

Overexpression of Cytochrome P450 Epoxygenases Prevents Development of Hypertension in Spontaneously Hypertensive Rats by Enhancing Atrial Natriuretic Peptide^[S]

Bin Xiao, Xuguang Li, Jiangtao Yan, Xuefeng Yu, Guangtian Yang, Xiao Xiao, James W. Voltz, Darryl C. Zeldin, and Dao Wen Wang

The Institute of Hypertension and Department of Internal Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, People's Republic of China (B.X., X.L., J.Y., X.Y., G.Y., X.X., D.W.W.); Molecular Pharmaceutics, University of North Carolina School of Pharmacy, Chapel Hill, North Carolina (X.X.); and Division of Intramural Research, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina (J.W.V., D.C.Z.)

Received February 22, 2010; accepted May 24, 2010

ABSTRACT

Cytochrome P450 (P450)-derived epoxyeicosatrienoic acids (EETs) exert well recognized vasodilatory, diuretic, and tubular fluid-electrolyte transport actions that are predictive of a hypotensive effect. The study sought to determine the improvement of hypertension and cardiac function by overexpressing P450 epoxygenases in vivo. Long-term expression of CYP102 F87V or CYP2J2 in spontaneously hypertensive rats (SHR) was mediated by using a type 8 recombinant adeno-associated virus (rAAV8) vector. Hemodynamics was measured by a Millar Instruments, Inc. (Houston, TX) microtransducer catheter, and atrial natriuretic peptide (ANP) mRNA levels were tested by real-time polymerase chain reaction. Results showed that urinary excretion of 14,15-EET was increased at 2 and 6 months after injection with rAAV-CYP102 F87V and rAAV-CYP2J2 compared with controls ($p < 0.05$). During the course of the 6-month study, systolic blood pressure significantly decreased

in P450 epoxygenase-treated rats, but the CYP2J2-specific inhibitor C26 blocked rAAV-CYP2J2-induced hypotension and the increase in EET production. Cardiac output was improved by P450 epoxygenase expression at 6 months ($p < 0.05$). Furthermore, cardiac collagen content was reduced in P450 epoxygenase-treated rats. ANP mRNA levels were up-regulated 6- to 14-fold in the myocardium, and ANP expression was significantly increased in both myocardium and plasma in P450 epoxygenase-treated rats. However, epidermal growth factor (EGF) receptor antagonist 4-(3'-chloroanilino)-6,7-dimethoxyquinazoline (AG-1478) significantly attenuated the increase in the EET-induced expression of ANP in vitro. These data indicate that overexpression of P450 epoxygenases attenuates the development of hypertension and improves cardiac function in SHR, and that these effects may be mediated, at least in part, by ANP via activating EGF receptor.

Introduction

Cytochrome P450 (P450) epoxygenases metabolize arachidonic acid to biologically active eicosanoids referred to as

This work was supported in part by the National Natural Science Foundation of China [Grants 30430320, 30930039]; National "973" projects [Grant 2007CB512004]; International Collaboration and Wuhan City projects; and in part by the Intramural Research Program of the National Institutes of Health National Institute of Environmental Health Sciences.

B.X. and X.L. contributed equally to this work.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

doi:10.1124/jpet.110.167510.

[S] The online version of this article (available at <http://jpet.aspetjournals.org>) contains supplemental material.

cis-epoxyeicosatrienoic acids (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET) (Capdevila et al., 2000). Early studies identified EETs as endothelium-derived hyperpolarizing factors that can activate calcium-sensitive potassium channels, resulting in hyperpolarization of resting membrane potential and relaxation of vascular smooth muscle cells (Cohen and Vanhoutte, 1995). Subsequent work has shown that EETs have diverse biological effects within the cardiovascular system. Indeed, EETs diminish cytokine-induced endothelial cell adhesion molecule expression and inhibit leukocyte adhesion to vessel walls (Node et al., 1999) and also show fibrinolytic effects by increasing tissue plasminogen activator expression and activity in endothelial cells (Node et al.,

ABBREVIATIONS: P450, cytochrome P450; EET, epoxyeicosatrienoic acid; eNOS, endothelial nitric-oxide synthase; sEH, soluble epoxide hydrolase; DHET, dihydroxyeicosatrienoic acid; rAAV, recombinant adeno-associated viral vector; SHR, spontaneously hypertensive rat; ANP, atrial natriuretic peptide; GFP, green fluorescent protein; NE, norepinephrine; ACh, acetylcholine; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcriptase-polymerase chain reaction; MMP, matrix metalloproteinase; AG-1478, 4-(3'-chloroanilino)-6,7-dimethoxyquinazoline; EGFR, epithelial growth factor receptor; CRM, cross-reacting material; HB-EGF, heparin-binding epidermal growth factor-like growth factor; GW9662, 2-chloro-5-nitro-N-phenylbenzamide; PPAR- γ , peroxisome proliferator-activated receptor- γ ; CO, cardiac output; Ea, arterial elastance; CRE, cAMP-response element.

2001). Exogenous EETs or overexpression of various P450 epoxygenases also protect endothelial cells from apoptosis (Yang et al., 2007), up-regulate endothelial nitric-oxide synthase (eNOS), elevate eNOS activity (Wang et al., 2003), and enhance angiogenesis in vivo and in vitro by activating Akt/PKB and mitogen-activated protein kinases (Wang et al., 2005; Jiang et al., 2007b). Furthermore, CYP2J2 overexpression has been shown to protect against postischemic myocardial dysfunction in mice (Seubert et al., 2004).

EETs have natriuretic, vasodilatory actions and renal tubular fluid-electrolyte transport actions that are predictive of a hypotensive effect (McGiff, 1991; Capdevila and Falck, 2002; Roman, 2002; Fleming and Busse, 2006). Recently published single nucleotide polymorphism analyses reveal that a CYP2J2 G-50T variant (CYP2J2*7) is associated with hypertension in humans (McGiff, 1991; King et al., 2005). Soluble epoxide hydrolase (sEH) metabolizes EETs to the less active dihydroxyeicosatrienoic acids (DHETs) (Imig, 2006). Oral administration of the sEH inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid significantly increases the urinary excretion ratio of EET/DHET and reduces blood pressure in rats infused with angiotensin and fed either a normal salt diet or a high salt diet (Imig et al., 2005). The cumulative data described above suggest that P450 epoxygenases and EETs may play important roles in regulating blood pressure as originally proposed more than a decade ago (McGiff, 1991), but to date, no direct evidence showed their hypotensive effects.

In the present study, an in vivo model of hypertension was used to test the hypothesis that P450 epoxygenases could induce a long-term increase in circulating EET concentrations and a resultant improvement in blood pressure and cardiac function. A recombinant adeno-associated virus vector (rAAV) was used to mediate long-term expression of either CYP2J2 or a mutant isoform of bacterial CYP102 (F87V) in spontaneously hypertensive rats (SHRs). Consistently elevated EET levels were observed with long-term CYP2J2 or CYP102 F87V overexpression and were associated with significant improvement in cardiovascular endpoints. Additional biochemical, immunohistochemical, and cell culture assessments indicate that the beneficial effects of P450 epoxygenase overexpression may be mediated via induction of atrial natriuretic peptide (ANP) production.

Materials and Methods

rAAV Vector Production. Type 8 rAAV vectors containing human CYP2J2, CYP102 F87V (a mutant form of CYP102 that has high arachidonic acid epoxygenase activity and produces exclusively the active 14,15-EET enantiomer) (Graham-Lorence et al., 1997), or green fluorescent protein (GFP) were prepared by triple plasmid cotransfection in human embryonic kidney 293 cells as described previously (Xiao et al., 1998; Wang et al., 2004).

Animals and Vector Administration. Male SHRs weighing 200 to 220 g were obtained from the Experimental Animal Center of Beijing (People's Republic of China). Experimental protocols were approved by the Institutional Animal Research Committee of Tongji Medical College and complied with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). Twenty-four animals were randomized to four groups (six animals/group) as follows: saline control, rAAV-GFP control, rAAV-CYP102 F87V, and rAAV-CYP2J2. Animals received a single injection of either saline or rAAV (1×10^{12}

vector genomes/rat) via tail vein. In addition, we administered rAAV-CYP2J2-treated SHR with C26, a selective CYP2J2 inhibitor, which can decrease EET production without effect on CYP2J2 mRNA or protein expression (Chen et al., 2009). In brief, 24 male SHRs were divided to four groups: control group, control+C26 group, rAAV-2J2 group, and rAAV-2J2+C26 group. Animals received a single intravenous injection of either saline or rAAV-CYP2J2. C26 was orally treated at a dose of 1.5 mg/kg/day for 2 months.

Measurement of Blood Pressure. After vector injection, systolic blood pressures were measured every 2 months for 6 months at room temperature by a photoelectric tail-cuff system (PowerLab; ADInstrument Pty Ltd, Bella Vista, NSW, Australia) as described previously (Yayama et al., 1998).

Hemodynamic Study. Six months after injection, rats were anesthetized with pentobarbital (40 mg/kg), and a microtransducer catheter (SPR-838; Millar Instruments, Inc.) was inserted via the right carotid artery into the left ventricle. After stabilization for 20 min, the data were continuously recorded by using conductance data acquisition (MPVS-400; Millar Instruments, Inc.). The cardiac function parameters were calculated by the analysis software PVAN3.6 (Millar Instruments, Inc.) as described previously (Xu et al., 2008). Before the catheter was inserted into the left ventricle, intra-arterial (carotid artery) blood pressure was recorded.

Isolation of Thoracic Aortic Rings and Determination of Epoxygenase-Induced Relaxation. Thoracic aortic rings were prepared as follows: briefly, thoracic aortas were rapidly isolated and immersed in Krebs-Ringer HCO₃ buffer (NaCl, 118.3 mM; KCl, 4.7 mM; CaCl₂, 2.5 mM; MgSO₄, 1.2 mM; KH₂PO₄, 1.2 mM; NaHCO₃, 25.0 mM; Ca-EDTA, 0.026 mM; glucose, 11.1 mM), which was aerated with 95% O₂/5% CO₂, pH 7.4. The vessel was carefully trimmed of surrounding tissues and cut into 2- to 3-mm rings. The rings were mounted on specimen holders and placed in glass organ chambers containing 6 ml of aerated Krebs-Ringer HCO₃ buffer at 37°C. Whereas one holder remained fixed, the other was connected to an isometric force-displacement transducer (model FTO3; Grass Instruments, Quincy, MA) coupled to a polygraph (model 7D; Grass Instruments). The aortic rings were incubated for 60 min at a tension of 2.0 g, during which time the chamber was rinsed every 15 min with aerated Krebs-Ringer HCO₃ buffer. We examined the responsiveness of aortic rings from rats overexpressing P450 epoxygenases to norepinephrine (NE) and acetylcholine (ACh) using a multichannel physiologic recorder (ML-840 PowerLab; ADInstrument Pty Ltd.) (Guan et al., 2009).

14,15-DHET Determination in Urine and Tissues. The 14,15-DHET enzyme-linked immunosorbent assay (ELISA) kit (Detroit R&D Inc., Detroit, MI) was used to measure 14,15-DHET according to the manufacturer's instructions as described previously (Jiang et al., 2005; Yang et al., 2007). EETs can be hydrolyzed to DHETs by acid treatment; thus, DHET in acidified urine represents total DHETs. The difference between total 14,15-DHET and 14,15-DHET before acidification will be 14,15-EET levels. The concentrations of 14,15-DHET and 14,15-EET were expressed as nanogram per milliliter of urine or picogram per milligram of tissue specimen.

Real-Time Polymerase Chain Reaction for ANP. Total RNA was prepared by TRIzol using the manufacturer protocols (Invitrogen, Carlsbad, CA). cDNA was produced using reverse transcriptase (Takara, Kyoto, Japan). A LightCycler reverse transcriptase-polymerase chain reaction (RT-PCR) system (Roche Diagnostics, Basel, Switzerland) was used with an automated sequence detection instrument for the real-time monitoring of nucleic acid green dye fluorescence (SYBR Green; Toyobo Engineering, Osaka, Japan) as described previously (Wang et al., 2006b). Primers and conditions of PCR are shown in Supplemental Table S1.

Western Blotting. Western blot was performed according to the method described previously (Wang et al., 2003). CYP102 F87V antibody was a gift from Dr. Jorge H. Capdevila (Vanderbilt University, Nashville, TN). Specific polyclonal antibodies raised against CYP2J2 were developed as described previously (King et al., 2002). The horse-

radish peroxidase-conjugated secondary antibody (goat anti-rabbit) was bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Immunohistochemical Detection of ANP in Heart. Immunohistochemistry was performed as described previously (Capdevila and Falck, 2002) using ANP antibody (Santa Cruz Biotechnology, Inc.).

Analysis of Myocardial and Renal and Arterial Morphology. Four-micrometer-thick heart and artery sections were stained with Sirius red (to stain collagen) using a previously described method (Wei et al., 2005). Cardiomyocyte diameter and percentage of extracellular matrix production were quantified using the HAIPS Pathological Imagic Analysis System (Tongjiqianping Image Company, Wuhan, China). Heart and kidney sections were stained with hematoxylin and eosin and were detected under microscope.

In Vitro Effects of EETs on ANP Production from Cultured Cardiomyocytes. Primary culture of neonatal rat cardiomyocytes was carried out as described previously (De Windt et al., 2000). More than 90% of cells were identified as cardiomyocytes by the detection of α -actin protein in the cells stained with 3,3'-diaminobenzidine. 11,12- and 14,15-EET (0.1 and 1.0 μ M) were added to the cultured cells. To elucidate the relevant mechanisms, different inhibitors were added to the cultures of neonatal rat cardiomyocytes [1,10-phenanthroline, matrix metalloproteinase (MMP) inhibitor, 100 μ M; 4-(3'-chloroanilino)-6,7-dimethoxy-quinazoline (AG-1478), epithelial growth factor receptor (EGFR) inhibitor, 100 nM; cross-reacting material (CRM)-197, heparin-binding epidermal growth factor-like growth factor (HB-EGF) inhibitor, 10 μ g/ml; 2-chloro-5-nitro-*N*-phenylbenzamide (GW9662), peroxisome proliferator-activated receptor- γ (PPAR- γ) inhibitor, 1 μ M], respectively, with or without 1.0 μ M 14,15-EET. After incubation for 24 h, cardiomyocytes and culture medium were collected for Western blots and determination of ANP using an ELISA kit, respectively.

Determination of ANP and cGMP and Albumin Levels by ELISA. ANP levels in serum and cell culture medium samples and albumin level in urine samples were determined with ELISA kits (Phoenix Pharmaceuticals, Belmont, CA and Immunology Consultants Laboratory, Newberg, OR) according to the manufacturers' instructions, respectively. cGMP levels in urine and cultured cardiomyocytes were measured by ELISA kits (Cayman Chemical, Ann Arbor, MI).

Statistical Analysis. Data are presented as mean \pm S.E.M. Multiple comparisons between two groups were performed with unpaired *t* tests; between three or more groups they were carried out with one-way analysis of variance and Newman-Keuls tests for post hoc analyses. Significance was accepted at a value of $p < 0.05$.

Results

P450 Epoxygenase Overexpression Induces Prolonged Production of EETs In Vivo. Western blot analyses for expression of P450 epoxygenases indicated that a single administration of the respective rAAV vectors induced significant expression in vivo in the heart, kidney, liver, and aorta 6 months after a single treatment with the indicated rAAV constructs (Fig. 1A). Overexpression of P450 epoxygenases was associated with a significant increase in urinary 14,15-DHET and 14,15-EET levels at both 2 and 6 months compared with levels in rats injected with saline or AAV-GFP ($p < 0.05$; Fig. 1, B and C). Furthermore, we measured 14,15-DHET and 14,15-EET levels in the heart, kidney, and aorta. Results showed that both 14,15-DHET and 14,15-EET levels were increased in rats injected with rAAV-CYP102 F87V and rAAV-CYP2J2 ($p < 0.05$; Fig. 1, D and E). These results indicate that a single injection of rAAV-CYP102 F87V or rAAV-CYP2J2 in rats induced significant and prolonged increases in both P450 epoxygenase protein expression and activity in vivo.

P450 Epoxygenase Overexpression Results in Hypotensive Effects In Vivo. Animals treated with rAAV-CYP102 F87V or rAAV-CYP2J2 showed a significant decrease in systolic blood pressure at 2 months postinjection corresponding with the peak 14,15-DHET levels (both $p < 0.05$ compared with control; Fig. 2A). This difference was still evident at the 6-month time point in the rAAV-CYP2J2-treated group ($p < 0.05$; Fig. 2A). Before sacrifice at the 6-month time point, the carotid intra-arterial pressure was measured. The data from this experiment were consistent with the noninvasive tail-cuff measurements ($p < 0.05$; Fig. 2B). However, only diastolic blood pressure of rAAV-CYP2J2-treated rats was decreased significantly at the end of the 6-month period (Supplemental Fig. S1). In addition, we observed effects of CYP2J2 inhibitor C26 on animal blood pressure, and results showed that rAAV-CYP2J2 significantly reduced blood pressure compared with controls (188.2 ± 6.6 mm Hg versus 219.35 ± 7.5 mm Hg; $p < 0.05$), but C26 administration exclusively blocked rAAV-CYP2J2-induced hypotension (Fig. 2C) and also the increase in EET and DHET production ($p < 0.05$; Fig. 2, D and E).

Overexpression of P450 Epoxygenases Improves Cardiac Function. Cardiac hemodynamics was measured 6 months after saline or rAAV injections to assess the long-term effects of the treatments on cardiac function. The results of these studies showed maximum cardiac pressure and end-systolic pressure, as well as both dP/dt_{\max} and dP/dt_{\min} , were reduced in rAAV-CYP102 F87V- and rAAV-CYP2J2-treated rats compared with saline and rAAV-GFP-treated rats ($p < 0.05$; Fig. 2F; Supplemental Table S2). However, the stroke volume and cardiac output (CO) were significantly increased compared with controls ($p < 0.05$; Fig. 2G; Supplemental Table S2), which were accompanied with the lower preload adjusted maximal power, suggesting that preload of left ventricle is reduced and increased stroke volume is attributable to reduction in afterload. There were no significant differences in heart rate and left ventricular end-diastolic pressure between groups (Supplemental Figs. S2 and S3). Combined, these results suggest that the overexpression of epoxygenases resulted in reduction in myocardial contractility in SHR but an increase in stroke volume and CO.

Overexpression of P450 Epoxygenases Improves Arterial Responsiveness. Recorded arterial elastance (Ea) in the rAAV-CYP102 F87V-treated and rAAV-CYP2J2-treated groups (1.6 ± 0.13 and 1.5 ± 0.19 mm Hg/ μ l, respectively) was significantly lower than in the saline-treated control group (2.6 ± 0.2 mm Hg/ μ l; $p < 0.05$; Fig. 3A), suggesting that the P450 epoxygenase overexpression improved Ea. Furthermore, rAAV-CYP2J2 and rAAV-CYP102 F87V treatments significantly enhanced the responsiveness of aortic rings to ACh and attenuated responsiveness to NE (Fig. 3, B and C), further suggesting that P450 epoxygenase overexpression results in altered responsiveness to endogenous vasoconstrictors and vasodilators.

Overexpression of P450 Epoxygenases Prevents Myocardial Hypertrophy, Cardiac Remodeling, and Renal Damage. We evaluated the preventive effects of epoxygenase overexpression on hypertension-induced myocardial hypertrophy by comparison of heart weight and cardiomyocyte diameter. Results showed that heart weight/body weight (mg/g) in epoxygenase-treated animals was remarkably lower than controls (Fig. 4A), and the cardiomyocyte

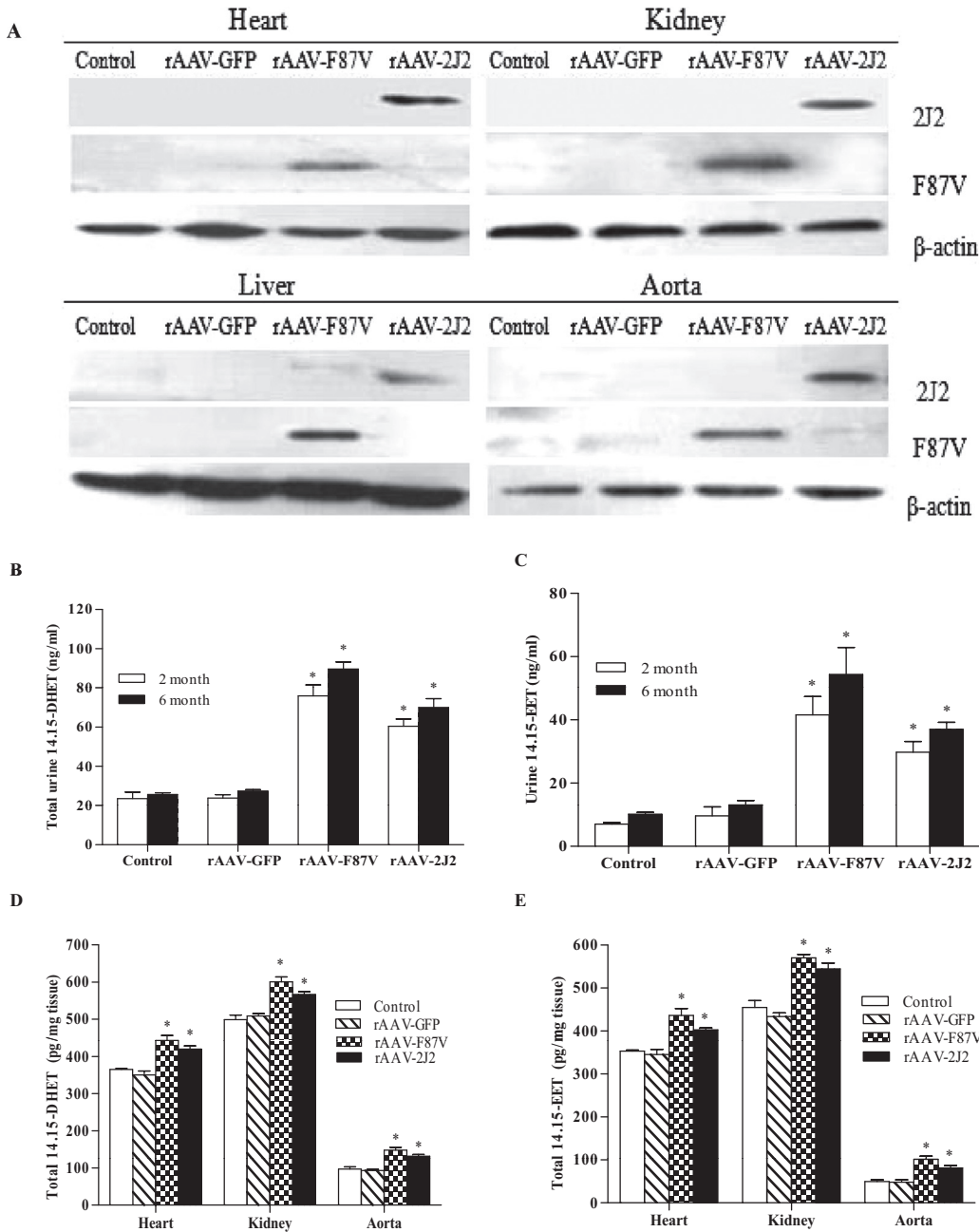


Fig. 1. P450 epoxygenase overexpression mediated by rAAV and quantitative analysis of total 14,15-DHET and 14,15-EET in urine and different tissues. **A**, Western blot analysis shows elevated levels of CYP2J2 in heart, kidney, liver, and aorta and CYP102 F87V and GFP in heart tissue 6 months after treatment of animals with rAAV-CYP2J2, rAAV-GFP, and rAAV-CYP102 F87V, respectively. **B** and **C**, total 14,15-DHET and 14,15-EET levels in urine of rats 2 and 6 months after injection of rAAV vectors; $n = 6/\text{group}$, *, $p < 0.05$ compared with control. **D** and **E**, total 14,15-DHET and 14,15-EET levels in heart, kidney, and aorta of rats 6 months after injection of rAAV vectors; $n = 5/\text{group}$, *, $p < 0.05$ compared with control.

diameter was significantly smaller in the gene-treated animals than controls (Fig. 4B), which suggest that epoxygenase overexpression efficiently attenuated hypertension-induced myocardial hypertrophy. The results of collagen staining showed that rAAV-CYP102 F87V- and rAAV-CYP2J2-injected groups had significantly reduced heart collagen content compared with the saline control group ($8.1 \pm 2.7\%$ and $5.3 \pm 0.6\%$ compared with $16.5 \pm 5.9\%$, respectively; both $p < 0.05$; Fig. 4, C and D). These results indicate CYP102 F87V and CYP2J2 overexpression reduced collagen deposition and attenuated hypertension-induced heart remodeling in vivo. We also studied the effects of epoxygenase overexpression on hypertension-induced renal damage by measuring albumin levels in urine and observing renal histology. Results showed that both rAAV-CYP102 F87V and rAAV-CYP2J2 treatments significantly reduced urinary albumin levels com-

pared with controls (40.47 ± 1.95 and 39.43 ± 1.34 ng/ml compared with 33.42 ± 3.23 and 32.71 ± 1.92 ng/ml, respectively; $p < 0.05$). Moreover, the histological analysis revealed atrophy in the glomerulus and renal tubules in control kidneys, and these effects were markedly attenuated by epoxygenase overexpression (Supplemental Fig. S4).

ANP Was Up-Regulated by Overexpression of P450 Epoxygenases. To assess potential mechanisms by which P450 epoxygenase overexpression conferred cardiovascular benefits in SHR, we measured ANP in serum and quantitatively analyzed levels of ANP mRNA in ventricular tissue by real-time PCR. Interestingly, serum ANP was significantly up-regulated in rAAV-CYP102 F87V- and rAAV-CYP2J2-treated rats (115.0 ± 13.0 pg/ml and 131.3 ± 27.3 pg/ml, respectively) compared with control and rAAV-GFP-treated groups (37.6 ± 7.0 pg/ml and 35.1 ± 6.3 pg/ml, respectively; $p < 0.05$; Fig. 5A).

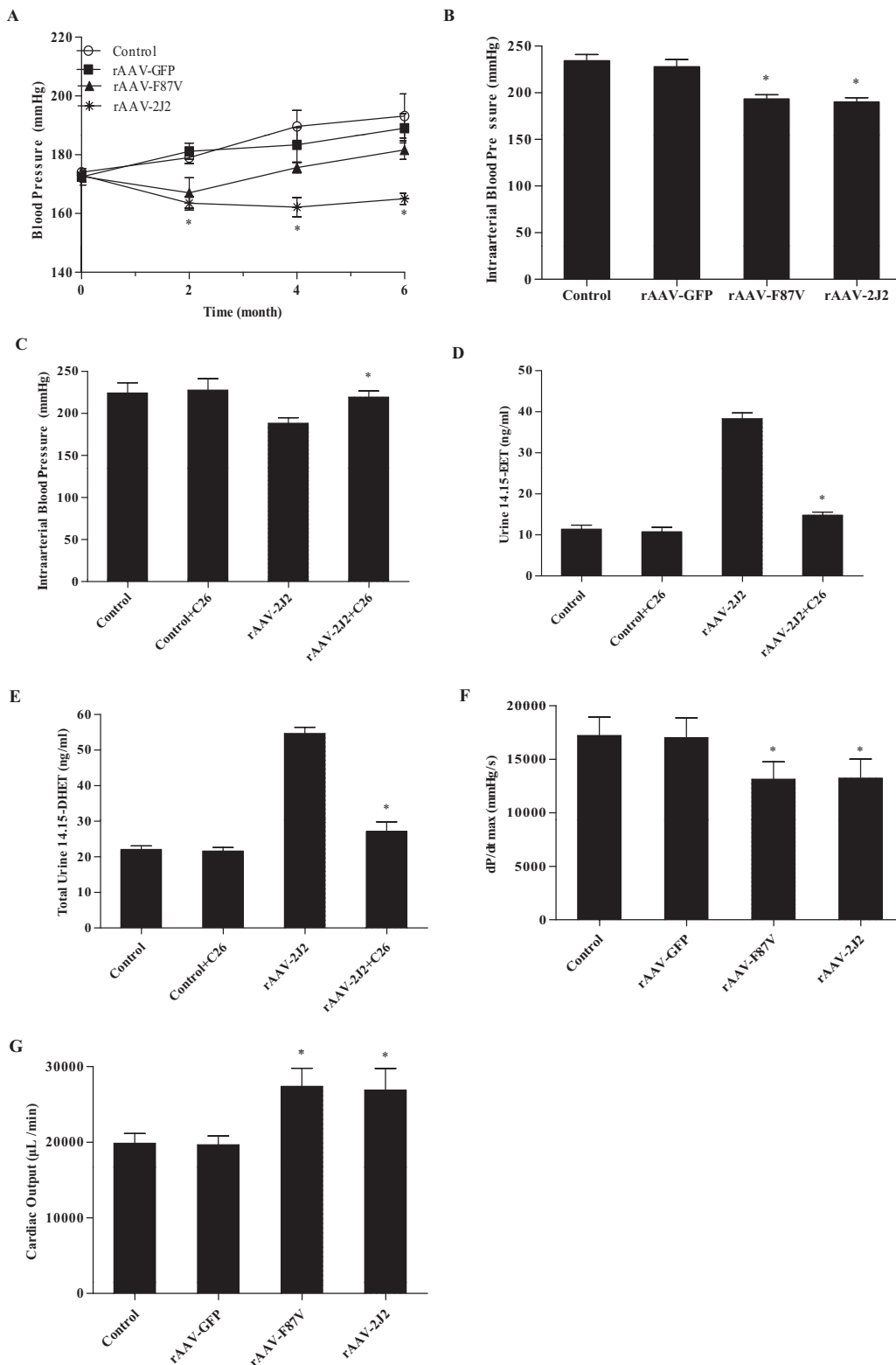


Fig. 2. The effects of rAAV-CYP2J2 and rAAV-CYP102 F87V treatments on blood pressure and cardiac function in SHR. A, systolic blood pressure measured by tail-cuff method; *, $p < 0.05$ compared with control group. B, carotid artery pressure measured invasively in rats 6 months after injections; *, $p < 0.05$ compared with control group. C, carotid artery pressure was measured invasively in rats 2 months after oral administration of C26; *, $p < 0.05$ compared with rAAV-CYP2J2 group. D and E, quantitative analysis of 14,15-EET and 14,15-DHET in urine of rats 2 months after injection of rAAV-CYP2J2 and C26 administration; $n = 5/\text{group}$, *, $p < 0.05$ compared with rAAV-CYP2J2 group. F and G, dP/dt_{max} and CO measured invasively in rats 6 months after injections; *, $p < 0.05$ compared with control group.

Moreover, ANP mRNA levels were also up-regulated by 14- and 18-fold in ventricular myocardium and 6- to 7-fold in atrial myocardium in rAAV-CYP2J2- and rAAV-CYP102 F87V-treated rats, respectively, compared with saline-treated control rats ($p < 0.01$; Fig. 5, B and C). Accordingly, urinary cGMP was increased in rAAV-CYP102 F87V- and rAAV-CYP2J2-treated rats (19.86 ± 1.23 nmol/24 h and 50.76 ± 1.68 nmol/24 h,

respectively) as ANP level up-regulated compared with control and rAAV-GFP-treated groups (11.31 ± 1.05 nmol/24 h and 13.33 ± 0.63 nmol/24 h, respectively; $p < 0.05$; Fig. 5D). Western blots show that ANP expression in ventricle tissues is significantly up-regulated in rAAV-CYP2J2- and rAAV-CYP102 F87V-treated rats ($p < 0.01$; Fig. 5, E and F). The expression levels of other vasoactive signaling molecules such as endothe-

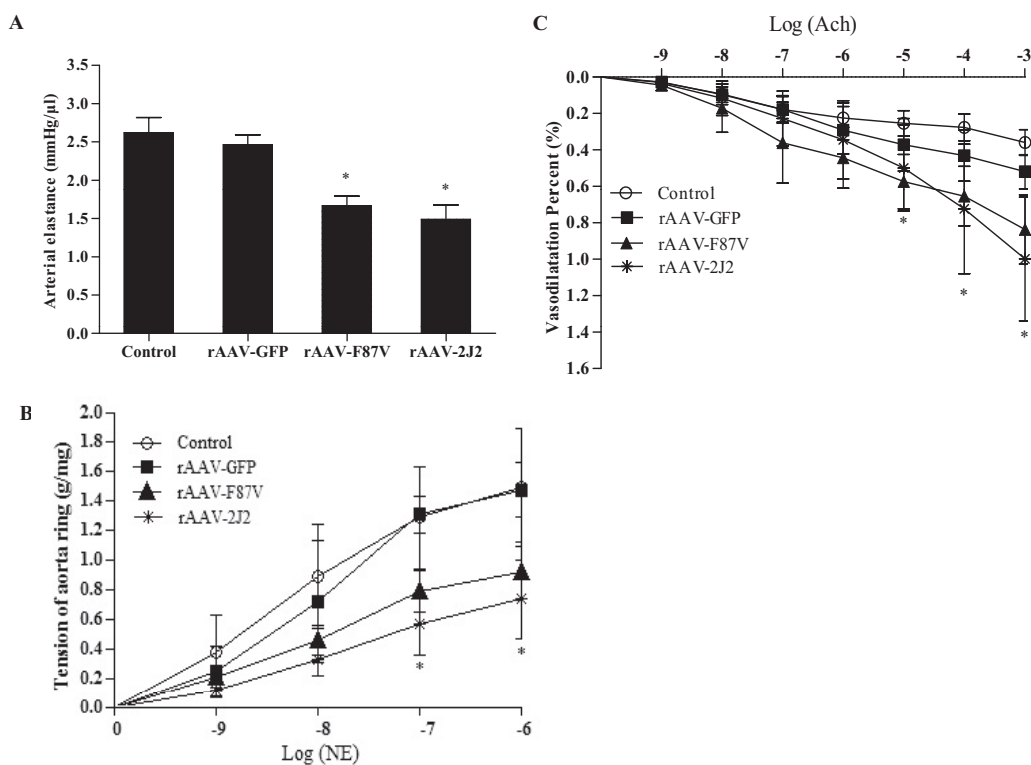


Fig. 3. The effects of rAAV-CYP2J2 and rAAV-CYP102 F87V treatments on aortic contractility. A, Ea index. B, reactivity of aortic rings to NE. C, reactivity of aortic rings to ACh. Contractility in response to NE decreased and dilation in response to ACh increased in aortic rings from rAAV-CYP102 F87V- and rAAV-CYP2J2-treated rats; *, $p < 0.05$ compared with controls.

lin-1 and adrenomedullin were also analyzed, and no significant changes were detected between the treatment groups (data not shown). Immunohistochemical staining using anti-ANP antibodies showed that the percentage of ANP-positive cells in myocardium increased by 1- to 2-fold in rAAV-CYP102 F87V- and rAAV-CYP2J2-treated rats compared with saline-treated controls in both ventricle (Fig. 6A) and atria (Fig. 6B). Finally, incubation with synthetic 14,15-EET increased secretion of ANP from cultured cardiomyocytes into the medium (Fig. 6C). Notably, 11,12-EET was without effects in this in vitro system. In agreement with increased ANP secretion from cardiomyocytes, cGMP levels in cardiomyocytes were also up-regulated (Fig. 6D). Together, these results show that the beneficial effects of P450 epoxygenase overexpression on cardiac function and blood pressure in SHR are associated with 14,15-EET-mediated secretion of ANP. We also found that epoxygenase overexpression increased the urine volume and urine Na^+ excretion (Supplemental Figs. S5 and S6).

Furthermore, we investigated possible mechanisms through which EETs induced secretion of ANP in cultured cardiomyocytes by using different molecular antagonists. Results showed that 14,15-EET markedly increased the expression of ANP, but EGFR antagonist AG-1478 significantly attenuated the increase in the EET-induced expression of ANP, and MMP inhibitor 1,10-phenanthroline and HB-EGF inhibitor CRM-197 also decreased the expression of ANP (Fig. 6, E and F).

Discussion

The regulation of blood pressure is a complex physiological process that involves multiple organs and systems and hundreds of genes and their products. EETs have endothelium-derived hyperpolarizing factor-like properties and natriuretic effects and up-regulate eNOS (Wang et al., 2003), all of which may contribute to the regulation of blood pressure.

Recently, sEH inhibitors were shown to lower arterial blood pressure in an angiotensin II-induced hypertension model (Imig et al., 2002). These observations cumulatively support the hypothesis that P450 epoxygenases and their EET metabolites exert hypotensive effects. In the present study, overexpression of CYP2J2 or CYP102 F87V epoxygenases in SHR resulted in significant increases in EET production and an associated reduction in systolic blood pressure. Moreover, the P450 epoxygenase inhibitor C26 reversed that change by decreasing production of EETs. Mechanistic studies revealed that P450 epoxygenase overexpression improved Ea, enhanced responsiveness of aortic rings to ACh, and attenuated responsiveness of aortic rings to NE. In addition, overexpression of P450 epoxygenases markedly up-regulated ANP levels in serum and enhanced the cardiac expression of ANP in vivo, whereas EETs enhanced ANP release in vitro in cultured cardiomyocytes. These data suggest a hypotensive effect of P450 epoxygenase-derived EETs that may be mediated, at least in part, by enhanced ANP activity.

Several mechanisms for the hypotensive effect of EETs have been described. EETs have been shown to cause hyperpolarization of smooth muscle cells by activation of Ca^{2+} -sensitive K^+ channels (Cohen and Vanhoutte, 1995) and to up-regulate eNOS, resulting in increased nitric oxide production (Wang et al., 2003). The data presented in this manuscript suggest that increases in ANP levels in response to P450 epoxygenase overexpression may account for some of the hypotensive effects attributed to EETs. ANP causes vasodilatation, decreased peripheral vascular resistance (Nishikimi et al., 2006), increased urinary sodium excretion (Nichols and Edwards, 2001; Anand-Srivastava, 2005), and decreased cardiac preload (Ohte et al., 1999). These characteristics, combined with the observations described in this manuscript, make increased ANP activity a possible mechanism for the hypotensive effects of EETs.

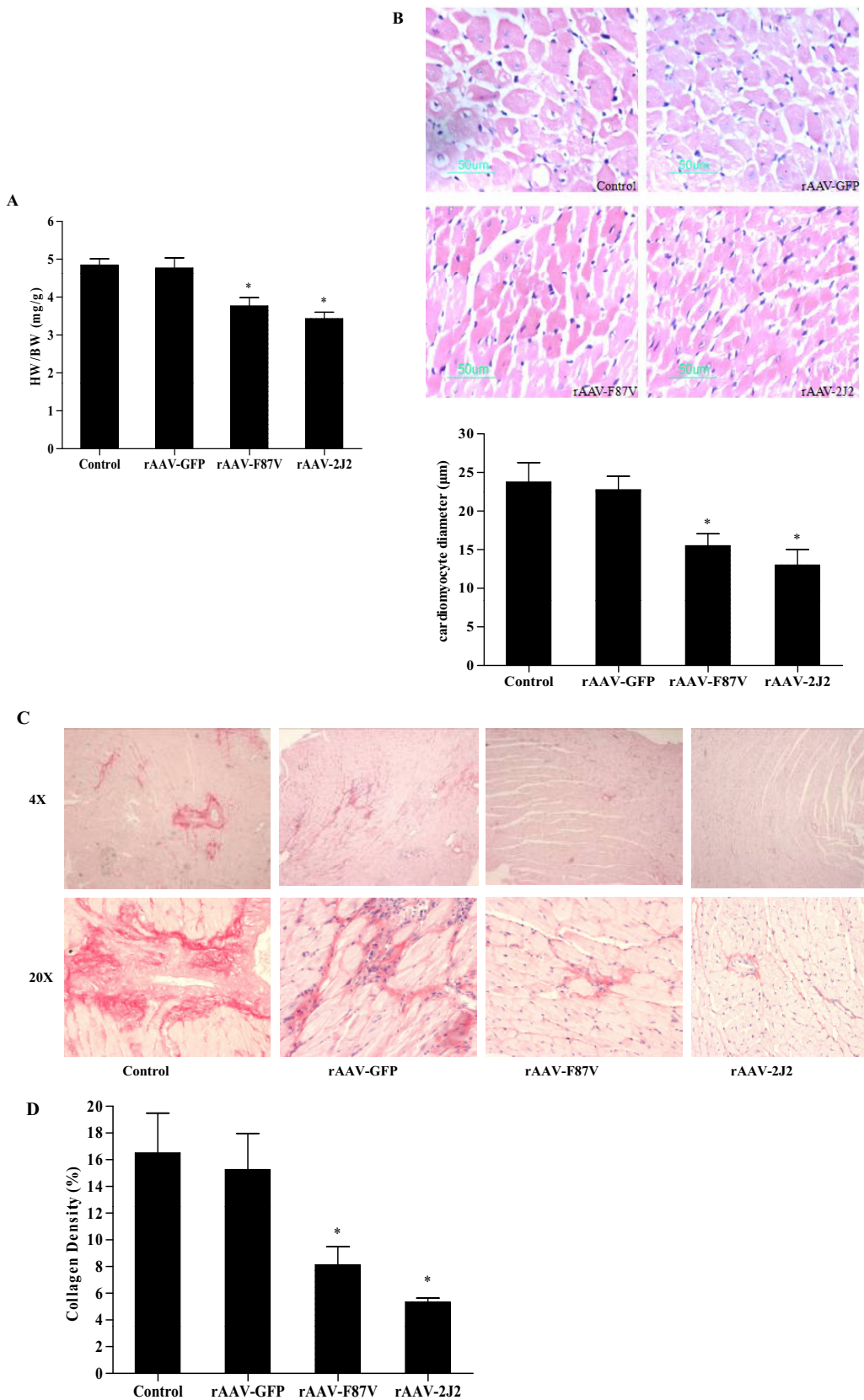


Fig. 4. The effects of rAAV-CYP102 F87V and rAAV-CYP2J2 treatments on heart remodeling. Ratio of whole heart weight to body weight (A) and microscope images of cross-section of ventricle (B) to show size of cardiomyocytes (top) and comparison of cardiomyocyte diameter (bottom), which suggest gene treatments prevent the hypertension-induced myocardial hypertrophy. C, heart collagen content assessed by Sirius red staining: representative photomicrographs (Sirius red staining for collagen content) of heart sections from the various treatment groups. D, quantification of collagen-positive areas showed reduced collagen deposition in hearts from rAAV-CYP2J2- and rAAV-CYP102 F87V-treated rats; *, $p < 0.05$ compared with controls.

In vivo cardiac hemodynamic measurements described herein suggest that P450 epoxygenase overexpression has negative inotropic effects. Published data indicate that EETs decrease the open probability of myocardial L-type Ca^{2+}

channels, decrease the intracellular Ca^{2+} concentration (Chen et al., 1999), and also induce activation of Ca^{2+} -dependent K^{+} channels (Giles and Imaizumi, 1988) and/or ATP-sensitive K^{+} channels (Lu et al., 2001; Wang et al., 2006a).

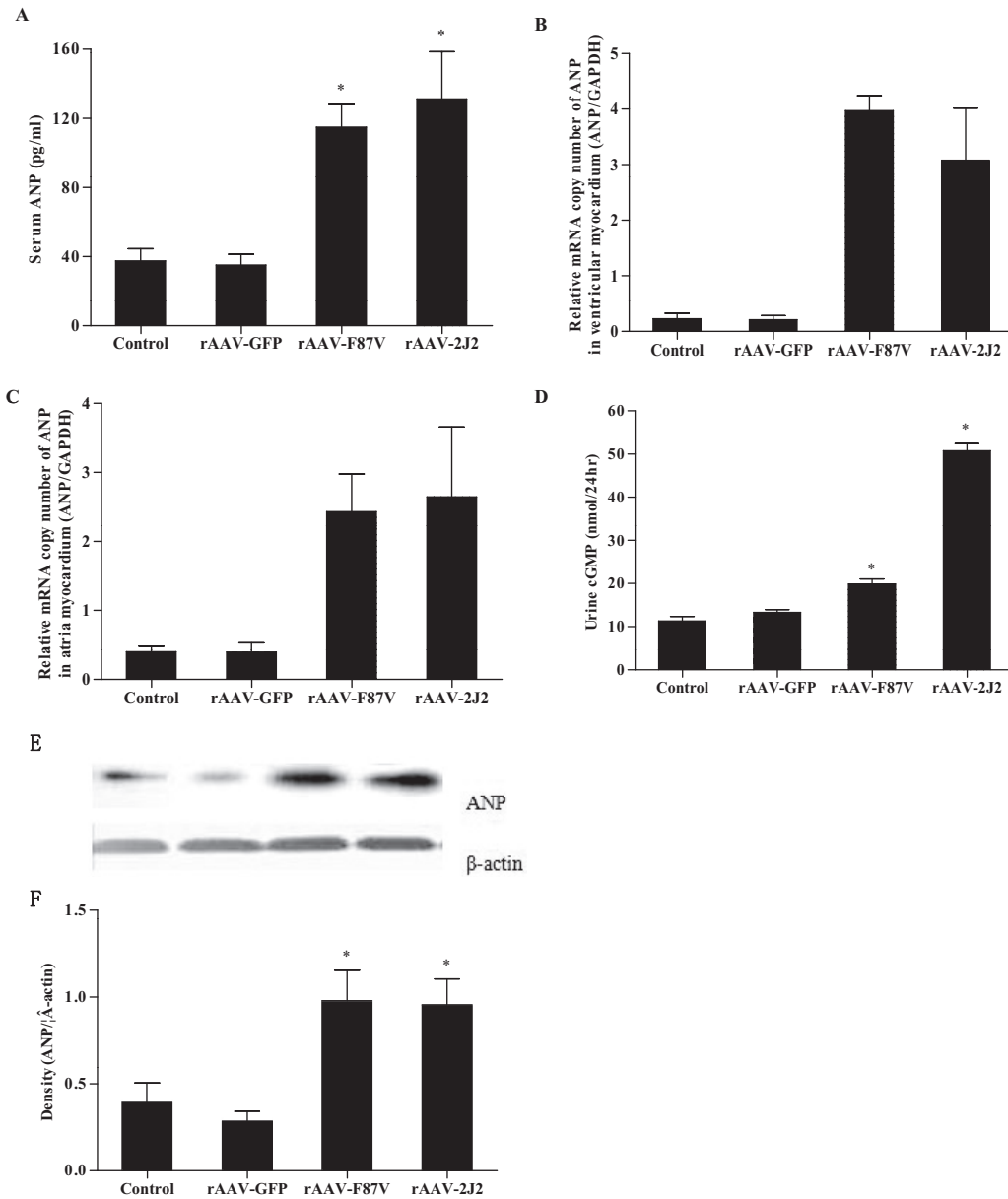


Fig. 5. The effects of rAAV-CYP102 F87V and rAAV-CYP2J2 treatments on secretion of ANP and expression of ANP. **A**, serum ANP in four groups; *, $p < 0.05$ compared with control groups. **B** and **C**, relative mRNA copy number of ANP relative to glyceraldehyde-3-phosphate dehydrogenase in ventricular and atrial myocardium, respectively, determined by real-time quantitative PCR. ANP mRNA levels were much greater in myocardium of the rAAV-CYP102 F87V and rAAV-CYP2J2 groups; *, $p < 0.01$ compared with control groups. **D**, urinary cGMP levels; *, $p < 0.05$ compared with control groups. **E** and **F**, Western blot of ANP expression in myocardium and relative density of ANP band against β -actin, showing that ANP level is significantly up-regulated in myocardium in rAAV-CYP102 F87V- and rAAV-CYP2J2-treated rats; *, $p < 0.01$ compared with controls.

These changes lead to shortening of the cardiac action potential, reduced Ca^{2+} entry, and suppression of cardiac systolic function. Our results are consistent with previously reported findings describing the capacity of ANP to directly depress cardiac contractility and produce negative inotropic effects (Wada et al., 1994; Kojda et al., 1996; Mohan et al., 1996; Lin et al., 1998), and we speculate that the negative inotropic effect of ANP induced by P450 epoxygenase overexpression may partially account for the observed hypotensive effect seen in the present study. To exclude the effect of cardiac atrium stretch on excretion of ANP, we applied exogenous EETs to cultured cardiomyocytes and found that addition of EETs resulted in increased ANP secretion. Thus, the excretion of ANP can be induced by EETs independent of cardiac atrium stretch. cGMP as the direct downstream messenger molecule of ANP receptor was up-regulated by increased ANP. In the study, the negative inotropic effects of P450 epoxygenase overexpression do not result in the decrease; in contrast, they induced a significant increase in stroke volume

and cardiac output, and simultaneously preload-adjusted maximal power is significantly reduced. These data suggest that preload of left ventricle is reduced and increased stroke volume is attributable to reduction in afterload, which is associated with both the vasodilation and diuretic effect derived directly from EETs and more importantly from ANP.

Previous studies showed that various rat models of hypertension developed myocardial hypertrophy with cardiac dysfunction (Yoshimoto et al., 1996). The present study found that overexpression of P450 epoxygenases prevented or attenuated hypertension-induced myocardial hypertrophy. Reduction in peripheral vascular resistance and resultant reduction in artery blood pressure may directly contribute to the antihypertrophy effect. Recent studies showed that sEH inhibitors could prevent cardiac hypertrophy via increasing EET level (Xu et al., 2006; Ai et al., 2009), supporting our conclusion. However, whether EETs can directly inhibit myocardial hypertrophy via their effects on cardiomyocytes remains to be elucidated in a future study. In addition, the

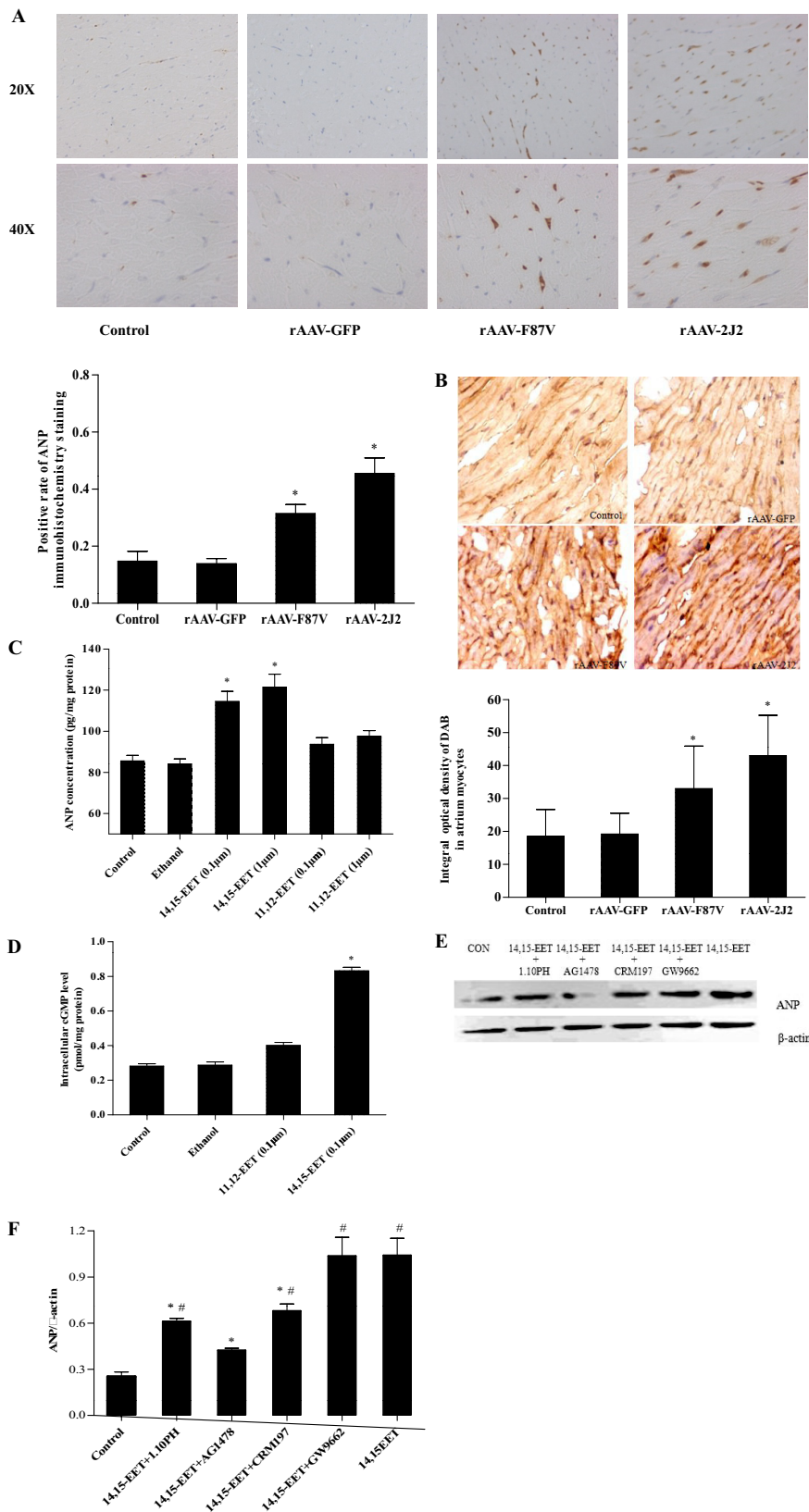


Fig. 6. Effects of epoxygenase overexpression or EET on expression of ANP. A and B, immunohistochemistry staining for ANP in ventricular myocardium and atria myocardium, respectively, reveals more positive cells and stronger overall staining in rAAV-CYP102 F87V- and rAAV-CYP2J2-treated groups (top) and quantification of ANP-positive cells (bottom); *, $p < 0.05$ compared with controls. C, ANP concentration in media of cultured cardiomyocytes incubated with synthetic EETs; *, $p < 0.05$ compared with control. D, intracellular cGMP levels in cultured cardiomyocytes; *, $p < 0.05$ compared with control. E, representative Western blot and relative density of ANP band: effects of inhibitors of MMP (1,10-phenanthroline), HBEGF (CRM-197), and EGFR (AG-1478) on ANP expression in cultured cardiomyocytes. F, relative density of ANP band against β -actin; #, $p < 0.05$ compared with control (CON); *, $p < 0.01$ compared with EET treatment.

reduction in cardiac hypertrophy and collagen deposition in heart may facilitate improvement of cardiac function in epoxygenase gene therapy.

The mechanism whereby EETs up-regulate ANP expression is not known. Previous studies have shown that the

binding of EETs to a putative receptor leads to increases in cAMP levels and protein kinase A activity (Wong et al., 1997; Node et al., 2001). The regulation of gene transcription by cAMP is mediated by *trans*-acting factors that bind to the cAMP-response element (CRE) of target genes (Nagamine

and Reich, 1985). In this regard, a recent study showed that binding of activator protein-1 to the putative CRE site in the ANP promoter increases gene transcription by 17.5-fold (Cornelius et al., 1997a,b). These results are consistent with EET-mediated activation of CRE and/or CRE-binding protein(s) leading to induction of ANP. Previous study showed that EET significantly induced cleavage of HB-EGF and soluble HB-EGF release through activating MMPs and increasing their expression, and consequentially EET enhanced EGFR phosphorylation and its downstream signaling activation (Chen and Harris, 2002; Jiang et al., 2007a). This study showed that the EGFR antagonist, the MMP inhibitor, and the HB-EGF inhibitor, but not the PPAR- γ inhibitor, significantly attenuated the EET-induced expression of ANP, which suggests that EET-induced activation of EGFR may involve increased ANP secretion in heart.

The data presented in this study indicate that rAAV-CYP2J2 and rAAV-CYP102 F87V treatments improved aortic compliance by markedly decreasing Ea, an index which describes the elasticity of the large arteries. Furthermore, a reduction in the collagen content of aorta and myocardium was observed, which suggests that rAAV-CYP2J2 and rAAV-CYP102 F87V treatments attenuated cardiac and vessel remodeling (Supplemental Fig. S7). The precise mechanisms by which EETs reduced collagen deposition in target tissues are not known, but EETs can significantly increase expression and fibrinolytic activity of tissue plasminogen activator in endothelial cells (Node et al., 1999); this enhances collagen degradation and may contribute to the reduced remodeling of heart and vessel wall. In addition, the hypotensive effect of EETs may also reduce or delay remodeling within the cardiovascular system.

In summary, the present study provides *in vivo* evidence that P450 epoxygenase overexpression reduces arterial blood pressure and prevents cardiac dysfunction and remodeling in SHR. These effects are probably mediated by P450-derived EETs, particularly 14,15-EET, and appear to involve increases in the production of ANP. Together, these data suggest that studies to examine the potential benefits of targeting the P450 epoxygenase-ANP pathway may yield novel approaches to the treatment of hypertension and associated cardiovascular complications. This study has some limitations, such as we did not use ANP receptor antagonist *in vivo* to observe whether the hypotensive effect of epoxygenase overexpression was blocked to confirm the association of EET-induced ANP up-regulation with antihypertension; we found that epoxygenase overexpression induced elevation in cGMP level, but we did not tell the source, in response to increased NO-mediated activity or from up-regulated ANP or both. These need further study to elucidate.

Acknowledgments

We thank Dr. Jorge H. Capdevila (Department of Medicine, Vanderbilt University Medical Center) for the gift of CYP102 F87V antibody, and Dr. Jeffrey W. Card (Division of Intramural Research, National Institute of Environmental Health Sciences) for assistance with editing of this manuscript.

References

Ai D, Pang W, Li N, Xu M, Jones PD, Yang J, Zhang Y, Chiamvimonvat N, Shyy JY, Hammock BD, et al. (2009) Soluble epoxide hydrolase plays an essential role in angiotensin II-induced cardiac hypertrophy. *Proc Natl Acad Sci U S A* **106**:564–569.

Anand-Srivastava MB (2005) Natriuretic peptide receptor-C signaling and regulation. *Peptides* **26**:1044–1059.

Capdevila JH and Falck JR (2002) Biochemical and molecular properties of the cytochrome P450 arachidonic acid monooxygenases. *Prostaglandins Other Lipid Mediat* **68–69**:325–344.

Capdevila JH, Falck JR, and Harris RC (2000) Cytochrome P450 and arachidonic acid bioactivation. Molecular and functional properties of the arachidonate monooxygenase. *J Lipid Res* **41**:163–181.

Chen C, Li G, Liao W, Wu J, Liu L, Ma D, Zhou J, Elbekai RH, Edin ML, Zeldin DC, et al. (2009) Selective inhibitors of CYP2J2 related to terfenadine exhibit strong activity against human cancers *in vitro* and *in vivo*. *J Pharmacol Exp Ther* **329**:908–918.

Chen JK, Capdevila J, and Harris RC (2002) Heparin-binding EGF-like growth factor mediates the biological effects of P450 arachidonate epoxygenase metabolites in epithelial cells. *Proc Natl Acad Sci U S A* **99**:6029–6034.

Chen JK, Wang DW, Falck JR, Capdevila J, and Harris RC (1999) Transfection of an active cytochrome P450 arachidonic acid epoxygenase indicates that 14,15-epoxyicosatrienoic acid functions as an intracellular second messenger in response to epidermal growth factor. *J Biol Chem* **274**:4764–4769.

Cohen RA and Vanhoutte PM (1995) Endothelium-dependent hyperpolarization. Beyond nitric oxide and cyclic GMP. *Circulation* **92**:3337–3349.

Cornelius T, Holmer SR, Müller FU, Riegger GA, and Schunkert H (1997a) Regulation of the rat atrial natriuretic peptide gene after acute imposition of left ventricular pressure overload. *Hypertension* **30**:1348–1355.

Cornelius T, Holmer SR, Müller FU, Riegger GA, and Schunkert H (1997b) Regulation of the rat atrial natriuretic peptide gene after acute imposition of left ventricular pressure overload. *Hypertension* **30**:1348–1355.

De Windt LJ, Lim HW, Haq S, Force T, and Molkenkin JD (2000) Calcineurin promotes protein kinase C and c-Jun NH2-terminal kinase activation in the heart. Cross-talk between cardiac hypertrophic signaling pathways. *J Biol Chem* **275**:13571–13579.

Fleming I and Busse R (2006) Endothelium-derived epoxyeicosatrienoic acids and vascular function. *Hypertension* **47**:629–633.

Giles WR and Imaizumi Y (1988) Comparison of potassium currents in rabbit atrial and ventricular cells. *J Physiol* **405**:123–145.

Graham-Lorence S, Truan G, Peterson JA, Falck JR, Wei S, Helvig C, and Capdevila JH (1997) An active site substitution, F87V, converts cytochrome P450 BM-3 into a regio- and stereoselective (14S,15R)-arachidonic acid epoxygenase. *J Biol Chem* **272**:1127–1135.

Guan H, Wang P, Hui R, Edin ML, Zeldin DC, and Wang DW (2009) Adeno-associated virus-mediated human C-reactive protein gene delivery causes endothelial dysfunction and hypertension in rats. *Clin Chem* **55**:274–284.

Imig JD (2006) Cardiovascular therapeutic aspects of soluble epoxide hydrolase inhibitors. *Cardiovasc Drug Rev* **24**:169–188.

Imig JD, Zhao X, Capdevila JH, Morisseau C, and Hammock BD (2002) Soluble epoxide hydrolase inhibition lowers arterial blood pressure in angiotensin II hypertension. *Hypertension* **39**:690–694.

Imig JD, Zhao X, Zaharis CZ, Olearczyk JJ, Pollock DM, Newman JW, Kim IH, Watanabe T, and Hammock BD (2005) An orally active epoxide hydrolase inhibitor lowers blood pressure and provides renal protection in salt-sensitive hypertension. *Hypertension* **46**:975–981.

Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals*, 7th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington DC.

Jiang JG, Chen CL, Card JW, Yang S, Chen JX, Fu XN, Ning YG, Xiao X, Zeldin DC, and Wang DW (2005) Cytochrome P450 2J2 promotes the neoplastic phenotype of carcinoma cells and is up-regulated in human tumors. *Cancer Res* **65**:4707–4715.

Jiang JG, Chen RJ, Xiao B, Yang S, Wang JN, Wang Y, Cowart LA, Xiao X, Wang DW, and Xia Y (2007a) Regulation of endothelial nitric-oxide synthase activity through phosphorylation in response to epoxyeicosatrienoic acids. *Prostaglandins Other Lipid Mediat* **82**:162–174.

Jiang JG, Chen RJ, Xiao B, Yang S, Wang JN, Wang Y, Cowart LA, Xiao X, Wang DW, and Xia Y (2007b) Regulation of endothelial nitric-oxide synthase activity through phosphorylation in response to epoxyeicosatrienoic acids. *Prostaglandins Other Lipid Mediat* **82**:162–174.

King LM, Gainer JV, David GL, Dai D, Goldstein JA, Brown NJ, and Zeldin DC (2005) Single nucleotide polymorphisms in the CYP2J2 and CYP2C8 genes and the risk of hypertension. *Pharmacogenet Genomics* **15**:7–13.

King LM, Ma J, Srettabunjong S, Graves J, Bradbury JA, Li L, Spiecker M, Liao JK, Mohrenweiser H, and Zeldin DC (2002) Cloning of CYP2J2 gene and identification of functional polymorphisms. *Mol Pharmacol* **61**:840–852.

Kojda G, Kottenberg K, Nix P, Schlüter KD, Piper HM, and Noack E (1996) Low increase in cGMP induced by organic nitrates and nitrovasodilators improves contractile response of rat ventricular myocytes. *Circ Res* **78**:91–101.

Lin KF, Chao J, and Chao L (1998) Atrial natriuretic peptide gene delivery attenuates hypertension, cardiac hypertrophy, and renal injury in salt-sensitive rats. *Hum Gene Ther* **9**:1429–1438.

Lu T, Hoshi T, Weintraub NL, Spector AA, and Lee HC (2001) Activation of ATP-sensitive K(+) channels by epoxyeicosatrienoic acids in rat cardiac ventricular myocytes. *J Physiol* **537**:811–827.

McGiff JC (1991) Cytochrome P-450 metabolism of arachidonic acid. *Annu Rev Pharmacol Toxicol* **31**:339–369.

Mohan P, Brutsaert DL, Paulus WJ, and Sys SU (1996) Myocardial contractile response to nitric oxide and cGMP. *Circulation* **93**:1223–1229.

Nagamine Y and Reich E (1985) Gene expression and cAMP. *Proc Natl Acad Sci U S A* **82**:4606–4610.

Nichols WW and Edwards DG (2001) Arterial elastance and wave reflection augmentation of systolic blood pressure: deleterious effects and implications for therapy. *J Cardiovasc Pharmacol Ther* **6**:5–21.

- Nishikimi T, Maeda N, and Matsuoka H (2006) The role of natriuretic peptides in cardioprotection. *Cardiovasc Res* **69**:318–328.
- Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, and Liao JK (1999) Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science* **285**:1276–1279.
- Node K, Ruan XL, Dai J, Yang SX, Graham L, Zeldin DC, and Liao JK (2001) Activation of Galpha s mediates induction of tissue-type plasminogen activator gene transcription by epoxyeicosatrienoic acids. *J Biol Chem* **276**:15983–15989.
- Ohte N, Cheng CP, Suzuki M, and Little WC (1999) Effects of atrial natriuretic peptide on left ventricular performance in conscious dogs before and after pacing-induced heart failure. *J Pharmacol Exp Ther* **291**:589–595.
- Roman RJ (2002) P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev* **82**:131–185.
- Seubert J, Yang B, Bradbury JA, Graves J, Degraff LM, Gabel S, Gooch R, Foley J, Newman J, Mao L, et al. (2004) Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K⁺ channels and p42/p44 MAPK pathway. *Circ Res* **95**:506–514.
- Wada A, Tsutamoto T, Matsuda Y, and Kinoshita M (1994) Cardiorenal and neurohumoral effects of endogenous atrial natriuretic peptide in dogs with severe congestive heart failure using a specific antagonist for guanylate cyclase-coupled receptors. *Circulation* **89**:2232–2240.
- Wang H, Lin L, Jiang J, Wang Y, Lu ZY, Bradbury JA, Lih FB, Wang DW, and Zeldin DC (2003) Up-regulation of endothelial nitric-oxide synthase by endothelium-derived hyperpolarizing factor involves mitogen-activated protein kinase and protein kinase C signaling pathways. *J Pharmacol Exp Ther* **307**:753–764.
- Wang T, Li H, Zhao C, Chen C, Li J, Chao J, Chao L, Xiao X, and Wang DW (2004) Recombinant adeno-associated virus-mediated kallikrein gene therapy reduces hypertension and attenuates its cardiovascular injuries. *Gene Ther* **11**:1342–1350.
- Wang XL, Lu T, Cao S, Shah VH, and Lee HC (2006a) Inhibition of ATP binding to the carboxyl terminus of Kir6.2 by epoxyeicosatrienoic acids. *Biochim Biophys Acta* **1761**:1041–1049.
- Wang Y, Wang Y, Yang Q, Yan JT, Zhao C, Cianflone K, and Wang DW (2006b) Effects of bezafibrate on the expression of endothelial nitric oxide synthase gene and its mechanisms in cultured bovine endothelial cells. *Atherosclerosis* **187**:265–273.
- Wang Y, Wei X, Xiao X, Hui R, Card JW, Carey MA, Wang DW, and Zeldin DC (2005) Arachidonic acid epoxygenase metabolites stimulate endothelial cell growth and angiogenesis via mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways. *J Pharmacol Exp Ther* **314**:522–532.
- Wei X, Zhao C, Jiang J, Li J, Xiao X, and Wang DW (2005) Adrenomedullin gene delivery alleviates hypertension and its secondary injuries of cardiovascular system. *Hum Gene Ther* **16**:372–380.
- Wong PY, Lai PS, Shen SY, Belosludtsev YY, and Falck JR (1997) Post-receptor signal transduction and regulation of 14(R),15(S)-epoxyeicosatrienoic acid (14,15-EET) binding in U-937 cells. *J Lipid Mediat Cell Signal* **16**:155–169.
- Xiao X, Li J, and Samulski RJ (1998) Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* **72**:2224–2232.
- Xu D, Li N, He Y, Timofeyev V, Lu L, Tsai HJ, Kim IH, Tuteja D, Mateo RK, Singapuri A, et al. (2006) Prevention and reversal of cardiac hypertrophy by soluble epoxide hydrolase inhibitors. *Proc Natl Acad Sci U S A* **103**:18733–18738.
- Xu X, Wan W, Ji L, Lao S, Powers AS, Zhao W, Erikson JM, and Zhang JQ (2008) Exercise training combined with angiotensin II receptor blockade limits post-infarct ventricular remodeling in rats. *Cardiovasc Res* **78**:523–532.
- Yang S, Lin L, Chen JX, Lee CR, Seubert JM, Wang Y, Wang H, Chao ZR, Tao DD, Gong JP, et al. (2007) Cytochrome P-450 epoxygenases protect endothelial cells from apoptosis induced by tumor necrosis factor-alpha via MAPK and PI3K/Akt signaling pathways. *Am J Physiol Heart Circ Physiol* **293**:H142–H151.
- Yayama K, Wang C, Chao L, and Chao J (1998) Kallikrein gene delivery attenuates hypertension and cardiac hypertrophy and enhances renal function in Goldblatt hypertensive rats. *Hypertension* **31**:1104–1110.
- Yoshimoto T, Naruse M, Naruse K, Shionoya K, Tanaka M, Tanabe A, Hagiwara H, Hirose S, Muraki T, and Demura H (1996) Angiotensin II-dependent down-regulation of vascular natriuretic peptide type C receptor gene expression in hypertensive rats. *Endocrinology* **137**:1102–1107.

Address correspondence to: Dao Wen Wang, Department of Internal Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan 430030, People's Republic of China. E-mail: dwwang@tjh.tjmu.edu.cn
