Arachidonate CYP hydroxylases of kidney contribute to formation of hypertension and maintenance of blood pressure[1]

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KEY WORDS arachidonic acid; monophenol monooxygenase; hydroxylases; cytochrome P-450 CYP4A1; hypertension; 20-hydroxyeicosatetraenoic acids; kidney

ABSTRACT

AIM: To investigate the relationship of kidney-specific expression of cytochrome P-450 (CYP) 4A1 and the blood pressure. **METHODS**: The specific sense and antisense CYP4A1 cDNA was administered respectively with the help of eukaryotic expression vector pcDNA3.1 to the Sprague-Dawley (SD) rats *via* sublingual vein (2 mg/kg). The systolic blood pressure of rats was assessed by the tail-cuff method, and the relative tissue expression of CYP4A1 was analyzed by Western blot and Northern blot at RNA and protein levels in the brain, heart, lung, liver, and kidney of control, sense, and antisense CYP4A1 cDNA-treated rats. **RESULTS**: Two weeks after the injection of the sense and antisense CYP4A1 cDNA recombinants respectively, the mean systolic pressure of the transgenic rats increased by 1.8 kPa ± 0.3 kPa (13.2 mmHg ± 2.5 mmHg) or decreased by 1.7 kPa ± 0.3 kPa (13.0 mmHg ± 2.2 mmHg) compared with control. At the levels of transcription and translation, the Northern and Western blots all demonstrated that CYP4A1 preferentially overexpressed in the kidney. **CONCLUSION**: The administration of sense and antisense CYP4A1 cDNA induced hypertension and hypotension, respectively, which indicated that renal arachidonate hydroxylase contributed to the formation of hypertension and maintenance of blood pressure in normotensive rats.

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INTRODUCTION

It has been well known that the arachidonic acid (AA) exists extensively in eukaryotic cells, and its metabolism is catalyzed by cycloxygenases and lipoxygenases to produce prostaglandins (PGs) and hydroperoxyeicosatetraenoic acid (HPETE)[1]. Recently the third pathway of arachidonic acid metabolism confirmed[2] is metabolic pathway of cytochrome P-450-dependent monoxygenases, which include epoxygenases and hydroxylases. The epoxygenases catalyze AA to produce epoxyeicosatraenoic acids (EETs). The hydroxylase metabolize AA to 19, 20-hydroxyeicosatetraenoic acid (19, 20-HETE), the later is critically important in blood pressure regulation[3]. 20-HETE can not only constrict renal artery by inhibiting Ca^{2+}-activated K^+ channels (K_{Ca}) in smooth muscle, but also affect renal tubular transport of electrolytes. The metabolism of AA by CYP hydroxylase may be important in the control of sodium and chloride ions balance[4-6], through which HETE affects the blood pressure. However, it is still controversy how they do so *in vivo*. Ma *et al*[7] reported the production of 20-HETE by outer medullary microsomes was lower in prehypertensive Dahl salt-sensitive (SS/Jr) than in Dahl salt-resistant (SR/Jr) rats, and considered that the deficiency in the production of 20-HETE in the outer medulla of SS/Jr rats might contribute to the development of hypertension in these animals. The fact that induction of renal P-450 fatty acid ω-hydroxylase activity with clofibrate prevented the hypertension in SS/Jr rats supports this possibility[8]. In contrast, Schwartzman *et al* reported that P-450 4A1 and 4A3 could be detected in the kidney of 3-week-old male spontaneous hypertension rats (SHR), increasing with age, peaking at 5-to 7-week, and the production of 20-HETE was at its maximum when severe hypertension occurred[1,9,10]. Renal arachidonic acid ω-hydroxylase activity inhibited by 1-aminobenzotriazole (ABT) (an inhibitor of CYP activity that can spare ω-hydroxylase)

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can reduce blood pressure in the SHR[11], which suggested that excessive 19,20-HETE production in the kidney could cause hypertension. These paradoxical results about the role of ω^-, ω^{-1} hydroxylases in the development and regulation of hypertension require further investigations to figure out. Therefore in the present paper, we studied the role of CYP4A1 in the development of hypertension and the regulation of blood pressure by using gene transfer technology to overexpress or block the expression of CYP4A1.

MATERIALS AND METHODS

Materials TRIzol reagent for protein and total RNA isolation was purchased from Life Technologies (USA). Western blotting kit (supersignal substrate, an enhanced chemiluminescent substrate for detection of HRP) was from Pierce Company (USA). Protein molecular weight markers were obtained from Bio-Rad Laboratories (USA). Hybrisol solution was from Intergen Company. Polyvinylidene difluoride (PVDF) and nylon transfer membranes were purchased from Schleicher and Schuell (Dassel, Germany). [α-^{32}P] dCTP was purchased from Beijing Yahui Company, Primer-It Ⅱ random primer labeling kit was obtained from Stratagene Company (USA) and QIAquick™ nucleotide removal kit for purification of probe was from Qiagen Company (Germany). Mammalian expression vector pcDNA3.1 was obtained from Invitrogen Corporation (USA), anti-CYP4A1 antisera was purchased from Gentest Corporation (USA), and Sprague-Dawley (SD) rats were provided by the Medical Experimental Center of Tongji Medical College of Huazhong University of Science and Technology (Grade Ⅱ, Certificate № 19-053).

Cloning of CYP4A1 cDNA Reverse transcription was performed in 20 μL reaction mixture containing 2 μL of 10 × buffer, 2 μL of dNTPs 1 mmol/L, 2.5 U/μL of reverse transcriptase (Gibco BRL), 5 μg of total RNA isolated from male SD rat's kidney by Trizole reagent according to manufacturer's instruction, and 2.5 μmol/L of 16-mer oligo-dT (Perkin-Elmer) After 1 h at 40 ℃, the reactions were terminated by heating (100 ℃, 2 min), and then the product was utilized as PCR template.

PCR was carried out in 50 μL reaction mixture containing 5 μL of 10 × PCR buffer, 5 μL of dNTPs 2 mmol/L, each of the following primers at 50 pmol/L: sense primer (5′-GTATAGAATTCCGAGGAGTGGCTGCACC-3′) and antisense primer (5′-GTTACGTCGACACCACCAACTCAGCTT-3′), which were designed from the published cDNA sequences[12], and 2.5 U of *Taq* DNA polymerase. Thirty cycles of PCR were performed in a thermal cycler with a denaturing phase of 1 min at 94 ℃, annealing phase of 40 s at 65 ℃, and extension phase of 1 min at 72 ℃. The resulting PCR products were gel-purified, and about 1600 bp of the fragment was subcloned into PBS vector by *Eco*R Ⅰ/*Sal* Ⅰ, and sequenced by the dideoxy chain termination method. Sequence analysis demonstrated that the sequence of the cloned CYP4A1 cDNA was identical to that of Genbank[12].

Construction of sense and antisense CYP4A1/pcDNA3.1 The *Eco*R Ⅰ/*Xho* Ⅰ and *Kpn* Ⅰ/*Eco*R Ⅰ fragment of CYP4A1 (about 1600 bp) was subcloned into pcDNA3.1 to construct pcDNA·4A1 (sense) and pcDNA·anti4A1 (antisense), respectively.

Effects of pcDNA·4A1 and pcDNA·anti4A1 on systolic blood pressure in normotensive rats SD rats (n = 24, weighing 210 – 225 g) were used in this study. Rats were housed in an air-conditioned room with a 12-h light/dark cycle, received a standard rat chow (0.4 % sodium chloride), and drunk tap water. One week before gene delivery, the rats were randomly divided into 3 groups which were administered with pcDNA·4A1, pcDNA·anti4A1, and pcDNA3.1 (control) plasmid *via* sublingual vein (2 mg/kg), respectively. The systolic blood pressure of rats was assessed by the tail-cuff method after the rats were warmed at 37 ℃ for 10 min. Blood pressure was measured under conscious conditions at the beginning of the experiment and at 1, 2, 3, and 4 weeks after delivery of plasmid.

Preparation of protein and RNA At 2 weeks after plasmid injection, two animals were sacrificed for each group. Brain, heart, lung, liver, and kidney samples were collected and frozen in liquid nitrogen followed by storage at −80 ℃ until analysis. Total RNA and protein from brain, heart, lung, liver, and kidney samples were extracted by Trizole reagent according to manufacturer's instructions. The RNA concentration was determined spectrophotometrically at 260 nm, and ethidium bromide-stained agrose gels were used to check its integrity. Protein concentrations of the five tissues were determined by Bradford method.

Northern blot analysis[13] CYP4A1 cDNA (1600 bp) and 500 bp of GAPDH cDNA were labeled

with [α-^{32}P]dCTP, using prime-a-gene labeling system (Promega, USA). Total RNA 20 μg for each sample were separated by 1.2 % formaldehyde-agarose gel electrophoresis and transferred onto hybond nylon membrane overnight. The blot was baked for 2 h at 80 ℃, following by prehybridization for 2 h with Hybrisol I (Intergene Co, USA). Then the labeled probe was added to hybridize in same conditions. All blots were subjected to stringent washing condition (2 XSSC/0.1 % SDS and 0.5 XSSC/0.1 % SDS 20 min two times for each washing solution), prior to autoradiography to X-ray film with intensifying screen at −80 ℃ for 3 − 4 d.

Western blot analysis[14] The tissue proteins (20 μg/well) were resolved by 10 % SDS/PAGE and transferred onto PVDF membrane in a transfer buffer consisting of Tris-HCl 25 mmol/L, glycine 192 mmol/L, and 20 % methanol at 4 ℃ overnight. After blocked by immersion in a buffer (TBS-T) containing Tris-HCl 10 mmol/L, sodium chloride 150 mmol/L, 0.05 % Tween 20, and 5 % milk power with nonfat for 2 h at room temperature, the membrane was incubated overnight at 4 ℃ with primary antibodies of goat anti-CYP4A1 antisera (Daiichi Pure Chemicals, USA) in a 1:800 dilution in TBS-T. Then it was incubated with donkey anti-goat horseradish peroxidase antibodies at room temperature after washing out primary antibodies. Labeled bands were visualized by using an enhanced chemiluminescence kit (supersignal substrate, Pierce, USA).

RESULTS

Effect of CYP4A1 and antiCYP4A1 cDNA injection on rat blood pressure The results showed that the basal level of the blood pressure in all groups had no significant difference and pcDNA3.1 injection did not lead significant change in blood pressure. However, pcDNA·4A1 significantly increased the blood pressure one week after injection and to the maximum by 1.8 kPa ± 0.3 kPa (13.2 mmHg ± 2.5 mmHg) at 2 weeks, then returned to baseline at 4 weeks after injection. In contrast, pcDNA · anti4A1 significantly decreased the blood pressure one week after injection and to the lowest level by 1.7 kPa ± 0.3 kPa (13.0 mmHg ± 2.2 mmHg) at 2 weeks, then returned to baseline at 4 weeks after injection (Fig 1).

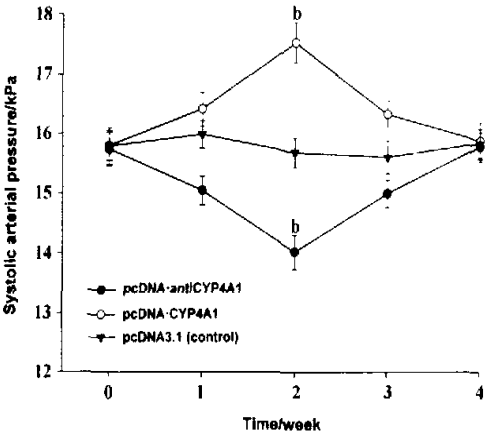

Fig 1. The time curve of SD rat's systolic blood pressure in response to treatment with pcDNA·4A1, pcDNA·anti4A1 and pcDNA3.1 (control). $n = 6$. $\bar{x} \pm s$. $^{b}P < 0.05$ *vs* control.

Northern blot analysis after gene delivery To determine dynamics and expression level of CYP4A1 and antiCYP4A1 in various organs, Northern blot was performed. The results demonstrated that the transcriptional levels of CYP4A1 and antiCYP4A1 were different in the tissues of brain, heart, lung, liver, and kidney. CYP4A1 and antiCYP4A1 preferentially transcripted in the liver and kidney, especially much more highly in the kidney, compared with the control (Fig 2). While in

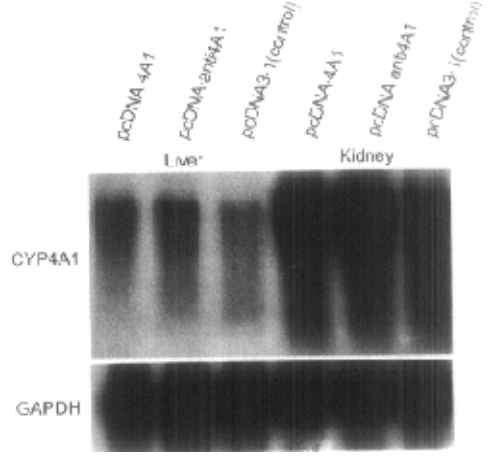

Fig 2. Northern blot (total RNA per lane 20 μg) showed CYP4A1 and antiCYP4A1 mRNA levels in kidney and liver of rats 2 weeks after the injection of pcDNA·4A1, pcDNA·anti4A1, and pcDNA3.1 (control) respectively. Equal RNA loading for each sample was verified by hybridization with labeled GAPDH. This is a representative picture of 3 individual experiments.

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the brain, heart, and lung, the transcriptional level of CYP4A1 was almost the same as the control (data not shown).

Western blot analysis Western blot was performed to compare expression levels of CYP4A1 in various organs of different groups at 2 weeks after the injection. The results showed that CYP4A1 expression increased in kidney of pcDNA·4A1-treated rats, and in pcDNA·anti4A1-treated animal, CYP4A1 expression was almost completely blocked in kidneys (Fig 3), while the translation level of CYP4A1 in brain, heart, and lung was almost the same as the control (data not shown).

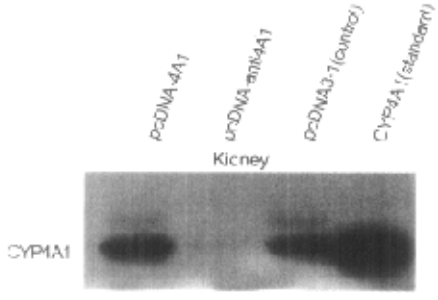

Fig 3. Western blot of kidney (20 μg protein per lane) in rats treated with pcDNA·4A1, pcDNA·anti4A1, and pcDNA3.1 (control, 2 mg/kg). This is a representative picture of 3 individual experiments.

DISCUSSION

Four isoforms of cytochrome P-450 in 4A family (4A1, 4A2, 4A3, and 4A8) have been identified in rats now. CYP4A1, 4A2, and 4A3 all catalyze the ω^- and ω^{-1} hydroxylation of fatty acids and produce 19, 20-HETE. The 20-HETE is the main product in both the quantity and physiological actions. Using RT-PCR with isoform-specific primers, the expression of CYP4A1 mRNA was very low but CYP4A2 and 4A3 mRNA were constitutively expressed throughout various nephron segments and the renal vascular of male SD rats[15]. Schwartzman *et al* also had discovered that by Northern blot analysis CYP4A2 and 4A3 mRNA were constitutively expressed in the rat kidneys, whereas the expression of CYP4A1 mRNA was nearly undetectable in the kidney of 7-week-old male SD rats. These reports suggest that the expression of CYP4A1 mRNA is not very high in the normal SD rats. Additionally, CYP4A1 expresses a little in arterial system but almost undetectable in other organs.

To investigate the relationship of CYP4A1 expression level and blood pressure in rats, we used eukaryotic expression vector pcDNA3.1 as vehicle to overexpress or block the expression of CYP4A1 by injection of pcDNA·4A1 and pcDNA·anti4A1 *via* sublingual vein respectively. To our surprising, Northern blot and Western blot demonstrated that CYP4A1 preferentially expressed in the kidney and poorly expressed in the brain, heart, and lung. Meanwhile, the expression of CYP4A1 on the protein level was almost blocked by antiCYP4A1 in the kidney of antiCYP4A1-treated rats. It is well known that pcDNA3.1 vector carrying the CYP4A1 and antiCYP4A1 driven by the ubiquitous cytomegalovirus (CMV) promoter. The reason why CYP4A1 and antiCYP4A1 can highly kidney-specifically express is further exploring. However, This kind of expression characterization facilitates us to investigate the relationship of CYP4A1 level of the kidney and the blood pressure variation.

The pattern of CYP4A1 expression determined the production rate of 20-HETE. Therefore, we consider that the elevation of mean systolic pressure in the CYP4A1-treated rats related to the kidney-specific overexpression of CYP4A1. All these suggest that overexpression of CYP4A1 plays an important role in the development of hypertension. This result is consistent with that of Schwartzman *et al*[1].

In SHR the expression pattern of CYP4A1 was similar to that of CYP4A1 transfected SD rats, which typically exhibited a higher level of 20-HETE productions in the kidney than that of normotensive rats.

The decrease of mean systolic pressure in antisense CYP4A1-delivered rats was due to the CYP4A1 expression blocked by antisense CYP4A1 mRNA production which can hybridize CYP4A1 to form double-strand RNA, the later is easily digested by RNase in the cells. This result suggests that the CYP4A1 also plays an important role in the maintenance of normal blood pressure.

20-HETE increased the blood pressure of rat in the kidney through at least two ways. Namely, 20-HETE might constrict renal artery by inhibiting Ca^{2+}-activated K^+ channels in smooth muscle directly and also act indirectly as second messenger for ET-1. It was discovered in the model of unx/salt/DOCA of which the rat was uninephrectomized, given excess salt and treated with DOCA, the blood pressure increased progressively

over the 3-week period. Between the second and third week, urinary excretion of ET-1 and HETE had increased by three to four fold. Blockade of the ETA receptor could lower blood pressure and attenuate organ hypertrophy and proteinuria while decreasing the excretion of 20-HETE.

In summary, in normotensive SD rats, the delivery of CYP4A1 *via* sublingual vein could lead CYP4A1 overexpression in the kidney and elevated the blood pressure two weeks after injection, while the delivery of antisense CYP4A1 specifically blocked CYP4A1 expression in the kidney and significantly decreased the blood pressure. Therefore we make the conclusion that cytochrome P-450 arachidonic acid hydroxylases in rats substantially contribute to the formation of high blood pressure and the maintenance of normal blood pressure.

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肾脏花生四烯酸 CYP 羟化酶参与高血压的形成及维持正常血压的稳定[1]

R96 A

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关键词 花生四烯酸；一元酚单氧化酶；羟化酶；细胞色素 P-450 CYP4A1；高血压；20-羟花生四烯酸；肾

目的：研究肾脏特异性表达 CYP4A1 与血压变化的关系。**方法**：将含有开放阅读框的 CYP4A1 cDNA 克隆到真核表达载体 pcDNA3.1，构建 4A1/pcDNA3.1 和反义 4A1/pcDNA3.1 重组体，舌下静脉注射转染 SD 雄性大鼠，经尾动脉测量收缩压。用 Western blot 及 Northern blot 分析转染对照 pcDNA3.1 质粒及正、反义 CYP4A1 重组体后，CYP4A1 在大鼠的脑、心、肺、肝、肾组织表达水平。**结果**：转染正义、反义 CYP4A1 两周后，大鼠的血压与对照组相比分别增加 1.8 kPa ± 0.3 kPa (13.2 mmHg ± 2.5 mmHg)和减少了 1.7 kPa ± 0.3 kPa (13.0 mmHg ± 2.2

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mmHg). Western blot 及 Northern blot 结果均显示 CYP4A1 可选择性地在肾脏表达，而在脑、心、肺、肝几乎无表达，且在给予反义 CYP4A1 的大鼠肾脏中 CYP4A1 蛋白表达几乎被完全阻断. **结论**：在正常的 SD 大鼠肾脏中，转染正、反义 CYP4A1 可引起血压升高和降低，说明肾脏花生四烯酸 CYP 羟化酶参与高血压的形成及维持正常血压的稳定.

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Acta Pharmacologica Sinica will publish a special issue about Traditional Chinese Medicine on the 12th issue this year. Focal topic will be on "The pharmacology of tetrandrine and related alkaloids". *In addition to invited reviews*, we will also accept submission of excellent original research papers subjected to peer review.

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