Xueying Zhao, John D. Imig, Anne M. Dorrance

Abstract—The incidence of essential hypertension increases with obesity; however, the mechanisms that link obesity with hypertension are unclear. Renal cytochrome P450 (CYP)-derived eicosanoids—hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs), and dihydroxyeicosatrienoic acids (DHETs)—have been shown to play an important role in the regulation of renal function, vascular tone, and blood pressure. The objective of this study was to examine CYP-derived eicosanoid synthesis in the different renal zones (cortex, medulla, and papilla) of rats fed a high fat diet (HF). Male Sprague-Dawley rats were fed a HF diet or regular rat chow for 10 weeks. After 10 weeks, HF rats showed significantly higher systolic blood pressure, body weight, and fat:body weight ratio. The renal \( \omega \)-hydroxylase activity was decreased by 46% in cortex, 43% in medulla, and 46% in papilla of HF rats. The renal epoxygenase activity was decreased by 46% in cortex, 31% in medulla, and 56% in papilla of HF rats. Interestingly, the changes in the rate of 20-HETE and EET formation in different renal zones were consistent with the levels of expression of CYP4A and CYP2C23 proteins, respectively. Furthermore, there were no significant changes in the synthesis of these metabolites in the renal microvessels. These results demonstrate that HF diet causes the downregulation of CYP4A and CYP2C23 in renal tubules, and these proteins are responsible for renal 20-HETE and EET formation. The reduction in the synthesis of these eicosanoids may play an important role in the regulation of renal function and blood pressure in obesity-induced hypertension. (Hypertension. 2003;42:594-599.)

Key Words: arachidonic acids | hypertension, obesity | blood pressure | kidney | ions | obesity

Cytochrome P450 (CYP) enzymes constitute a major metabolic pathway for arachidonic acid in rat kidney. CYP enzymes are primarily located in the renal cortex with high concentrations in proximal tubules, the thick ascending limb of the loop of Henle (TALH), and the vasculature, where these enzymes metabolize arachidonic acid mainly into hydroxyeicosatetraenoic acids (19 and 20-HETE) and epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14, 15 EETs).1–4 In the renal vasculature, 20-HETE causes vasoconstriction, whereas EETs cause vasodilation of renal arterioles.5–7 In renal tubular segments, 20-HETE and the EETs play an important role in the regulation of tubular reabsorption of sodium. For example, 20-HETE inhibits Na\(^+\)-K\(^+\)-ATPase activity in the proximal tubule,8 inhibits Na\(^+\)-K\(^+\)–2Cl\(^-\) co-transporter activity in TALH segment,9 and blocks a 70-pS K\(^+\) channel in the apical membrane of the TALH cells.10 Similarly, EETs also inhibit sodium transport in the cortical collecting duct.11 Based on biological actions of these compounds, these metabolites have been linked to the regulation of renal function and blood pressure in many animal models of hypertension.5–7

20-HETE synthesis is catalyzed primarily by isoforms of the CYP4A gene family.12 In the rat, four isoforms have been identified: CYP4A1, CYP4A2, CYP4A3, and CYP4A8. Several reports have demonstrated that \( \omega \)-hydroxylation of arachidonic acid is carried out by these CYP4A isoforms.13–15 On the other hand, EET synthesis is less specific and can be carried out by numerous CYP enzymes from different CYP gene families, including 1A, 2B, 2C, 2D, 2E, 2J, and 4A families.7 However, several reports have demonstrated that epoxidation of arachidonic acid in rat kidney is attributed to CYP 2C and 2J isoforms.7,16–18 In addition, by using an antibody inhibition approach, Holla et al19 have concluded that CYP2C23 is the major epoxygenase enzyme expressed in rat kidney.

It has been estimated that approximately 97 million people in the United States are obese.20 Obesity is considered a serious health problem because it is an important factor for essential hypertension.21 However, the precise mechanisms by which obesity causes hypertension are still not clear. It has been postulated that obesity can cause an abnormality in renal function such as increased renal sodium reabsorption and an impaired pressure natriuresis relation resulting in hypertension.21 Since CYP-derived eicosanoids have been shown to have biological effects including inhibition of ion transport along the nephron and vasoconstriction or vasodilation of...
renal arterioles, we hypothesize that renal CYP-derived eicosanoid synthesis is affected during obesity and that these metabolites are involved in the regulation of renal function and blood pressure in obesity. To test our hypothesis, we used rats fed a high-fat diet as our experimental obesity model. Previous studies from Dobrian et al22 have demonstrated that this animal model has characteristics similar to human obesity-induced hypertension such as increased plasma noradrenaline response to intravenous glucose, increased plasma leptin concentration, and decreased growth hormone secretion and synthesis. Hence, the present study was designed to compare control rats and rats fed a high-fat diet in terms of the activity and the expression of CYP4A, CYP2C, and CYP2J isoforms in the different renal zones as well as arachidonic acid metabolism in renal microvessels. This study provides valuable information for evaluating the role of these CYP-derived metabolites in the regulation of renal function and blood pressure in obesity.

Methods

Materials

[1-14C]-arachidonic acid (56 mCi/mmol) was obtained from DuPont-New England Nuclear. All reagents for Western blot analysis were purchased from Amersham Bioscience. All solvents were HPLC grade. All EETs and 20-HETE standards were purchased from Cayman Chemicals.

Animals

Three-week-old male Sprague-Dawley rats were purchased from Harlan (Indianapolis, Ind). Rats were divided into two groups: high fat (HF) rats were fed a modified chow containing 30% fat (15.2% saturated and 20.8% unsaturated), 35% carbohydrate, and 0.4% salt (Bio-Serv) and control rats were fed normal rat chow containing 4.4% fat (2.5% saturated and 1.9% unsaturated), 46.6% carbohydrate, and 0.3% salt. The studies were carried out on 13-week-old rats, and animals were fasted overnight before use. Blood pressure was measured after 10-week treatment; only HF rats confirmed to be hypertensive were used in this study. All animals were maintained on a 12-hour light/dark cycle and were housed 2 to a cage. All animal protocols were approved by the Institutional Animal Care and Use Committee and were in accordance with the protocols for animal use outlined by the American Physiological Society.

Systolic Blood Pressure

The onset and development of hypertension were assessed with the tail-cuff method. Rats were warmed at 40°C for 5 minutes and allowed to rest quietly in a Lucite chamber before assessment of blood pressure; 5 pressure measurements were recorded for each rat, and the average systolic blood pressure was calculated.

Isolation of Renal Microsomal Fractions and Microvessels

Control and HF rats were anesthetized with sodium pentobarbital (50 mg/kg IP) after 10 weeks of treatment. The visceral fat was removed and weighed and expressed as a ratio with the fasting body weight. The kidneys were removed and dissected into three zones: cortex, medulla, and papilla. The tissues were homogenized in buffer containing 100 mmol/L Tris-HCl and 1.15% KCl, pH 7.4. Homogenates were centrifuged at 10 000 g for 30 minutes. Microsomes were obtained by centrifugation of the supernatant at 100 000g for 90 minutes and resuspended in 0.25 mol/L sucrose buffer and stored at −80°C. For the isolation of renal microvessels, kidneys were excised, placed in ice-cold Tyrode buffer, and coronally sectioned. The renal papilla was removed to expose the microvessels. The segments of the interlobular artery were microdissected and freed from cortical and connective tissue. The purity of the microdissected microvessel preparation was determined as described previously.23

Activities of Arachidonic Acid Metabolism in Renal Microsomes and Renal Microvessels

Microsomes (150 μg) from renal cortex, medulla, or papilla isolated from control and HF rats were incubated with [1-14C]-arachidonic acid (0.4 μCi, 7 nmol) and NADPH (1 mmol/L) in 0.3 mL potassium phosphate buffer (pH 7.4) containing 10 mmol/L MgCl2 for 30 minutes at 37°C. The reaction was terminated by acidification to pH 3.5 to 4.0 with 2 mol/L formic acid, and arachidonic acid metabolites were extracted with ethyl acetate. The ethyl acetate was evaporated under nitrogen, and the metabolites were resuspended in 50 μL of methanol and injected onto the HPLC column. The detailed procedure for reverse-phase HPLC was described in our previous publication.23 The activity of the formation of these metabolites was estimated on the basis of the specific activity of the added [1-14C]-arachidonic acid and was expressed as picomoles per milligram of protein per minute.

The production of arachidonic acid metabolites in renal microvessels was determined in whole renal microvessels. Isolated renal interlobular arteries (18 segments, 2 mm per segment) were preincubated with 0.1% Tween 80 in a 100 mmol/L potassium phosphate buffer (pH 7.4) containing 10 mmol/L MgCl2 and 1 mmol/L EDTA for 15 minutes at 4°C. This step results in the permeabilization of the tissue and ensures free access of exogenous arachidonic acid and NADPH to CYP enzymes located in the endoplasmic reticulum.24 Renal microvessels were washed twice with buffer, spun down by centrifugation, and incubated with [1-14C]-arachidonic acid (50 μCi/μmol, 30 μmol/L final concentration) and indomethacin (10 μmol/L, final concentration) in 500 μL of potassium phosphate buffer containing 1 mmol/L NADPH in a shaking bath for 60 minutes at 37°C as described previously.24 The reactions were terminated by acidification to pH 4.0 with 2 mol/L formic acid, and renal microvessels were homogenized. Extraction and HPLC analysis were carried out as described above. Enzyme activity in renal microvessels was normalized to the protein concentration of microdissected microvessels. Six segments (2 mm per segment) of renal microvessels from each sample were collected and suspended in 300 μL of 1 mol/L NaOH, in which they were left overnight to dissolve. Any intact vessels remaining were manually ground with the use of a glass homogenizer, and the protein concentration was determined with a Bio-Rad protein assay. To assess the consistency of microdissection, the protein concentration of different isolations was measured, and we found the protein concentration among different isolations was within ±5%.

Western Blot Analysis

Renal microsomes (10 μg) from HF and control were separated by electrophoresis on a 10×20-cm, 8% SDS-PAGE at 25 mA/gel at 4°C for 18 to 20 hours. The detailed procedure for transfer, blocking, and washing was described in our previous publication.23 The membranes were incubated for 10 hours with goat anti-rat CYP4A1 (1:2000; Gentest), goat anti-rat CYP2C11 (1:500; Gentest), rabbit anti-rat CYP2C23 antibody (1:1000; a gift from Dr D.C. Zeldin, National Institute of Environmental Health Science, Research Triangle Park, NC) at room temperature. The membranes were washed several times with TBS solution and further incubated with 1:5000 dilution of horseradish peroxidase–coupled, rabbit anti-goat secondary antibody for CYP4A1 and CYP2C11 and 1:5000 dilution of donkey anti-rabbit secondary antibody for CYP2C23 and CYP2J2. The incubation period for the secondary antibody was 1 hour. The immunoblots were developed with the use of an ECL detection kit (Amersham). To normalize the expression of CYP4A1, CYP2C, and 2J isoforms, renal microsomes (10 μg) from HF and control rats were incubated with a 1:5000 dilution of mouse anti-chicken β-actin antibodies (Sigma) for 10 hours. The secondary antibody was horseradish peroxidase–coupled, rabbit anti-mouse secondary antibody (1:5000). Immuno-
reactive β-actin was detected as described above. The ECL films of Western blot analysis were scanned, and densitometry analysis was performed with Scion Image software, with gray color scale used as standard.

Statistical Analysis
Data are expressed as mean±SEM. All data were analyzed by 1-way ANOVA or Student t test for unpaired samples. Statistical significance was set at P<0.05.

Results
Effect of High Fat Diet on Systolic Blood Pressure, Body Weight, and Fat:Body Ratio
Consistent with reports in the literature, systolic blood pressure was significantly increased in HF rats (Table). After 10 weeks of treatment, HF rats showed significantly higher systolic blood pressure (160±4 versus 139±2 mm Hg, P<0.01, n=10). Moreover, HF rats became obese because both body weight and fat:body weight ratio were significantly higher in HF rats compared with the values of control rats (Table).

Arachidonic Acid Metabolism in HF Rats
To characterize CYP-derived arachidonic acid metabolism pathways in HF rats, renal cortical, medulla, and papilla microsomes were prepared from HF and control rats. As shown in Figure 1, incubation of cortical microsomes with [14C]-arachidonic acid produced DHETs, EETs, and 20-HETE. Cortical microsomal DHET, EET, and 20-HETE formation from HF rats was significantly decreased relative to control rats. Because of the presence of epoxide hydrolase activities in renal cortex, then in papilla, and the lowest activities in medulla (Figure 2).

CYP4A, 2C, 2J Isoform Expression in the Kidney of HF and Control Rats
To examine CYP enzymes responsible for the ω-hydroxylase and epoxygenase activities, we conducted Western blot analysis for CYP4A, 2C, and 2J isoforms in the different renal zones of HF and control rats. A representative Western blot of CYP4A, 2C11, 2C23, and 2J isoforms in the different renal zones of HF rats is shown in Figure 3. The expression of CYP4A isoforms in the cortex, medulla, and papilla of HF rats was lower than in control rats. Densitometry analysis normalized with β-actin revealed a significant decrease of 58% (0.98±0.02 versus 0.42±0.01 arbitrary unit, n=3, P<0.05), 34% (0.57±0.01 versus 0.36±0.003 arbitrary unit, n=3, P<0.05), and 53% (0.68±0.02 versus 0.32±0.01 arbitrary unit, n=3, P<0.05) of CYP4A expression in the cortex, medulla, and papilla microsomes of HF rats. Similarly, a high fat diet decreased CYP2C23 protein by 67% (2.03±0.04 versus 0.67±0.02 arbitrary unit, n=3, P<0.05) in the cortex, by 35% (0.72±0.01 versus 0.47±0.01 arbitrary unit, n=3, P<0.05) in medulla, and by 66% (1.02±0.02 versus 0.34±0.02 arbitrary unit, n=3, P<0.05) in papilla, whereas renal cortical, medulla, and papilla CYP2C11 and CYP2J protein levels were similar between HF and control rats.
Effect of High Fat Diet on Renal Microvessel CYP-Derived Eicosanoid Synthesis

To study renal microvessel CYP-derived eicosanoid synthesis, we established a method adapted from recent publications by Nakagawa et al.25 and Ito et al.26 Whole renal microdissected microvessels permeabilized with 0.1% Tween 80 and then incubated with [14C]-arachidonic acid (30 μmol/L in final concentration) in the presence of NADPH resulted in the production of detectable levels of DHETs, 20-HETE, and EETs. To validate this methodology, preliminary experiments were carried out in male rats treated with CYP4A inhibitor-1-aminobenzotriazole (ABT, 25 mg/kg per day, IP). Renal microvessel arachidonic acid metabolism was determined after 4 days of treatment. Both arachidonic acid ω-hydroxylase and epoxygenase activities were decreased by 58% (52 ± 6 versus 22 ± 1 pmol/mg per minute, P < 0.05, n = 3) and by 21% (23 ± 2 versus 18 ± 1 pmol/mg per minute, n = 3), respectively, after treatment. These results are consistent with our previous report that ABT can significantly decrease renal microvessel homogenate 20-HETE synthesis.27 After establishing the validity of the method, we determined renal microvessel arachidonic acid metabolism in HF and control rats. As shown in Figure 4, a high fat diet treatment has no significant effect on renal microvessel ω-hydroxylase and epoxygenase activities.

Discussion

This study demonstrates that the activities of arachidonic acid metabolic pathways that yield DHETs, EETs, and 20-HETE are decreased in renal tubular sites during the treatment of rats with a high fat diet for 10 weeks. This conclusion is based on the observation that the ability of microsomes isolated from different renal zones to catalyze ω-hydroxylation of arachidonic acid to 20-HETE and epoxidation of arachidonic acid to EETs is significantly decreased in rats fed a high fat diet. The expression levels of CYP4A and CYP2C23 are also significantly decreased in the renal microsomes of HF rats, and there is no significant change of the activities of CYP metabolism in renal microvessels between HF and control rats. Since arachidonic acid metabolites have been shown to play an important role in the inhibition of ion transport along the nephron,5,7 the downregulation of the synthesis of these metabolites may cause the augmentation of ion reabsorption in the kidneys and result in the elevation of blood pressure in HF rats.

Both 20-HETE and EETs are produced in nephron segments and have renal actions that are relevant to the operation of renal mechanisms controlling blood pressure. For example, 20-HETE inhibits renal Na⁺-K⁺-ATPase activity and inhibits sodium transport in isolated perfused rabbit proximal tubules.28 In medullary TALH, McGiff and coworkers have demonstrated the inhibitory effect of 20-HETE on the Na⁺-K⁺-2Cl⁻ cotransporter.9,29 20-HETE has been shown to inhibit K⁺ efflux through a large-conductance (70 ps) K⁺ channel.10 This limits the amount of K⁺ available for transport through the Na⁺-K⁺-2Cl⁻ cotransporter and reduces the lumen positive potential, which is the main driving force for passive reabsorption of cations in this portion of the nephron. With regard to EETs, 5,6-EET has been shown to inhibit sodium transport in the cortical collecting duct.11 and 14,15-EET inhibited apical and basolateral Na⁺-K⁺-2Cl⁻ cotrans-
port in renal epithelial cells. All of these results demonstrate that the upregulation of the synthesis of these eicosanoids causes natriuresis and lowers blood pressure. Therefore, downregulation of the synthesis of these eicosanoids favors the elevation of blood pressure. Actually, the change in tubular 20-HETE synthesis has been suggested to be responsible for resetting renal function and the regulation of blood pressure in hypertensive animal models and human hypertension. For example, Holla et al have demonstrated that CYP2C11 has the highest, CYP2C23 is in the middle, and CYP2C24 has the lowest epoxygenase activity. On the basis of kidney epoxygenase profiles and antibody inhibition studies, it has been established that CYP2C23 is the major arachidonic acid epoxygenase in rat kidney. Our present results indicate that downregulation of CYP4A and CYP2C23 along with the decreasing activities of ω-hydroxylation and epoxidation of arachidonic acid suggest CYP 4A and 2C23 isoforms contribute significantly to the synthesis of these metabolites, and these isoforms may play an important role in the regulation of renal function and blood pressure in HF rats.

Obesity hypertension is characterized by the increases in arterial blood pressure, cardiac output, glomerular filtration rate, and heart rate. Although the exact mechanisms that mediate obesity hypertension are still unknown, a recent review paper by Hall et al suggests a direct link between altered renal function and obesity-induced hypertension. For example, research results by Hall et al have demonstrated that an increase in cumulative sodium balance is associated with the elevation of mean arterial blood pressure in an obese dog model, and the increase of sodium retention is due to an augmentation of tubular reabsorption. In this study, we have shown the downregulation of 20-HETE and EETs in the tubular sites of HF rats. The downregulation of the synthesis of these metabolites in the tubular sites of HF rats may produce an increase of sodium reabsorption and sodium retention because 20-HETE and EETs are known to inhibit sodium transport from lumen of proximal tubule, TALH, and collecting duct into peritubular fluid. Further studies are required to elucidate the downregulation sites of the synthesis of these metabolites in the nephron of HF rats.

**Perspectives**

This study is the first to demonstrate that 20-HETE and EETs and the expression of enzymes that catalyze their formation are altered in the renal tubular sites after the treatment with a high fat diet. The change in the synthesis of 20-HETE and EETs is associated with the elevation of blood pressure in hypertensive animal models and human hypertension. For example, in Dahl salt-sensitive rats, renal medullary synthesis of 20-HETE is much lower than that in Dahl salt-resistant rats, and induction of its tubular synthesis by clofibrate prevents the development of hypertension.

Many results have shown that the CYP4A enzymes are the major arachidonic acid ω-hydroxylases in the rat kidney and thereby the primary contributors of 20-HETE synthesis. For example, several reports have demonstrated that CYP4A isoforms, in their recombinant or purified forms, can catalyze the ω-hydroxylation of arachidonic acid into 20-HETE, and CYP4A1 is the low Km form for ω-hydroxylase. More interestingly, besides ω-hydroxylation of arachidonic acid, CYP4A2 and 4A3 carry out epoxidation of arachidonic acid to 11,12-EET. Moreover, studies with CYP4A isoform-specific antisense oligonucleotides have suggested that CYP4A contributes to renal 20-HETE synthesis in tubular and vascular structures. Although many CYP enzymes can carry out the epoxidation of arachidonic acid, several reports have suggested that CYP2C and 2J isoforms are responsible for renal EETs formation. For example, Holla et al have demonstrated that CYP2C11 has the highest, CYP2C23 is in the middle, and CYP2C24 has the lowest epoxygenase activity. On the basis of kidney epoxygenase profiles and antibody inhibition studies, it has been established that CYP2C23 is the major arachidonic acid epoxygenase in rat kidney. Our present results indicate that downregulation of CYP4A and CYP2C23 along with the decreasing activities of ω-hydroxylation and epoxidation of arachidonic acid suggest CYP 4A and 2C23 isoforms contribute significantly to the synthesis of these metabolites, and these isoforms may play an important role in the regulation of renal function and blood pressure in HF rats.

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In the article by Wang et al (Downregulation of renal CYP-derived eicosanoid synthesis in rats with diet-induced hypertension), which appeared in the October 2003 issue (Hypertension. 2003;42:594–599), there was an error in Figure 4B. The measurements in the Y axis should have been 0 to 70 pmol/mg/min, instead of 0 to 700, as published. Figure 4B is shown correctly here. The author regrets this error.

**Figure 4.** Effect of HF diet on renal microvessel epoxygenase and ω-hydroxylase activities. A, Representative reverse-phase HPLC elution profiles of metabolites formed in incubation of arachidonic acid with whole renal microvessels isolated from control and HF rats. B, Bar graph shows effect of HF diet on epoxygenase and ω-hydroxylase activities. Results are mean±SEM (n=3).