Epoxyeicosatrienoic Acid Inhibition Alters Renal Hemodynamics During Pregnancy

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In this study we examined the expression of cytochrome P450 (CYP) 2C and CYP2J isoforms in renal proximal tubules and microvessels isolated from rats at different stages of pregnancy. We also selectively inhibited epoxyeicosatrienoic acid (EET) production by the administration of *N*-methanesulfonyl-6-(2-proparyloxyphenyl)hexanamide (MSPPOH 20 mg/kg/day iv) to rats during Days 14–17 of gestation and to age-matched virgin rats and determined the consequent effects on renal function. Western blot analysis showed that CYP2C11, CYP2C23, and CYP2J2 expression was significantly increased in the renal microvessels of pregnant rats on Day 12 of gestation. In the proximal tubules, CYP2C23 expression was significantly increased throughout pregnancy, while the expression of CYP2C11 was increased in early and late pregnancy and the expression of CYP2J2 was increased in middle and late pregnancy. MSPPOH treatment significantly increased pregnant rats' mean arterial pressure, renal vascular resistance, and sodium balance but significantly decreased renal blood flow, glomerular filtration rate, and urinary sodium excretion, as well as fetal pups' body weight and length. In contrast, MSPPOH treatment had no effect on renal hemodynamics or urinary sodium excretion in age-matched virgin rats. In pregnant rats, MSPPOH treatment also caused selective inhibition of renal cortical EET production and significantly decreased the expression of CYP2C11, CYP2C23, and CYP2J2 in the renal cortex, renal microvessels, and proximal tubules. These results suggest that upregulation of renal vascular and tubular EETs contributes to the control of blood pressure and renal function during pregnancy. Exp Biol Med 231:1744–1752, 2006

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Introduction

In rat kidneys, cytochrome P450 (CYP) enzymes constitute a major metabolic pathway for arachidonic acid. Epoxidation of arachidonic acid generates four epoxyeicosatrienoic acids (EETs): 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. EETs can be hydrolyzed by epoxide hydrolase to the corresponding dihydroxyeicosatrienoic acids (DHETs): 5,6-DHET, 8,9-DHET, 11,12-DHET, and 14,15-DHET, which have less physiologic activity than their EET counterparts. Based on the results obtained thus far, it is thought that in rat kidneys EET synthesis is catalyzed by CYP2C and CYP2J isoforms (1). In the rat, three major isoforms of the CYP2C family have been identified (CYP2C11, CYP2C23, and CYP2C24), and messages of these isoforms have been detected in the kidneys (2). These isoforms, although sharing 60%–76% homology and a common catalytic activity, are regulated differently by high-salt treatment (2). For example, a previous study by Holla *et al.* (2) showed that renal CYP2C23 is upregulated during excess salt treatment. These authors also found that CYP2C23 is the predominant arachidonic acid epoxygenase in the rat kidney. Besides members of the CYP2C subfamily, CYP2J isoforms are implicated in EET synthesis in the rat kidney (1).

During the past decade it has been recognized that EETs have important biologic effects in the kidneys, including inhibition of sodium transport in the nephron and vasodilation of renal arterioles (1, 3). EETs are produced in the proximal tubules, where they inhibit Na^+-K^+-ATPase activity (4) and sodium transport (1). Wei *et al.* demonstrated that in the cortical collecting duct 11,12-EET has a direct inhibitory effect on the activity of epithelial sodium channels

(5). In renal microvessels, 11,12-EET and 14,15-EET cause vasodilation (6); 11,12-EET can also activate potassium channels in vascular smooth muscle cells and act as an endothelium-derived hyperpolarizing factor (7, 8). In addition, several studies have demonstrated the participation of EETs in regulating renal hemodynamics (9, 10).

Normal pregnancy in rats is associated with significantly decreased blood pressure and total peripheral resistance but also with increased cardiac output, glomerular filtration rate, and renal blood flow (11, 12). These maternal changes during pregnancy are important to accommodate the growth of the fetus (13). Since the biologic activities of EETs have various effects on the renal tubules and microvessels (1, 3), we hypothesize that these eicosanoids also contribute to the regulation of renal function and blood pressure during pregnancy. We previously demonstrated that renal cortical EET production and the expression levels of CYP epoxygenases (CYP2C11, CYP2C23, and CYP2J2) are significantly elevated in pregnant rats and that the inhibition of EET production by a selective epoxygenase inhibitor, 6-(2-propargyloxyphenyl)hexanoic acid, during late pregnancy causes hypertension and a reduction in the body weight of fetal pups (14). However, the sites at which the upregulation of renal EET synthesis occurs have not been identified. It also remains unclear whether or not elevated EET synthesis has any effects on the regulation of renal function in pregnant rats.

In the present study, we examined the expression of CYP epoxygenases in renal microvessels and proximal tubules during early, middle, and late pregnancy. We also examined the effects of another selective CYP epoxygenase inhibitor, *N*-methanesulfonyl-6-(2-proparyloxyphenyl)hexanamide (MSPPOH), on various renal functional parameters, including mean arterial pressure (MAP), renal blood flow (RBF), renal vascular resistance (RVR), glomerular filtration rate (GFR), and urinary sodium excretion. Age-matched virgin rats served as controls. This study provides valuable information regarding the contribution of EETs to the regulation of renal function in pregnant rats.

Materials and Methods

Materials. We obtained [1-^{14}C]-arachidonic acid (56 mCi/mmol) from DuPont-New England Nuclear (Boston, MA). All HPLC solvents and buffer chemicals were from Sigma-Aldrich (Milwaukee, WI). We purchased 20-HETE, EETs, and DHETs standard from Cayman Chemicals (Ann Arbor, MI).

Animals. All animals were purchased from Charles River Laboratories (Wilmington, MA). We conducted experiments on pregnant (timed pregnancy) and age-matched virgin Sprague-Dawley rats. All rats were maintained on a 12-hour light-dark cycle and were housed two to a cage. All animal protocols were approved by the Institutional Animal Care and Use Committee and were in accordance with the requirements for animal use stated in the *Guide for the Care and Use of Laboratory Animals.*

Protocol to Evaluate the Expression of CYP2C and CYP2J in Renal Microvessels and Proximal Tubules of Pregnant and Control Rats. Virgin ($n = 4$) and pregnant rats ($n = 4$) on the 6th, 12th, or 19th gestational day were anesthetized with 2% isoflurane. These gestational days, respectively, represent early, middle, and late pregnancy. We anesthetized the rats, removed their kidneys, and isolated the renal microvessels and proximal tubules. To isolate the renal microvessels, we placed the excised kidneys in ice-cold Tyrode's buffer and sectioned them coronally. The renal papilla was removed to expose the microvessels. We microdissected the segments of the interlobar artery free from the cortical and connective tissues. The purity of the microdissected microvessel preparation was determined as described previously (15). The proximal tubules were isolated according to the method of Hatzinger *et al.* (16) and Chaudhari *et al.* (17), which uses proteolytic digestion and Percoll gradient centrifugation. These procedures yielded a preparation of proximal tubules that was about 95% pure as measured by alkaline phosphatase staining (18). We examined the expression of CYP2C and CYP2J isoforms in the renal microvessels and proximal tubules by Western blot analysis.

Protocol to Evaluate the Effect of *N*-Methane-Sulfonyl-6-(2-Proparyloxyphenyl)Hexanamide on Renal Function in Pregnant and Virgin Rats. We placed rats at the 12th gestational day ($n = 12$) and age-matched virgin rats ($n = 12$) in metabolic cages to obtain the basal levels of 24-hour urinary sodium excretion and sodium balance. The sodium intake was calculated from rats' daily consumption of food; sodium excretion was calculated from the volume of urine. Sodium balance was calculated as the differences between sodium intake and urinary sodium excretion. We then divided the pregnant rats (14th day of gestation) and age-matched virgin rats into two groups ($n = 6$). We treated one group with a vehicle (2-hydroxypropyl-β-cyclodextrin) and the other group with MSPPOH, 20 mg/kg/day, administered intravenously through a tail vein, for 4 days. The dosage of MSPPOH was based on our previous study and a literature search (14, 19). On Day 17 of pregnancy we returned these rats to the metabolic cages to measure 24-hour urinary sodium excretion and sodium balance again. We determined urinary sodium concentrations using the Beckman Synchron EL-ISE Electrolyte System (Brea, CA).

We then used these MSPPOH- and vehicle-treated pregnant rats (18th day of pregnancy) and age-matched virgin rats for a renal functional study. After finishing that study we immediately removed their kidneys for renal cortical, renal microvessel, and proximal tubular preparation. These renal tissues were used to assess renal EET production and CYP2C and CYP2J expression. Fetal pups were removed from the pregnant rats ($n = 6$); the body weight and length of each pup was recorded.

Renal Functional Measurements. For the renal functional study of pregnant rats and age-matched virgin rats from the MSPPOH- and vehicle-treated groups, we weighed each rat before surgery and anesthetized with 2% isoflurane delivered by an anesthesia apparatus. We then placed one polyethylene cannula in the trachea (PE-205) to allow free breathing, one in the bladder (PE-240) to collect urine, one in the femoral artery (PE-50) for measuring and recording MAP with a pressure transducer, and one (PE-50) in the femoral vein for the infusion of agents. We then began infusing saline (3 ml/hr, iv). A priming dose of 0.5 ml of fluorescein isothiocyanate (FITC) inulin (8 mg/ml in PBS; Sigma-Aldrich) was administered over 2 min. We performed a left laparotomy and placed a transonic flow probe (Transonic System, Inc., Ithaca, NY) over the left renal artery to measure RBF. Throughout these procedures the rat's body temperatures were maintained at 37°C by a temperature controller (Cole Palmer Instrument, Vernon Hills, IL) connected to a heating mat and a rectal temperature probe. We infused a saline solution containing 6.2% bovine serum albumin equal to 1.25% of body weight for the replacement of volume loss during surgery. We then infused saline (3 ml/hr, iv) and FITC inulin (12 mg/hr, iv) to maintain isotonic NaCl and a constant concentration of FITC inulin.

After surgery we allowed at least 45 min for equilibration before beginning 30-min urine collections. Arterial blood (0.4 ml) was drawn from the femoral artery in the middle of each 30-min clearance period for measurement of GFR. An equal volume of normal saline was infused for volume replacement. We obtained MAP, RBF, and RVR from a computerized data collection and analysis system (EMKA Technologies, Falls Church, VA). We determined the concentration of FITC inulin in the plasma and urine using a GENios Plus Fluorescent Plate Reader (Tecan, Research Triangle Park, NC) at 485-nm excitation and 538-nm emission. As previously described, we used the concentration of FITC inulin in the plasma and urine to calculate the GFR (20).

Arachidonic Acid Metabolism in Renal Cortical Homogenates. We incubated renal cortical homogenates (1 mg) isolated from treated and control rats with [1-^{14}C]-arachidonic acid (0.4 μCi, 7 nmol) and NADPH (1 m*M*, final concentration) in 1 ml of potassium phosphate buffer (100 m*M*, pH 7.4) containing 10 m*M* $MgCl_2$ for 15 min at 37°C. The reaction was terminated by acidification to pH 3.5–4.0 with 2 mol/l formic acid, after which we extracted arachidonic acid metabolites with ethyl acetate. We evaporated the ethyl acetate under nitrogen, resuspended the metabolites in 50 μl of methanol, and injected them into a high-performance liquid chromatography (HPLC) column. We performed reverse-phase HPLC on a 5-μm ODS-Hypersil Column, 4.6 × 200 mm (Hewlett Packard, Palo Alto, CA) using a linear gradient of acetonitrile:water:acetic acid ranging from 50:50:0.1 to 100:0:0.1 at a flow rate of 1 ml/min for 30 min. The elution profile of the radioactive products was monitored by a flow detector (IN/US Systems, Inc., Tampa, FL). We confirmed the identity of 20-HETE, DHETs, and EETs with authentic standards. The activity of 20-HETE formation was estimated based on the specific activity of the added [1-^{14}C]-arachidonic acid and expressed as pmol/mg protein/min as described previously (15, 21).

Western Blot Analysis. We separated renal samples (10 μg each) from pregnant and virgin rats and from MSPPOH- and vehicle-treated pregnant rats by electrophoresis on a 10 × 20 cm, 8% SDS-polyacrylamide gel at 25 mA/gel at 4°C for 18–20 hr. The proteins were electrophoretically transferred to an enhanced chemiluminescence (ECL) membrane. We blocked the membranes with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 10 m*M* Tris-HCl, 0.1% Tween-20, and 150 m*M* NaCl for 90 min and then washed three times with TBS. We incubated the membranes for 10 hr with goat anti-rat CYP2C11 (1:2000; Gentest; Woburn, MA), rabbit anti-rat CYP2C23 antibody (1:5000; a gift from Dr. J. H. Capdevila, Vanderbilt University, Nashville, TN), or rabbit anti-human CYP2J2 antibody (1:2000; a gift from Dr. D.C. Zeldin, National Institute of Environmental Health Science, Research Triangle Park, NC) at room temperature. We washed the membranes several times with TBS solution and further incubated them with a 1:5000 dilution of horseradish-peroxidase–coupled rabbit anti-goat secondary antibody (Sigma, St. Louis, MO) for CYP2C11 and a 1:5000 dilution of donkey anti-rabbit second antibody (Sigma) for CYP2C23 and CYP2J2. The incubation for the secondary antibody was 1 hr. The immunoblots were developed using an ECL detection kit (Amersham, Arlington Heights, IL). To normalize the expression of CYP isoforms, renal microsomes (10 μg) from treated and control rats were incubated with a 1:5000 dilution of mouse anti-chicken β-actin antibodies (Sigma) for 10 hr. The secondary antibody (Sigma) was horseradish-peroxidase–coupled rabbit anti-mouse antibody (1:5000). Immunoreactive β-actin was detected as described earlier. We scanned the ECL films of Western blot analyses and performed densitometry analysis with Scion Image software (Scion Corp., Frederick, MD) using the gray color scale as a standard.

Statistical Analysis. Data are expressed as mean ± SE. All data were analyzed by SPSS computer software (SPSS Inc., Chicago, IL). The significance of differences between groups was evaluated with analysis of variance ANOVA for repeated measurements followed by a Duncan's multiple-range post hoc test. All other data were analyzed by one-way ANOVA or an unpaired two-tailed *t*-test. Statistical significance was set at $P < 0.05$.

Results

Expression of CYP2C and CYP2J Isoforms in Renal Proximal Tubules and Microvessels During Pregnancy. To characterize EET production in these sites, we used Western blot analysis to examine the expression of CYP2C and CYP2J isoforms in renal microvessels and proximal tubules isolated from rats in

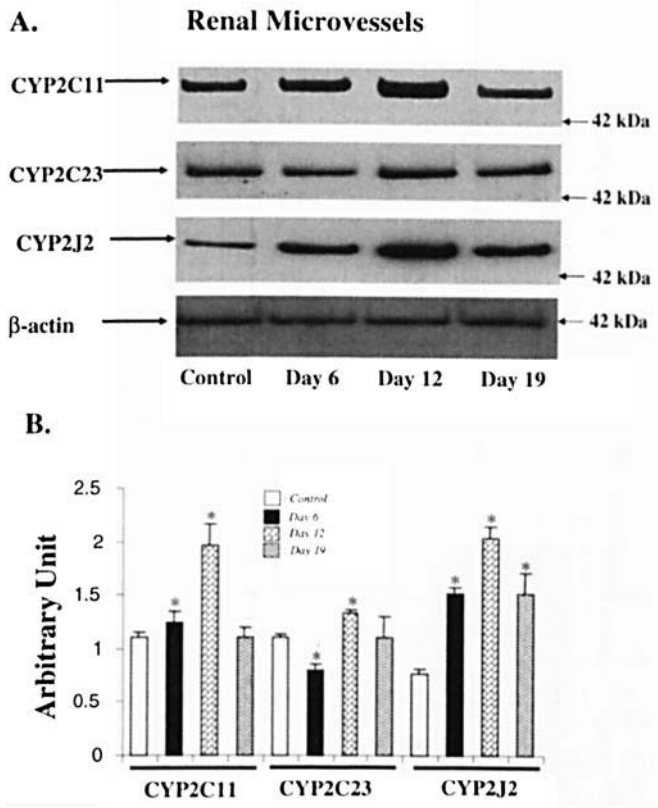

Figure 1. Representative immunoblots of CYP2C11, CYP2C23, and CYP2J2 isoforms and β-actin in renal microvessels (A) and densitometry analysis normalized with β-actin (B) from virgin rats and rats on the 6th, 12th, and 19th days of pregnancy. $n = 4$, $^*P < 0.05$ vs control virgin rats.

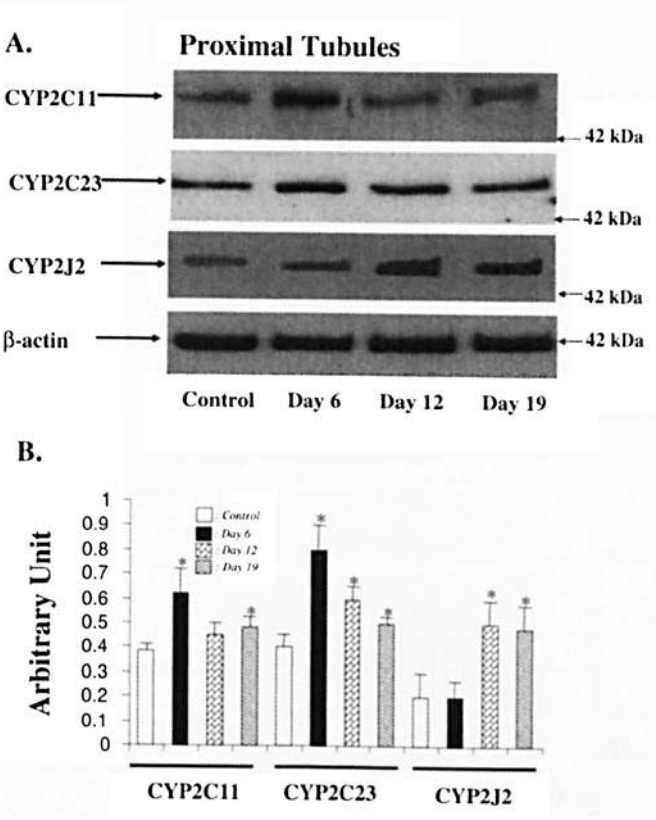

Figure 2. Representative immunoblots of CYP2C11, CYP2C23, and CYP2J2 isoforms and β-actin in the proximal tubules (A) and densitometry analysis normalized with β-actin (B) from virgin rats and rats on the 6th, 12th, and 19th days of pregnancy. $n = 4$, $^*P < 0.05$ vs control virgin rats.

early, middle, and late pregnancy. A representative Western blot of CYP2C and CYP2J isoforms in the renal microvessels is shown in Figure 1A. Densitometry analysis normalized with β-actin (Fig. 1B) showed that as compared to values in control rats, CYP2C11 expression on Day 6 of pregnancy was increased by 13% and on Day 12 of pregnancy was significantly increased by 97%. However, there was no significant change in CYP2C11 expression on Day 19 of pregnancy. CYP2C23 expression on Day 6 of pregnancy was significantly decreased by 23% as compared with that in control rats, as well as significantly increased by 21% on Day 12 of pregnancy. There was no significant change in CYP2C23 expression on Day 19 of pregnancy. On Days 6, 12, and 19 of pregnancy CYP2J2 expression was significantly increased by 97%, 164%, and 98%, respectively.

In the proximal tubules (Fig. 2B), densitometry analysis showed that on Day 6 of gestation CYP2C11 expression was significantly increased by 63%. Similarly, on Day 19 of pregnancy CYP2C11 was significantly increased by 18%. However, the levels of CYP2C11 expression on Day 12 of gestation did not significantly differ from that in virgin rats. CYP2C23 expression on Days 6, 12, and 19 of pregnancy was increased by 100%, 50%, and 25%, respectively; all of these increases were significant. The expression of CYP2J2 increased significantly on Day 12 of pregnancy by 150% and on Day 19 of pregnancy by 140%. However, the levels of CYP2J2 expression on Day 6 of pregnancy did not significantly differ from that in virgin rats.

Effects of MSPPOH on the Renal Function of Pregnant and Control Female Rats and on the Size of Fetal Pups. The preceding study showed upregulation of renal EET production in pregnant rats (14). To assess the effect of elevated EET production in pregnant rats, we examined renal functional parameters in pregnant and age-matched virgin rats treated with vehicle and MSPPOH. As shown in Figure 3, MAP was significantly lower on Day 18 of gestation than that in control rats. MSPPOH treatment from Days 14–17 of gestation caused a significant elevation of blood pressure, from 97 ± 2 to 117 ± 3 mm Hg. Pretreatment RBF values were slightly higher in pregnant rats than those in control rats (5.9 ± 0.3 vs 5.1 ± 0.3 ml/min). MSPPOH treatment of pregnant rats significantly decreased RBF from 5.9 ± 0.3 to 3.8 ± 0.1 ml/min. Pretreatment RVR values in pregnant rats were significantly lower than those in control rats (16 ± 1 vs 21 ± 1.3 mm Hg/ml/min, $P < 0.05$), but MSPPOH treatment significantly increased these values, from 16 ± 1 to 29 ± 1.3 mm Hg/ml/min. Conversely, as shown in Figure 3D, the pretreatment GFR values in pregnant rats were significantly higher

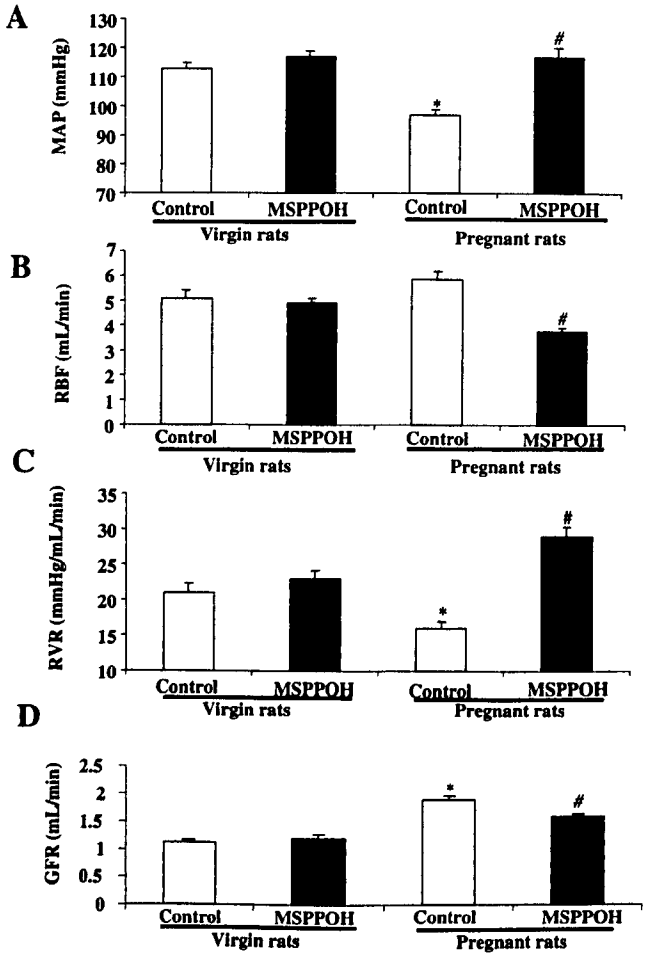

Figure 3. MAP (A), RBF (B), RVR (C), and GFR (D) in pregnant rats and age-matched virgin rats treated with MSPPOH (20 mg/kg/day, iv) or vehicle control for 4 days, beginning on the 14th day of pregnancy. Renal hemodynamics of pregnant rats was measured on the 18th day of pregnancy. Results are means ± SE ($n = 6$, $^{*}P < 0.05$ vs control virgin rats; $^{\#}P < 0.05$ vs vehicle-treated pregnant rats).

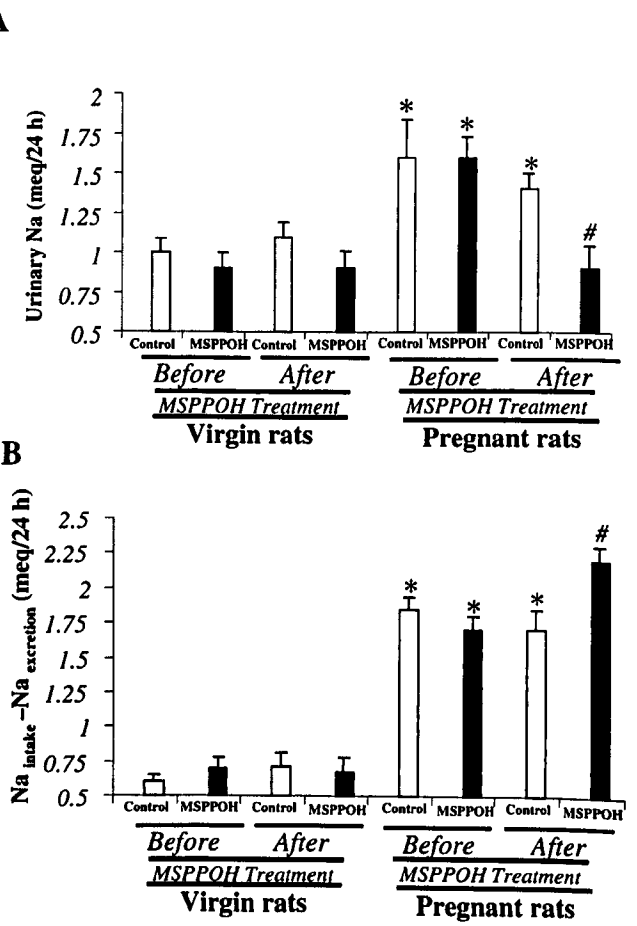

Figure 4. Urinary sodium excretion (A) and sodium balance (B) of age-matched virgin rats and pregnant rats before (Days 12–13 of pregnancy) and after (Days 17–18 of pregnancy) treatment with MSPPOH (20 mg/kg/day, iv) or vehicle control for 4 days, beginning on the 14th day of pregnancy. Results are means ± SE ($n = 6$, $^{*}P < 0.05$ vs control virgin rats; $^{\#}P < 0.05$ vs vehicle-treated pregnant rats).

than those in control rats (1.9 ± 0.08 vs 1.1 ± 0.04 ml/min, $P < 0.05$) but were significantly decreased by MSPPOH treatment (1.6 ± 0.05 vs 1.9 ± 0.08 ml/min, $P < 0.05$).

Urinary sodium excretion was significantly increased on Day 12 and Day 17 of gestation as compared with that in control rats (Fig. 4A). A significant decrease in urinary sodium excretion was noted 4 days after the beginning of MSPPOH treatment. Sodium intake was significantly increased in pregnant rats as compared with that of virgin rats (3.2 ± 0.1 vs 1.7 ± 0.1 mEq/24 hr, $P < 0.05$). $\text{Sodium}_{\text{intake}} - \text{sodium}_{\text{excretion}}$ values (an index for sodium balance) were significantly increased on Days 12 and 17 of gestation as compared with values in control rats. Moreover, a significant increase in $\text{sodium}_{\text{intake}} - \text{sodium}_{\text{excretion}}$ value was found 4 days after the beginning of MSPPOH treatment (Fig. 4B). In contrast, the MAP, RBF, RVR, GFR, and urinary sodium excretion in MSPPOH-treated virgin rats did not significantly differ from the corresponding values in vehicle-treated virgin rats (Figs. 3 and 4). Urine volume was significantly increased in pregnant rats as compared with that in control rats (36.7 ± 4 ml in pregnant group vs 16.7 ± 2 ml in control group, $P < 0.05$, $n = 6$).

MSPPOH treatment also significantly reduced both the body weight (0.9 ± 0.2 g in the MSPPOH-treated group vs 1.8 ± 0.2 g in the vehicle-treated pregnant group, $P < 0.05$, $n = 6$) and the body length of fetal pups (1.5 ± 0.2 cm in the MSPPOH-treated group vs 2.2 ± 0.3 cm in the vehicle-treated pregnant group, $P < 0.05$, $n = 6$). However, MSPPOH treatment had no significant effect on the number of pups (13 ± 2 for the vehicle-treated pregnant rats vs 12 ± 2 for the MSPPOH-treated pregnant rats, $n = 6$).

Effects of MSPPOH on Renal Cortical EET Production and CYP2C and CYP2J Expression in Renal Tissues of Pregnant Rats. To study the selectivity of the effect of MSPPOH on renal EET production, we used HPLC to examine renal cortical arachidonic acid metabolism in pregnant rats treated with MSPPOH and in vehicle-treated control pregnant rats.

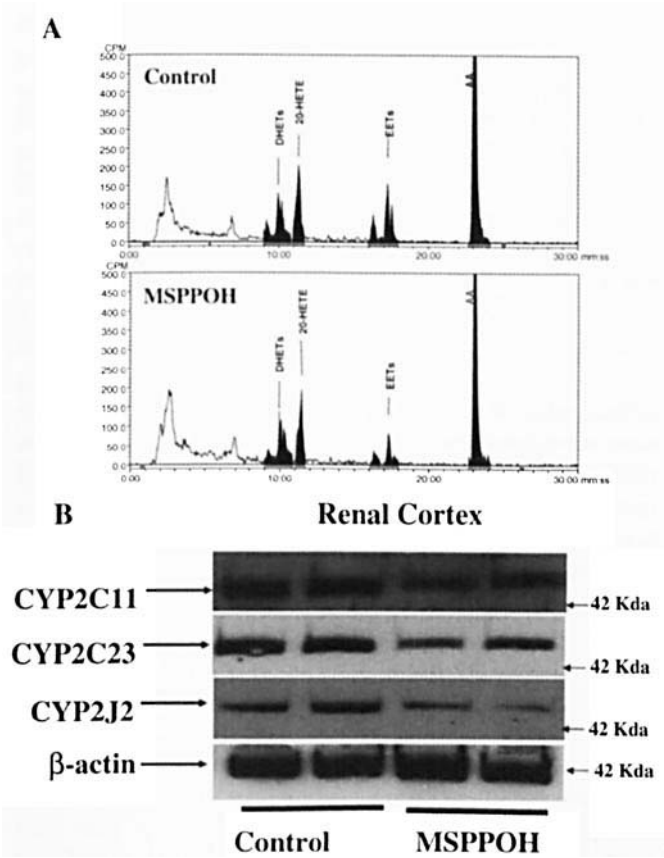

Figure 5. Effect of MSPPOH treatment on renal EET production and CYP2C and CYP2J2 isoform expression in the renal cortex of pregnant rats. (A) Representative reverse-phase HPLC elution profile of metabolites formed during incubation of arachidonic acid with renal cortical homogenates from vehicle- and MSPPOH-treated pregnant rats. (B) Representative Western blot of CYP2C11, CYP2C23, and CYP2J2 isoforms in renal cortical homogenates isolated from vehicle- and MSPPOH-treated pregnant rats.

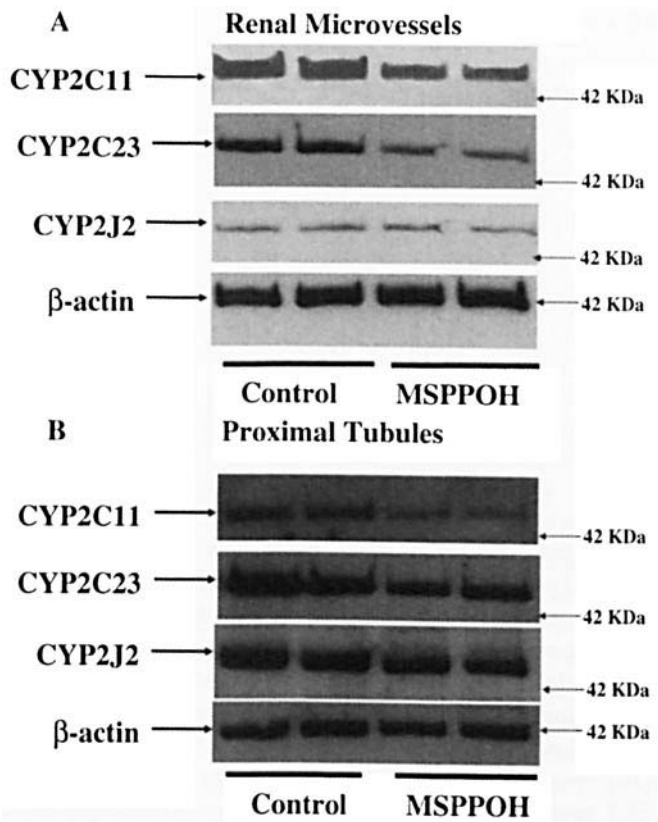

Figure 6. Effect of MSPPOH treatment on CYP2C and CYP2J2 isoform expression in the renal microvessel and proximal tubules of pregnant rats. (A) Representative Western blot of CYP2C11, CYP2C23, and CYP2J2 isoforms in renal microvessel homogenates isolated from vehicle- and MSPPOH-treated pregnant rats. (B) Representative Western blot of CYP2C11, CYP2C23, and CYP2J2 isoforms in renal proximal tubular homogenates isolated from vehicle- and MSPPOH-treated pregnant rats.

Incubation of renal cortical homogenates isolated from pregnant rats with [^{14}C]-arachidonic acid and NADPH produced DHETs, 20-HETE, and EETs (Fig. 5A). MSPPOH treatment of pregnant rats decreased EET and DHETs production by 28% (59.4 ± 4 vs 80 ± 6 pmol/mg/min, $n = 4$, $P < 0.05$), whereas MSPPOH treatment did not affect production of 20-HETE.

To examine the effect of MSPPOH treatment on CYP2C and CYP2J expression in different tissues, we performed Western blot analysis for CYP2C11, CYP2C23, and CYP2J2 in homogenates of the renal cortex, renal microvessels, and proximal tubules isolated from pregnant rats. As shown in Figure 5B, in the renal cortex MSPPOH treatment caused a 25% decrease in the expression of CYP2C11 (0.53 ± 0.05 vs 0.71 ± 0.04 arbitrary units, $n = 4$, $P < 0.05$), as well as a 30% decrease in the expression of CYP2C23 (0.53 ± 0.05 vs 0.76 ± 0.02 arbitrary units, $n = 4$, $P < 0.05$) and a 68% decrease in the expression of CYP2J2 (0.13 ± 0.06 vs 0.4 ± 0.04 arbitrary units, $n = 4$, $P < 0.05$); all of these decreases were significant. Similarly, as shown in Figure 6A, in renal microvessels MSPPOH treatment decreased the expression of CYP2C11 by 30% (0.7 ± 0.15 vs 1.0 ± 0.1 arbitrary units, $n = 4$), that of CYP2C23 by 50% (0.39 ± 0.1 vs 0.79 ± 0.1 arbitrary units, $n = 4$, $P < 0.05$), and that of CYP2J2 by 9% (0.09 ± 0.03 vs 0.1 ± 0.02 arbitrary units, $n = 4$). In the proximal tubules MSPPOH treatment decreased the expression of CYP2C11 by 45% (0.44 ± 0.06 vs 0.8 ± 0.1 arbitrary units, $n = 4$, $P < 0.05$), that of CYP2C23 by 30% (1.1 ± 0.2 vs 1.5 ± 0.1 arbitrary units, $n = 4$, $P < 0.05$), and that of CYP2J2 by 16% (1.34 ± 0.15 vs 1.6 ± 0.1 arbitrary units, $n = 4$, $P < 0.05$). In addition, we examined the effects of MSPPOH treatment on the expression of CYP2C11, CYP2C23, and CYP2J2 in the homogenate of renal cortex isolated from control virgin rats. As shown in Figure 7, MSPPOH treatment caused a 20% decrease in the expression of CYP2C11 (2.1 ± 0.2 vs 1.7 ± 0.3 arbitrary units, $n = 4$), as well as a 30% decrease in the expression of CYP2C23 (5.3 ± 0.3 vs 3.7 ± 0.2 arbitrary units, $n = 4$, $P < 0.05$) and a 27% decrease in the expression of CYP2J2 (1.5 ± 0.1 vs 1.1 ± 0.1 arbitrary units, $n = 4$, $P < 0.05$).

Discussion

This study demonstrated that the expression of CYP epoxygenases was increased in renal microvessels during middle pregnancy and was increased in proximal tubules at different stages of pregnancy. It also demonstrated that selective inhibition of renal EET synthesis in pregnant rats by MSPPOH treatment caused changes in renal function. Specifically, selective inhibition by MSPPOH treatment increased MAP, RVR, and sodium balance, while decreasing RBF, GFR, and urinary sodium excretion in pregnant rats. However, MSPPOH treatment had no significant effects on these renal-function parameters in the virgin rats. In addition, treatment with MSPPOH during Days 14–17 of gestation caused downregulation of the expression of CYP2C and CYP2J isoforms in both renal microvessels and proximal tubules.

Normal pregnancy in rats is associated with reductions in blood pressure (11, 22). Since EETs inhibit renal tubular sodium transport and affect blood pressure in hypertensive animals, we hypothesize that EETs may also be involved in the regulation of blood pressure during pregnancy in rats. In a previous study, we demonstrated that renal cortical CYP2C and CYP2J are elevated during pregnancy in rats (14). We also demonstrated that the augmentation of renal EET production is associated with lowered blood pressure during pregnancy (14). However, the relative expression of these isoforms in renal vascular and tubular sites during pregnancy still was not clear.

In the present study, we showed that the expression of CYP2C and CYP2J isoforms increased in the renal microvessels, whereas in the proximal tubules the expression of these isoforms tended to increase at different times during pregnancy. The exception to this tendency was that no significant changes in CYP2C11 expression occurred in middle pregnancy or in CYP2J2 expression in early pregnancy (Fig. 2B). Since CYP2C and CYP2J isoforms are the major enzymes for EET synthesis (1), these results suggest that EET synthesis is increased in the renal microvessels of middle pregnancy and in the proximal tubules at different stages of pregnancy. Nevertheless, the reasons for the differential increase of these CYP2C and CYP2J isoforms during pregnancy are still not clear. It is possible that this differential increase is a consequence of the increased circulation levels of some hormones during pregnancy. Pregnant rats, for example, have significantly increased levels of progesterone, estrogen, and relaxin (23). Whether or not the pregnancy-induced changes in hormonal background can regulate the expression of CYP epoxygenase will require further investigation.

The changes in EET production in the renal microvessels and proximal tubules at particular time during pregnancy have important functional implications. For example, Alexander *et al.* (11) showed that renal vasodilation and augmentation of GFR occurs in middle pregnancy (11). In renal microvessels EETs cause vasodilation, activate vascular smooth muscle cell potassium channels, and act as endothelium-derived hyperpolarizing factors (6–8). Therefore, the augmentation of renal vascular EET production can cause the observed vasodilation and contribute to the increased RBF and GFR in middle pregnancy. Moreover, during pregnancy in rats there is significantly increased sodium intake, which results in increased urinary sodium excretion (Fig. 4A). Interestingly, $\text{sodium}_{\text{intake}} - \text{sodium}_{\text{excretion}}$ is significantly increased in pregnant rats as compared with that of virgin rats (Fig. 4B). This is a good index of significantly increased sodium retention during pregnancy (24). In other words, the upregulation of EETs may serve as an adjustable mechanism to increase urinary sodium excretion and maintain sodium balance during pregnancy. Once EET production is inhibited by MSPPOH treatment in pregnant rats, their bodies tend to decrease urinary sodium excretion (Fig. 4A), which may cause extra sodium retention, elevation of blood pressure, and abnormal renal hemodynamics (Fig. 3). It should be noted, however, that this result is opposite to the results reported by Brand-Schieber *et al.* (19) who showed that MSPPOH treatment increased urinary sodium excretion and urine flow rates. The reasons for the inconsistency of their results and ours are still not known, but it could be a consequence of procedural differences. For example, we used pregnant rats, whereas Brand-Schieber *et al.* used male rats; we administered 20 mg/kg/day, while they used 5 mg/kg/day; and we injected MSPPOH once a day for 4 days, whereas they injected only one dose.

We also used MSPPOH to determine the effects of EET inhibition on blood pressure, renal hemodynamics, and urinary sodium excretion. The selective inhibition of EET production in the kidneys of pregnant rats is shown in Figure 5A. Our results indicate that MSPPOH treatment increases blood pressure in pregnant rats, and that this is associated with increased RVR, decreased RBF, decreased GFR, and decreased urinary sodium excretion (Figs. 3 and 4). Interestingly, MSPPOH decreases the expression of CYP epoxygenase in virgin rats (Fig. 7), but it has no significant effects on the renal function of virgin rats (Figs. 3 and 4). These findings suggest that the induction of renal vascular and proximal tubular EET synthesis or the overproduction of renal EETs in pregnant rats is a very important factor to mediate the maternal renal physiologic adaptations during pregnancy.

Several other studies have also demonstrated that EETs are involved in the regulation of renal hemodynamics. For instance, Takahashi *et al.* (10) showed that direct infusion of 5,6-EET (2 μg/kg/min) causes a reduction in RBF and GFR. However, in the presence of ibuprofen, a cyclooxygenase inhibitor, the effects of 5,6-EET treatment are reversed so that RBF and GFR are increased (10). This occurs because *in vivo* exogenous EET is easily metabolized by cyclooxygenase to a prostaglandin metabolite, which has vasoconstrictive activity (1). Interestingly, Ogungbade *et al.* (25) made similar observations and demonstrated that infusion of

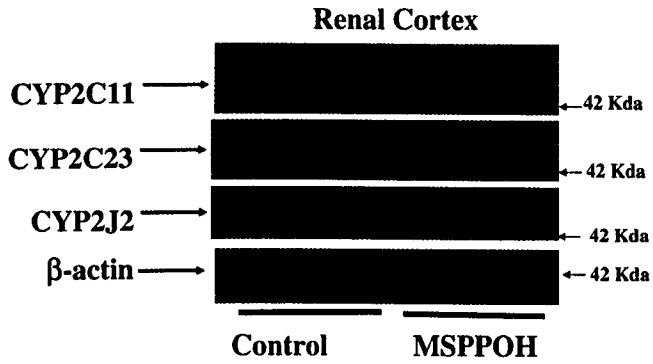

Figure 7. Effect of MSPPOH treatment on CYP2C and CYP2J2 isoform expression in the renal cortex of virgin rats. Representative Western blot of CYP2C11, CYP2C23, and CYP2J2 isoforms in renal cortical homogenates isolated from vehicle- and MSPPOH-treated virgin rats.

EETs reduces cortical blood flow in male rats. These studies not only indicate the complexity involved in interpreting the effects of treatment with exogenous EETs *in vivo* but also suggest the importance of studying the effects of EETs by means of selective inhibitors such as MSPPOH. Since we did not observe significant renal functional changes in MSPPOH-treated virgin rats (Figs. 3 and 4), the upregulation of renal EET production may be an important mechanism in the regulation of renal function during pregnancy. Taken together, the results of the present study solidly demonstrate that the upregulation of renal EET synthesis has many functional implications with respect to the regulation of blood pressure and renal hemodynamics in pregnant rats.

Previously we used 6-(2-propargyloxyphenyl)hexanoic acid (PPOH) to inhibit EET production *in vivo* (14). In the present study, we used another selective inhibitor of CYP epoxygenases, MSPPOH, which may be a better inhibitor for *in vivo* studies than PPOH because its methyl sulfonate group can make MSPPOH more resistant to β-oxidation (26). The reason for the selectivity of MSPPOH and PPOH on CYP epoxygenase activity could be the benzene ring moiety in these inhibitors. In this regard, Mancy *et al.* (27) proposed a model for the active site of CYP2C9 in which hydrophobic and/or π- π interaction is important for binding between the benzene ring of substrates and the aromatic amino acid residues of CYP2C9. It is possible that the benzene ring moiety of these two inhibitors is crucial for the binding of MSPPOH and PPOH to CYP epoxygenase and cause the selectivity of these two inhibitors on CYP epoxygenase activity. Besides their benzene rings, MSPPOH and PPOH contain a terminal acetylene moiety, an important chemical group that causes suicide-inhibition of CYP enzymes (28). Ortiz de Montellano *et al.* (28) demonstrated that the terminal acetylene moiety of inhibitors can be oxidized to a ketene moiety, which can then result in alkylation of the heme moiety of CYP epoxygenase and cause decreased expression of CYP epoxygenase.

Because of these specific chemical features, we can monitor the action of MSPPOH by examining the expression of CYP epoxygenases in rat kidneys. As shown in Figure 6, chronic treatment with MSPPOH caused decreased expression of renal microvessel and proximal tubular CYP2C and CYP2J2 isoforms. This finding demonstrated that intravenously injected MSPPOH can travel into both renal vascular and renal tubular sites to inactivate CYP2C and CYP2J2 isoforms localized in these renal tissues. Similar results were observed with PPOH treatment in pregnant rats (14). More interestingly, the downregulation of these isoforms by MSPPOH treatment is not only associated with changes in the renal vascular effect (increased RVR; Fig. 3) and the tubular effect (decreased urinary sodium excretion and increased $sodium_{intake} - sodium_{excretion}$; Fig. 4) but also causes the decrease in the size of pups (fetotoxic effects). The reasons for the MSPPOH-induced fetotoxic effects are not known. These effects could be caused by abnormal renal hemodynamics as this study suggests. Alternatively, they could be results of the suppressed effect of hemodynamics in reproductive organs after MSPPOH treatment. Interestingly, it has been reported that 14,15-EET is produced in human reproductive tissues (29). If EETs are also produced in rat reproductive tissues, it is possible that the inhibition of EET production in these tissues by MSPPOH treatment causes decreased blood flow and that this in turn causes the decrease in fetus size. Taken together, these findings demonstrate that the selective inhibition of EET production by MSPPOH *in vivo* (Fig. 6) is a useful approach to elucidating the mediating effects of EETs on physiologic function.

In summary, this study demonstrated that CYP epoxygenases are altered in renal microvessels and proximal tubules during pregnancy in rats. Our findings also demonstrated that MSPPOH, a selective CYP epoxygenase inhibitor, downregulates the expression of CYP epoxygenases at these sites in pregnant rats and causes increased MAP, RVR, and sodium balance; decreased RBF and GFR; and reduced size of fetal pups. This study calls attention to the possibility that augmentation of EET synthesis in renal vascular and tubular sites during pregnancy alters the regulation of renal function and blood pressure.

Perspectives. It has been shown that EETs dilate renal microvessels, inhibit renal tubular sodium transport, and affect renal blood flow and blood pressure in many animal models. This study demonstrates that renal vascular and tubular EET synthesis is induced during different stages of pregnancy and that malregulation of renal vascular and tubular EET synthesis can cause abnormalities in renal function and blood pressure. It appears that the changes of renal vascular and tubular EET synthesis may be an important factor in maintaining normal renal hemodynamics and sodium transport during gestation. Inhibition of renal microvessel and proximal tubular EET synthesis may contribute to the pathogenesis of hypertension during pregnancy by promoting renal vasoconstriction, decreasing renal blood flow, and decreasing urinary sodium excretion.

These alterations in renal hemodynamics can ultimately affect the development of the fetus. The present study establishes a basis for further investigations aimed at defining the regulatory mechanisms of EET synthesis in the renal microcirculation and tubular structures during pregnancy.

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1. Roman RJ. P-450 metabolites of arachidonic acid in the control of cardiovascular function. Physiol Rev 82:131–185, 2002.
2. Holla VR, Makita K, Zaphiropoulos PG, Capdevila JH. The kidney cytochrome P-450 2C23 arachidonic acid epoxygenase is upregulated during dietary salt loading. J Clin Invest 104:751–760, 1999.
3. Imig JD. Eicosanoid regulation of the renal vasculature. Am J Physiol 279:F965–F981, 2000.
4. Satoh T, Cohen HT, Katz AI. Intracellular signaling in the regulation of Na-K-ATPase: II. role of eicosanoids. J Clin Invest 91:409–415, 1993.
5. Wei Y, Lin DH, Kemp R, Yaddanapudi GS, Nasjletti A, Falck JR, Wang WH. Arachidonic acid inhibits epithelial Na channel via cytochrome P450 (CYP) epoxygenase-dependent metabolic pathways. J Gen Physiol 124:719–727, 2004.
6. Imig JD, Navar LG, Roman RJ, Reddy KK, Falck JR. Actions of epoxygenase metabolites on the preglomerular vasculature. J Am Soc Nephrol 7:2364–2370, 1996.
7. Fisslthaler B, Popp R, Kiss L. Cytochrome P450 2C is an EDHF synthase in coronary arteries. Nature 401:493–497, 1999.
8. Imig JD, Falck JR, Wei S, Capdevila JH. Epoxygenase metabolites contribute to nitric oxide-independent afferent arteriolar vasodilation in response to bradykinin. J Vasc Res 38:247–255, 2001.
9. Katoh T, Takahashi K, Capdevila J, Karara A, Falck JR, Jacobson HR, Badr KF. Glomerular stereospecific synthesis and hemodynamic actions of 8,9-epoxyeicosatrienoic acid in rat kidney. Am J Physiol 261:F578–F586, 1991.
10. Takahashi KJ, Capdevila A, Karara JR, Falck JR, Jacobson HR, Badr KF. Cytochrome P450 arachidonate metabolites in rat kidney: characterization and hemodynamic responses. Am J Physiol 258: F781–F789, 1990.
11. Alexander BT, Miller MT, Kassab S, Novak J, Reckelhoff JF, Kruckeberg WC, Granger JP. Differential expression of renal nitric oxide synthase isoforms during pregnancy in rats. Hypertension 33: 435–439, 1999.
12. Khalil RA, Granger JP. Vascular mechanisms of increased arterial pressure in preeclampsia: lessons from animal models. Am J Physiol 283:R29–R45, 2002.
13. Novak J, Reckelhoff J, Bumgarner L, Cockrell K, Kassab S, Granger JP. Reduced sensitivity of the renal circulation to angiotensin II in pregnant rats. Hypertension 30:580–584, 1997.
14. Zhou Y, Chang HH, Du J, Wang CY, Dong Z, Wang MH. Renal epoxyeicosatrienoic acid synthesis during pregnancy. Am J Physiol 288:F221–F226, 2005.
15. Wang MH, Smith A, Zhou Y, Chang HH, Lin S, Zhao X, Imig JD, Dorrance AM. Downregulation of renal CYP-derived eicosanoid synthesis in rats with diet-induced hypertension. Hypertension 42: 594–599, 2003.
16. Hatzinger PB, Stevens JL. Rat kidney proximal tubule cells in defined medium: the roles of cholera toxin, extracellular calcium and serum in cell growth and expression of gamma-glutamyltransferase. In Vitro Cell Dev Biol 25:205–212, 1989.
17. Chaudhari A, Kirschenbaum MA. A rapid method for isolating rabbit renal microvessels. Am J Physiol 254:F291–F296, 1988.
18. Lin F, Rios A, Falck JR, Belosludtsev Y, Laniado Schwartzman M. 20-Hydroxyeicosatetraenoic acid is formed in response to EGF and is a mitogen in rat proximal tubule. Am J Physiol 269:F806–F816, 1995.
19. Brand-Schieber E, Falck JF, Schwartzman M. Selective inhibition of arachidonic acid epoxidation in vivo. J Physiol Pharmacol 51:655–672, 2000.
20. Qi Z, Whitt I, Mehta A. Serial determination of glomerular filtration rate in conscious mice using FITC-inulin clearance. Am J Physiol 286: F590–F596, 2004.
21. Hoagland KM, Flasch AK, Roman RJ. Inhibitors of 20-HETE formation promote salt-sensitive hypertension in rats. Hypertension 42:669–673, 2003.
22. Khalil RA, Crews JK, Novak J, Kassab S, Granger JP. Enhanced vascular reactivity during inhibition of nitric oxide synthesis in pregnant rats. Hypertension 31:1065–1069, 1998.
23. Sherwood OD. Relaxin's physiological roles and other diverse actions. Endocr Rev 25:205–234, 2004.
24. Lindheimer MD, Katz AI. Renal physiology and disease in pregnancy. In: Seldin DW, Giebisch G, eds. The Kidney: Physiology and Pathophysiology. New York: Raven Press, pp3371–3431, 1992.
25. Ogungbade GO, Akinsanmi LA, Jiang H, Oyekan AO. Role of epoxyeicosatrienoic acids in renal functional response to inhibition of NO production in the rat. Am J Physiol 285:F955–F964, 2003.
26. Wang M-H, Brand-Schieber E, Zand BA. Cytochrome P450-derived arachidonic acid metabolism in the rat kidney: characterization of selective inhibitors. J Pharmacol Exp Ther 284:966–973, 1998.
27. Mancy A, Dijols S, Poli S, Guengerich FP, Mansuy D. Interaction of sulfaphenazole derivatives with human liver cytochrome P450 2C: molecular origin of the specific inhibitory effects of sulfaphenazole on CYP 2C9 and consequences for substrate binding site topology of CYP 2C9. Biochemistry 35:16205–16212, 1996.
28. Ortiz de Montellano PR, Reich NO. Specific inactivation of hepatic fatty acid hydroxylases by acetylenic fatty acids. J Biol Chem 259: 4136–4141, 1984.
29. Patel L, Sullivan MH, Elder MG. Production of epoxygenase metabolite by human reproductive tissues. Prostaglandins 38:615–624, 1989.