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

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Running Title: P450 Expoxygenases Prevent Hypertension by ANP

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Document statistics:

Number of text pages: 33.

Number of Figures: 6.

Number of References: 50.

The number of words in the Abstract: 243.

The number of words in the Introduction: 438.

The number of words in the Discussion: 1206.

Abbreviations: EETs, epoxyeicosatrienoic acids; CYP, Cytochrome P450; AA, arachidonic acid; DHET, Dihydroxyeicosatrienoic acid; ANP, atrial natriuretic peptide; rAAV, recombinant adeno-associated viral vector; RT-PCR, Reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGFR, epithelial growth factor receptor; eNOS, endothelial nitric-oxide synthase; PPAR gamma, peroxisome proliferator-activated receptor gamma; cGMP, cyclic guanosine monophosphate; LVEDP, left ventricular end-diastolic pressure; cAMP, cyclic adenosine monophosphate; MMP, matrix metalloproteinase. SBP, systolic blood pressure; GFP, green fluorescent protein.

ABSTRACT

Cytochrome P450 (CYP)-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 over-expressing CYP 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 Microtransducer Catheter, and atrial natriuretic peptide (ANP) mRNA levels were tested by real-time PCR. Results showed that urinary excretion of 14,15-EET was increased at 2 and 6 months following injection with rAAV-CYP102 F87V and rAAV-CYP2J2 compared with controls ($p<0.05$). During the course of the 6-month study, sytolic blood pressure significantly decreased in CYP epoxygenase-treated rats, but the CYP2J2 specific inhibitor C26 blocked rAAV-CYP2J2 induced-hypotension and increase in EET production. Cardiac output was improved by CYP-epoxygenase expression at 6 months ($p<0.05$). Furthermore, cardiac collagen content was reduced in CYP epoxygenase-treated rats. Atrial natriuretic peptide (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 CYP epoxygenase-treated rats. However, EGF receptor antagonist AG1478 significantly attenuated the increase in the EET-induced expression of ANP *in vitro*. These data indicate that overexpression of CYP 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.

Cytochrome P450 (CYP) epoxygenases metabolize arachidonic acid to biologically active eicosanoids referred to as *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., 2001). Exogenous EETs or overexpression of various CYP epoxygenases also protect endothelial cells from apoptosis(Yang et al., 2007), upregulate 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 AMP-activated protein kinase (AMPK)(Wang et al., 2005; Jiang et al., 2007b). Furthermore, CYP2J2 overexpression has been shown to protect against post-ischemic 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 CYP 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 utilized to test the hypothesis that CYP 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 (SHR). 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 CYP 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 GFP were prepared by triple plasmid cotransfection in HEK293 cells as described previously(Xiao et al., 1998; Wang et al., 2004).

ANIMALS AND VECTOR ADMINISTRATION

Male spontaneously hypertensive rats (SHR) weighing 200-220 g were obtained from the Experimental Animal Center of Beijing, PRC. 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. Twenty four animals were randomized to 4 groups (6 animals per group) as follows: saline control, rAAV-GFP control, rAAV-CYP102 F87V, and rAAV-CYP2J2. Animals received a single injection of either saline or of rAAV (1×10^{12} vector genomes per rat) via tail vein, respectively. Additionally, 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, twenty four male spontaneously hypertensive rats were divided to four groups: Control group, Control+C26 group, rAAV-2J2 group, 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 month for 6 months at room temperature by a photoelectric tail-cuff system (Millar, PowerLab) as described previously (Yayama et al., 1998).

HEMODYNAMIC STUDY

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

ISOLATION OF THORACIC AORTIC RINGS AND DETERMIATION OF EPOXYGENASE-INDUCED RELAXATION

Thoracic aortic rings were prepared as follows: Briefly, thoracic aortas were rapidly isolated and immersed in Krebs–Ringer HCO_3 buffer (NaCl, 118.3 mM; KCl, 4.7 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25.0 mM; Ca-EDTA, 0.026 mM; glucose, 11.1mM) which was aerated with 95% O_2:5% CO_2 (pH 7.4). The vessel was carefully trimmed of surrounding tissues and cut into 2-3 mm rings. The rings were mounted on specimen holders and placed in glass organ chambers

containing 6 ml of aerated Krebs–Ringer HCO_3 buffer at 37°C. While 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 HCO_3 buffer. We examined the responsiveness of aortic rings from rats overexpressing CYP epoxygenases to norepinephrine (NE) and acetylcholine (ACh) using a multichannel physiologic recorder (ML-840, PowerLab)(Guan et al., 2009).

14, 15-DHET DETERMINATION IN URINE AND TISSUES

The 14,15-DHET ELISA kit (Detroit R&D Inc., Detroit, MI) was used to measure 14,15-DHET according to the manufacturer's instructions as previously described(Jiang et al., 2005; Yang et al., 2007). EETs can be hydrolyzed to DHETs by acid treatment and 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 concentration of 14,15-DHET and 14,15-EET were expressed as ng/ml urine or pg/mg tissue specimen.

REAL-TIME PCR FOR ANP

Total RNA was prepared by Trizol using the manufacturer protocols (Life Technologies). cDNA was produced using reverse transcriptase (TaKaRa, Japan). A LightCycler RT-PCR System (Roche) was used with an automated sequence detection instrument for the real-time monitoring of nucleic acid green dye fluorescence (SYBR Green; TOYOBO) as described previously(Wang et al., 2006b). Primers and

conditions of PCR were shown in supplementary Table 1S.

WESTREN BLOTTING

Western blot was performed according to method described previously(Wang et al., 2003). CYP102 F87V antibody was a gift from Dr. Jorge H. Capdevila (Vanderbilt University). Specific polyclonal antibodies raised against CYP2J2 were developed as described(King et al., 2002). The horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit) was bought from Santa Cruz Biotechnology.

IMMUNOHISTOCHEMICAL DETECTION OF ANP IN HEART

Process of immunohistochemistry was performed as described previously(Capdevila and Falck, 2002) using ANP antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA).

ANALYSIS OF MYOCARDIAL AND RENAL AND ARTERIAL MORPHOLOGY

Four-μm 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 (ECM) production were quantified using the HAIPS Pathological Imagic Analysis System. Heart and kidney sections were stained with hematoxylin and eosin (H&E), 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 DAB.

11,12- and 14,15-EET (0.1 and 1.0 μM) were added to the cultured cells. In order to

elucidate the relevant mechanisms, different inhibitors were added to the cultures of

neonatal rat cardiomyocytes (1,10-phenanthroline, MMP inhibitor, 100μm; AG1478,

EGFR inhibitor, 100nm; CRM197, HBEGF inhibitor, 10μg/ml; GW9662, PPAR

gamma inhibitor, 1μm), respectively, with or without 1.0 μM 14.15-EET. After

incubation for 24hrs, 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, Burlingame,

CA and Immunology Consultants Laboratory, Newberg, OR) according to the

manufacturer's instructions, respectively. cGMP levels in urine and cultured

cardiomyocytes were measured by ELISA kits (Cayman. Inc, Ann Abor, MI, USA).

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Multiple comparisons between two groups were

performed with unpaired t tests; between three or more groups they were carried out

with One-way ANOVA and Newman-Keuls tests for post hoc analyses. Significance

was accepted at a value of $P<.05$.

Results

CYP EPOXYGENASE OVEREXPRESSION INDUCES PROLONGED PRODUCTION OF EETs IN VIVO

Western blot analyses for expression of CYP 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 (Figure 1A). Overexpression of CYP epoxygenases was associated with a significant increase in urinary 14,15-DHET and 14,15-EET levels at both 2 and 6 months compared to levels in rats injected with saline or with rAAV-GFP ($p<0.05$; Figure 1B 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 ($p<0.05$; Figure 1D 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 CYP epoxygenase protein expression and activity *in vivo*.

CYP EPOXYGENASE OVEREXPRESSION RESULTS IN HYPOTENSIVE EFFECTS IN VIVO

Animals treated with rAAV-CYP102 F87V or rAAV-CYP2J2 showed a significant decrease in SBP at 2 months post-injection corresponding with the peak 14,15-DHET levels (both $p<0.05$ compared to control, Figure 2A). This difference was still evident at the 6 month time point in the rAAV-CYP2J2-treated group ($p<0.05$, Figure 2A).

Prior to sacrifice at the 6 month time point, the carotid intra-arterial pressure was measured. The data from this experiment were consistent with the non-invasive tail-cuff measurements (p<0.05; Figure 2B). However, only diastolic blood pressure of rAAV-CYP2J2-treated rats was decreased significantly at the end of the 6 month period (supplementary data, Figure 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 mmHg versus 219.35 ± 7.5 mmHg, p<0.05), but C26 administration exclusively blocked rAAV-CYP2J2 induced-hypotension (Figure 2C) and also the increase in EET and DHET production (p<0.05; Figure 2D and E).

OVEREXPRESSION OF CYP EPOXYGENASES IMPROVES CARDIAC FUNCTION

Cardiac hemodynamics was measured 6 months following 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 to saline and rAAV-GFP-treated rats (p<0.05; Figure 2F, supplementary data, Table S2)). However, the stroke volume and cardiac output (CO) were significantly increased compared with controls (p<0.05; Figure 2G, supplementary data, Table S2), which were accompanied with the lower preload adjusted maximal power (PAMP), 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 LVEDP between groups (supplementary data,

Figures S2, S3). Combined these results suggest that the overexpression of

epoxygenases resulted in reduction in myocardial contractility in SHR but increase in

stroke volume and CO.

OVEREXPRESSION OF CYP 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/μL, respectively)

was significantly lower than in the saline-treated control group (2.6 ± 0.2 mm Hg/μL,

$p < 0.05$, Figure 3A), suggesting that the CYP epoxygenase overexpression improved

arterial elastance. Furthermore, rAAV-CYP2J2 and rAAV-CYP102 F87V treatments

significantly enhanced the responsiveness of aortic rings to Ach and attenuated

responsiveness to NE (Figures 3B, C), further suggesting that CYP epoxygenase

overexpression results in altered responsiveness to endogenous vasoconstrictors and

vasodilators.

OVEREXPRESSION OF CYP 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 that controls (Figure 4A), and the

cardiomyocyte diameter was significantly smaller in the gene-treated animals than

controls (Figure 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 $p<0.05$, Figures 4C, 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 compared 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 (supplementary Figure S4).

ANP was UPREGULATED BY OVEREXPRESSION OF CYP EPOXYGENASES

In order to assess potential mechanisms by which CYP 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 upregulated 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, Figure 5A).   Moreover, ANP mRNA levels were also upregulated by 14- and 18-fold in ventricular myocardium and 6-7 folds in atrial myocardium in rAAV-CYP2J2- and rAAV-CYP102 F87V-treated rats, respectively, compared to saline-treated control rats (p<0.01, Figures 5B, C). Accordingly, urinary cGMP were increased in rAAV-CYP102 F87V- and rAAV-CYP2J2-treated rats (19.86±1.23nmol/24hr and 50.76±1.68nmol/24hr, respectively) as ANP level upregulated, compared with control and rAAV-GFP-treated groups (11.31±1.05 nmol/24hr and 13.33±0.63nmol/24hr respectively, p<0.05, Figure 5D). Western blots show that ANP expression in ventricle tissues is significantly upregulated in rAAV-CYP2J2- and rAAV-CYP102 F87V-treated rats (p<0.01; Figure 5E, F). The expression levels of other vasoactive signaling molecules such as endothelin-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 percent of ANP positive cells in myocardium increased by 1-2 folds in rAAV-CYP102 F87V and rAAV-CYP2J2-treated rats compared with saline-treated controls in both ventricle (Figure 6A) and atria (Figure 6B). Finally, incubation with synthetic 14,15-EET increased secretion of ANP from cultured cardiomyocytes into the medium (Figure 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 upregulated (Figure 6D).

Together, these results demonstrate that the beneficial effects of CYP 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 (supplementary Figure 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 EGF receptor antagonist AG1478 significantly attenuated the increase in the EET-induced expression of ANP, and MMP Inhibitor 1.10PH and HB-EGF Inhibitor CRM197 also decreased the expression of ANP (Figure 6E 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 EDHF-like properties and natriuretic effects, and upregulate eNOS(Wang et al., 2003), all of which may contribute to the regulation of blood pressure. Recently, sEH inhibitors were demonstrated to lower arterial blood pressure in an angiotensin II-induced hypertension model(Imig et al., 2002). These observations cumulatively support the hypothesis that CYP 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 epoxygenases inhibitor C26 reversed that change by decreasing production of EETs. Mechanistic studies revealed that CYP epoxygenase overexpression improved arterial elastance, enhanced responsiveness of aortic rings to Ach, and attenuated responsiveness of aortic rings to NE. In addition, overexpression of CYP epoxygenases markedly up-regulated ANP levels in serum and enhanced the cardiac expression of ANP *in vivo*, while EETs enhanced ANP release *in vitro* in cultured cardiomyocytes. These data suggest a hypotensive effect of CYP 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

upregulate 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 CYP 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.

In vivo cardiac hemodynamic measurements described herein suggest that

CYP 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). 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 depresses 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 CYP epoxygenase overexpression may partially accounts for the observed

hypotensive effect seen in the present study. In order 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 upregulateded by increased ANP. In the study, the negative inotropic effects of CYP epoxygenase overexpression don't result in the decrease, in contrast, induced a significant increase in stroke volume and cardiac output, and simultaneously preload adjusted maximal power (PAMP) 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 demonstrated that various rat models of hypertension developed myocardial hypertrophy with cardiac dysfunction(Yoshimoto et al., 1996). The present study found that overexpression of CYP 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 anti-hypertrophy effect. Recent studies demonstrated that soluble epoxide hydrolase inhibitors could prevent cardiac hypertrophy via increasing EETs 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 future study. In addition, the reduction in cardiac hypertrophy as well as collagen deposition in heart may facilitate improvement of

cardiac function in epoxygenase gene therapy.

The mechanism whereby EETs upregulate 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 PKA 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 Y, 1985). In this regard, a recent study demonstrated that binding of AP-1 to the putative CRE site in the ANP promoter increases gene transcription by 17.5-fold (Cornelius et al., 1997a; Cornelius et al., 1997b). These results are consistent with EET-mediated activation of CRE and/or CRE-binding protein(s) leading to induction of ANP. Previous study demonstrated that EET significantly induced cleavage of heparin-binding epidermal growth factor-like growth factor (HB-EGF) and soluble HB-EGF release through activating metalloproteinases (MMP) and increasing their expression and consequentially EET enhanced EGF receptor phosphorylation and its downstream signaling activation(Chen JK, 2002; Jiang et al., 2007a). This study showed that the EGFR antagonist, the MMP inhibitor and the HB-EGF inhibitor but not the PPAR gamma inhibitor significantly attenuated the EET-induced expression of ANP, which suggest 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 (supplementary Figure 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. Additionally, 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 CYP epoxygenase overexpression reduces arterial blood pressure and prevents cardiac dysfunction and remodeling in SHR. These effects are likely mediated by CYP-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 CYP 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 can was blocked to confirm the association of EETs-induced ANP upregulation 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 upregulated ANP or both. These need further study to elucidate.

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Footnotes

FIGURE LEGENDS

Fig. 1. CYP 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, CYP102 F87V and GFP in heart tissue 6 months following 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 per group, * $p<0.05$ compared to 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 per group, * $p<0.05$ compared to control.

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 to control group. **B)** Carotid artery pressure measured invasively in rats 6 months following injections; * $p<0.05$ compared to control group. **C**) Carotid artery pressure was measured invasively in rats 2 months following oral administration of C26. * $p<0.05$ compared to 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 per group, * $p<0.05$ compared to rAAV-CYP2J2 group. **F** and **G**) dP/dt max and cardiac output (CO) measured invasively in rats 6 months following injections; * $p<0.05$ compared to control group.

Fig. 3. The effects of rAAV-CYP2J2 and rAAV-CYP102 F87V treatments on aortic

contractility. **A)** Arterial elastance (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 to controls.

Fig. 4. The effects of rAAV-CYP102 F87V and rAAV-CYP2J2 treatments on heart remodeling. **A**) Ratio of whole heart weight to body weight, and **B**) Microscope images of cross section of ventricle to show size of cardiomyocytes (upper panel) and comparasion of cardiomyocyte diameter (lower panel), 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 to controls.

Figure 5. The effects of rAAV-CYP102 F87V and rAAV-CYP2J2 treatments on secretion of ANP and expression of ANP and NPR-C. **A)** Serum ANP in four groups. * p<0.05 compared to control groups.   **B and C)** Relative mRNA copy number of ANP relative to GAPDH 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 to control groups. **D)** Urinary cGMP levels. * p<0.05 compared to control groups. **E** and **F)** Western Blot of ANP expression in myocardium and relative density

of ANP band against beta-actin, showing that ANP level is significantly upregulated in myocardium in rAAV-CYP102 F87V- and rAAV-CYP2J2-treated rats. * p < 0.01 compared to controls.

Figure 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 (upper panel) and quantification of ANP-positive cells (lower panel). * p < 0.05 compared to controls; **C)** ANP concentration in media of cultured cardiomyocytes incubated with synthetic EETs (* p < 0.05 compared to control); D) Intracellular cGMP levels in cultured cardiomyocytes (* p < 0.05 compared to control); **E)** Representative western blot and relative density of ANP band: effects of inhibitors of MMP (1.10PH), HBEGF (CRM197) and EGFR (AG1478) on ANP expression in cultured cardiomyocytes; F) relative density of ANP band against beta-actin. # p<0.05 compared with control (CON); * p <0.01 compared with EET treatment.

Figure 1

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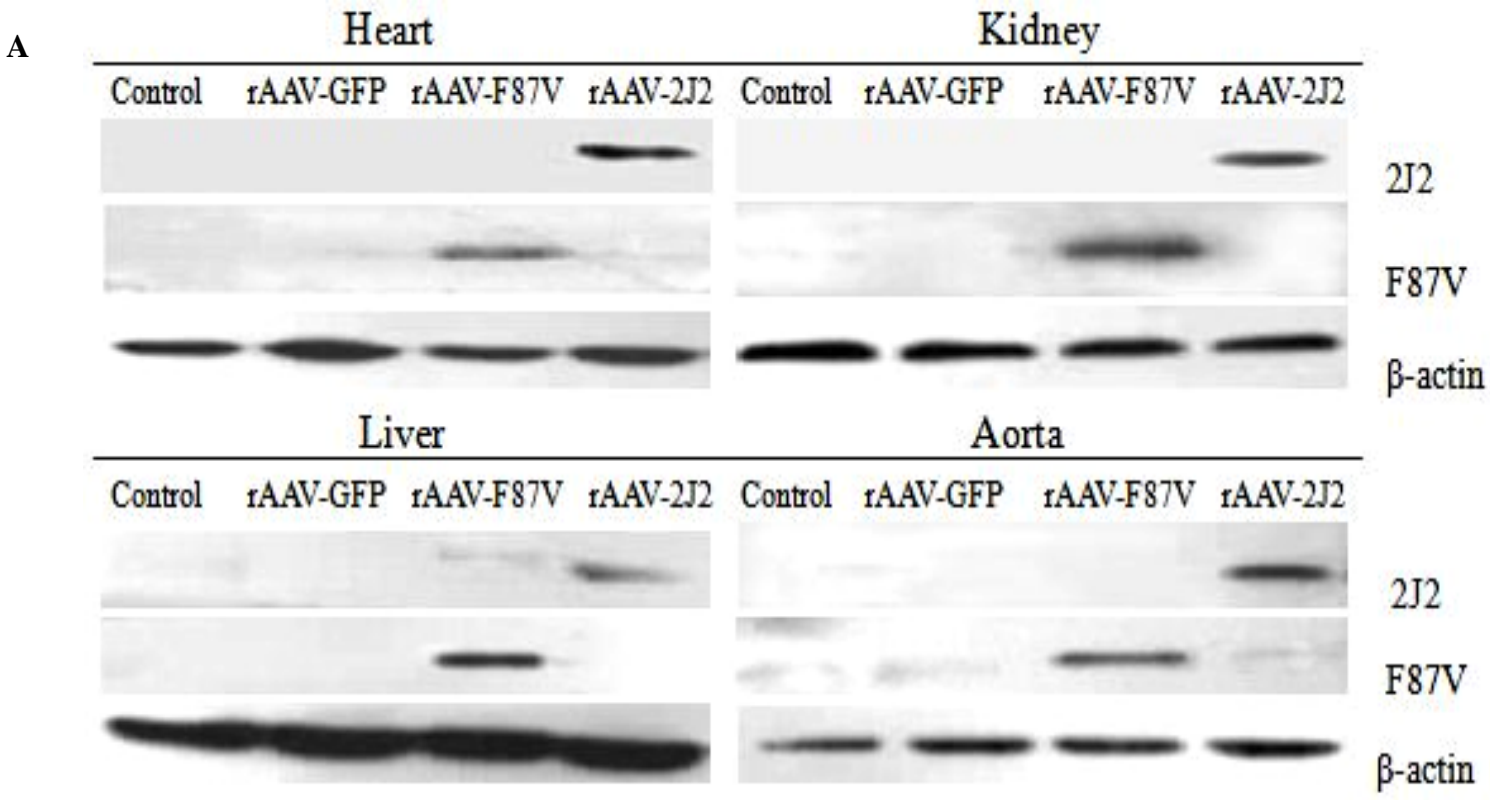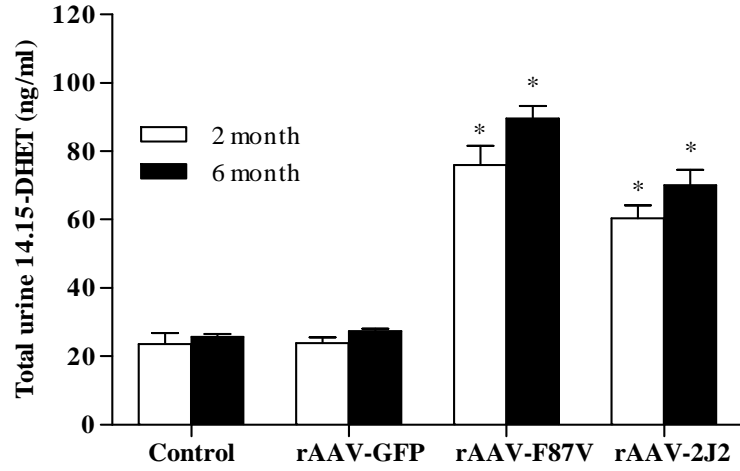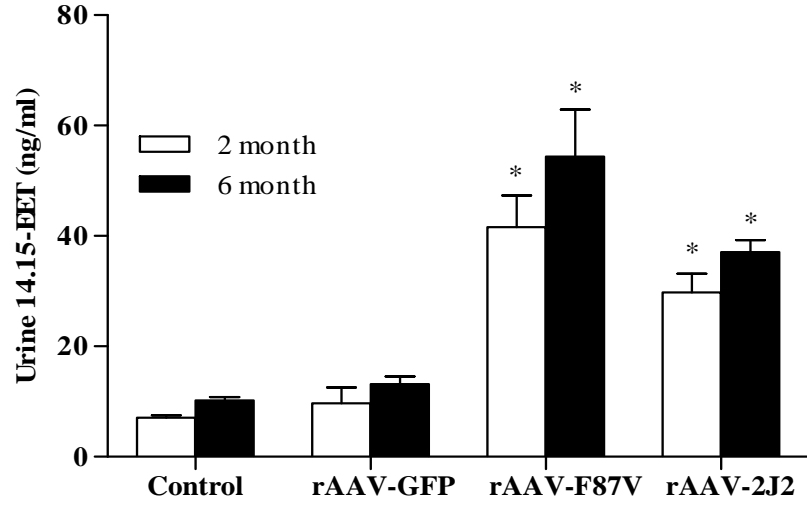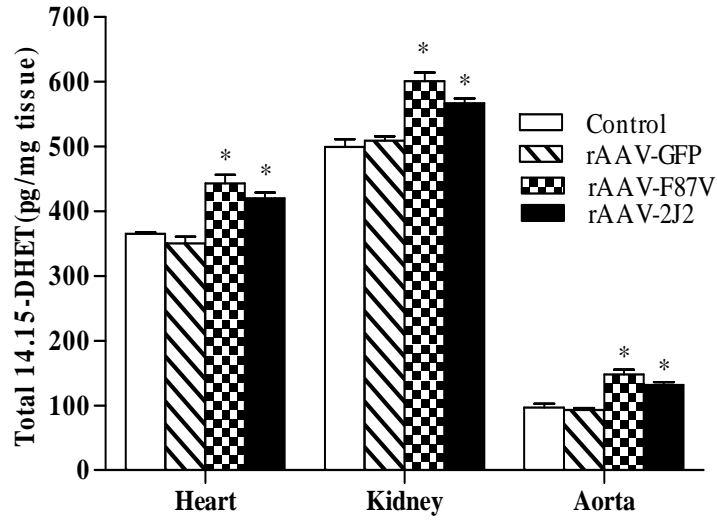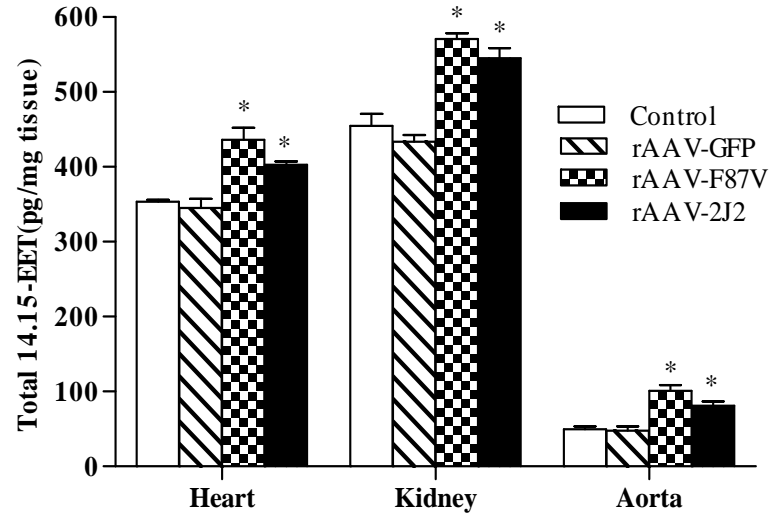
Figure 1

Figure 2

Figure 2

E

F

G

Figure 3

Figure 4

Figure 4

C

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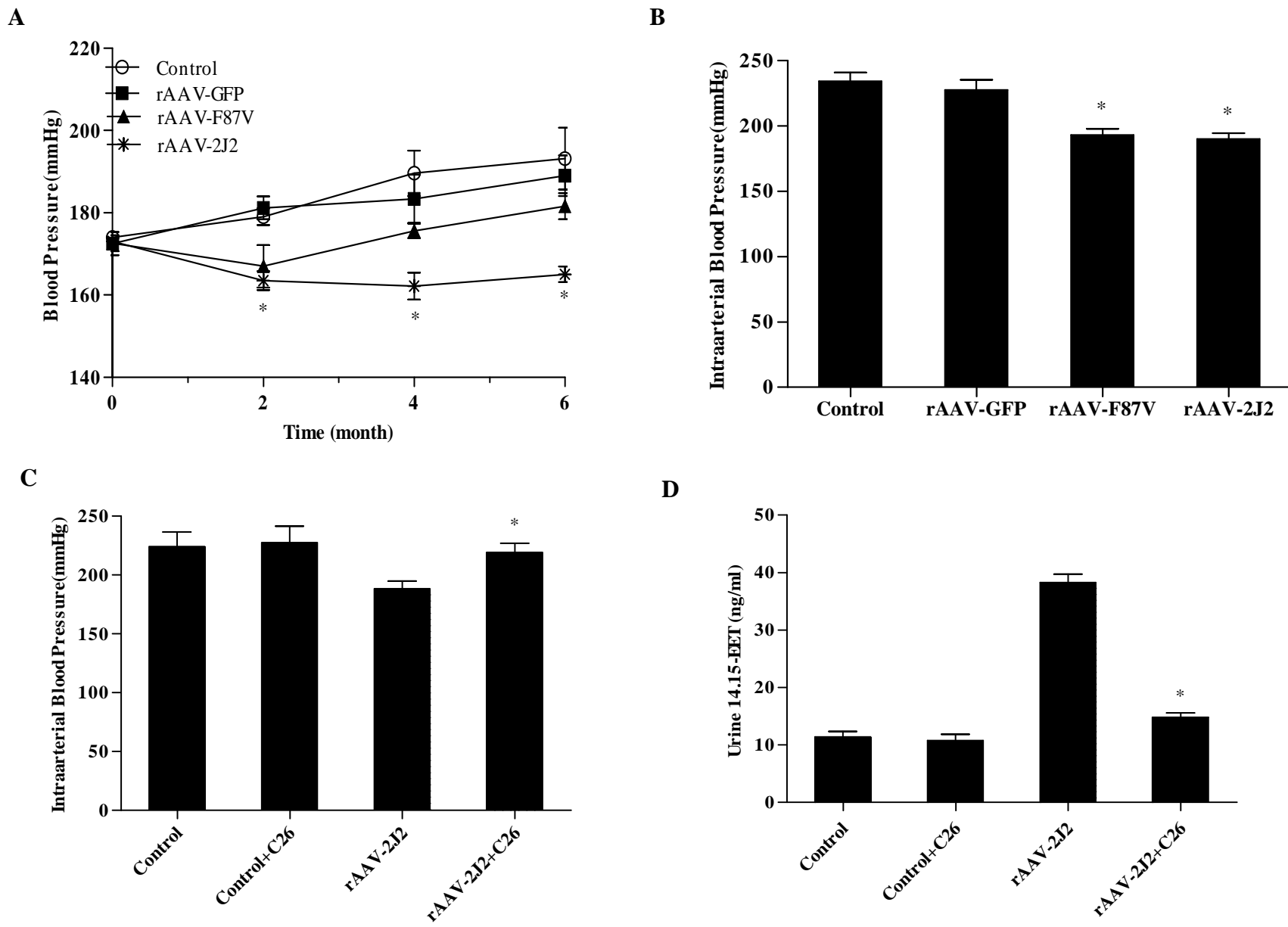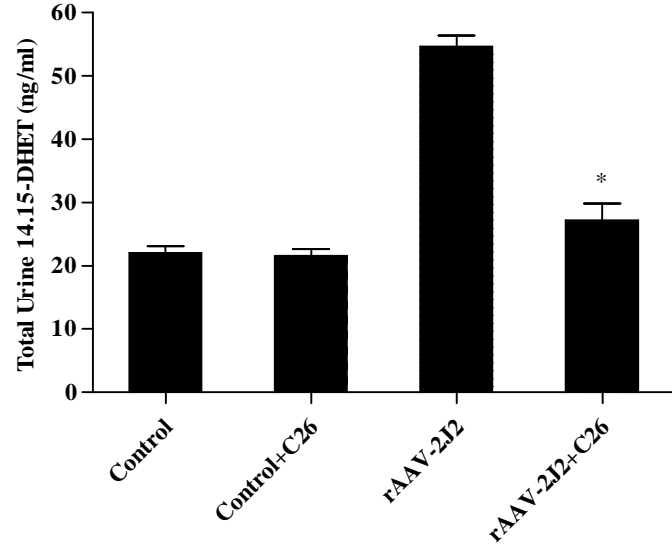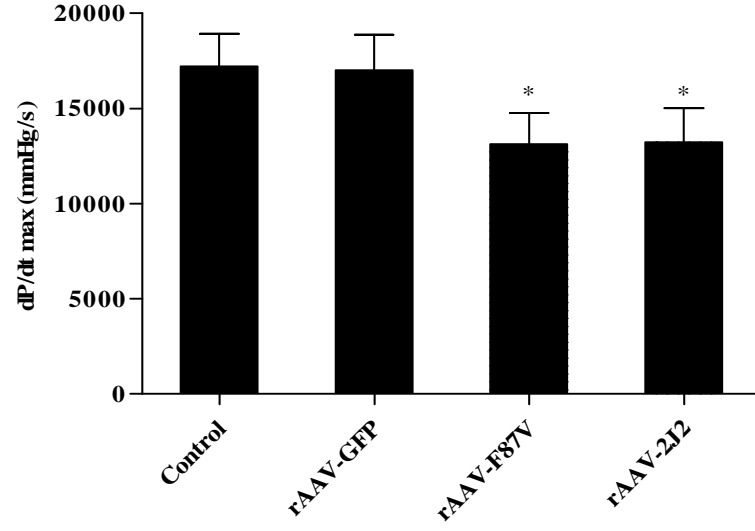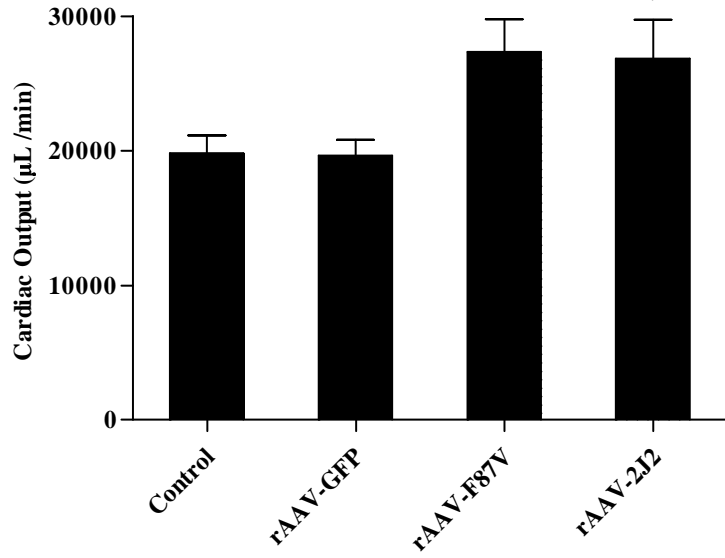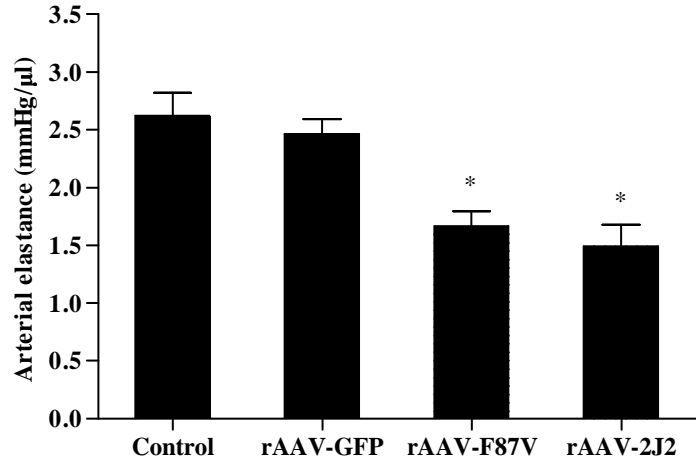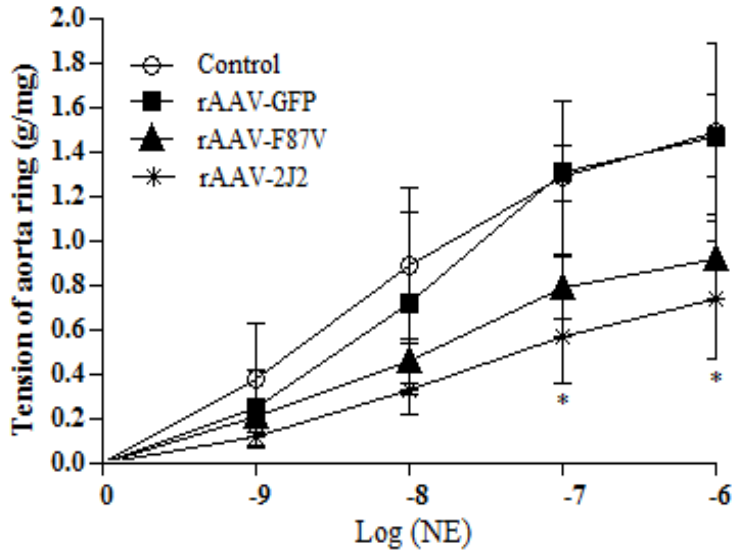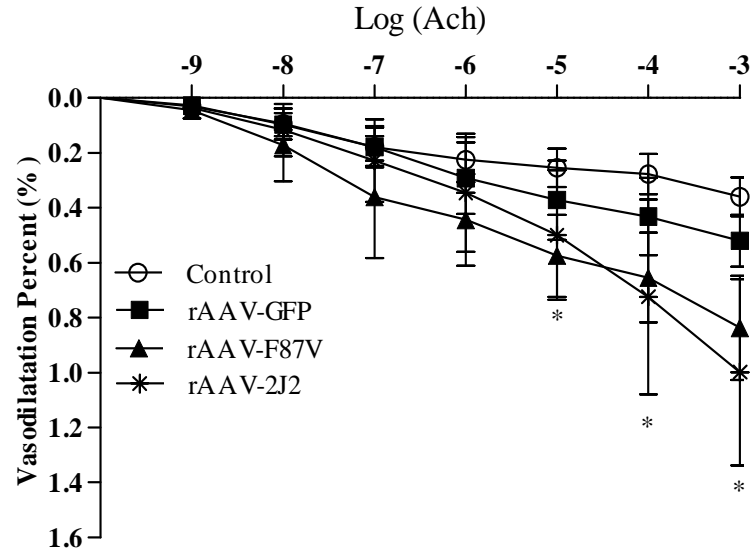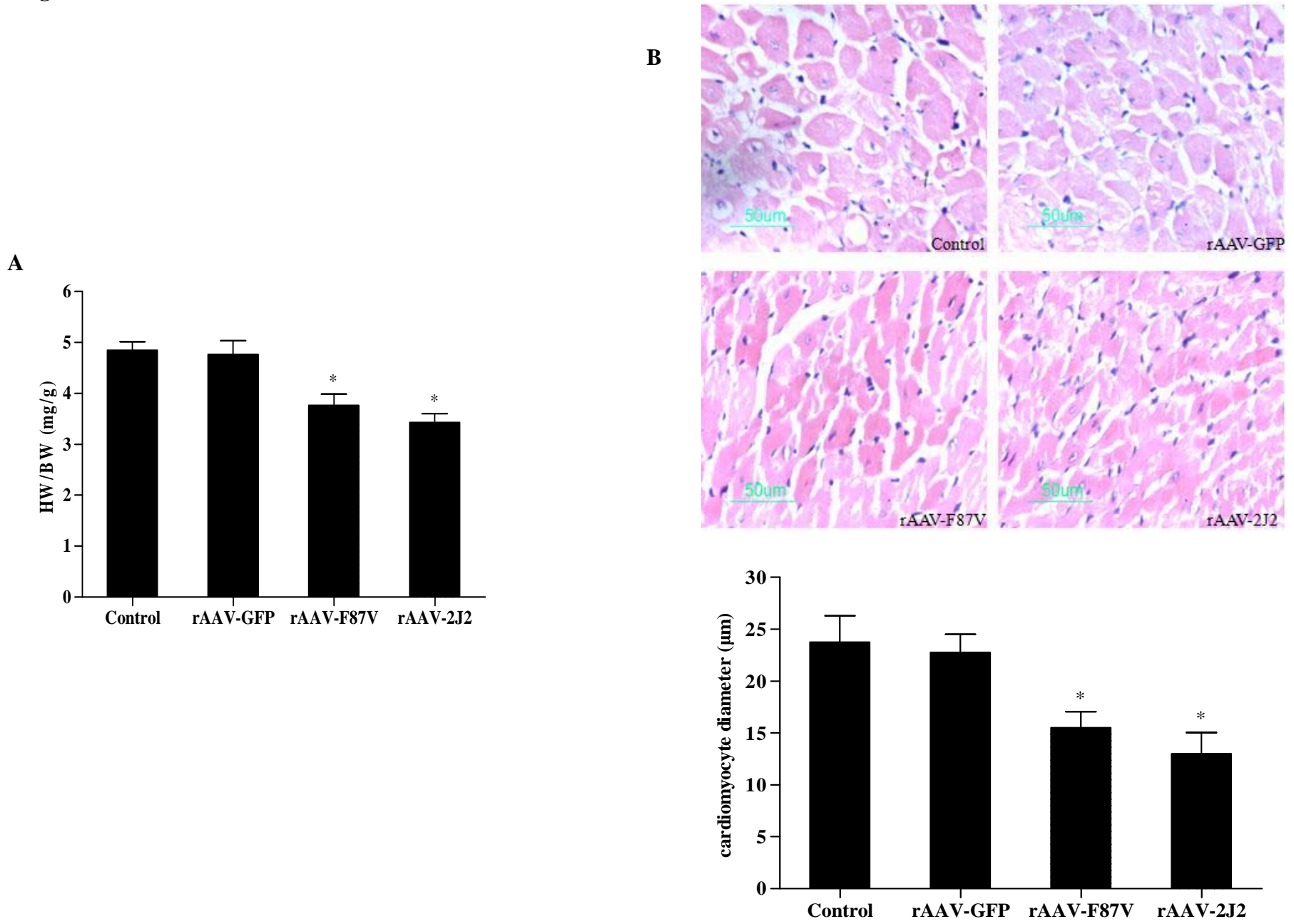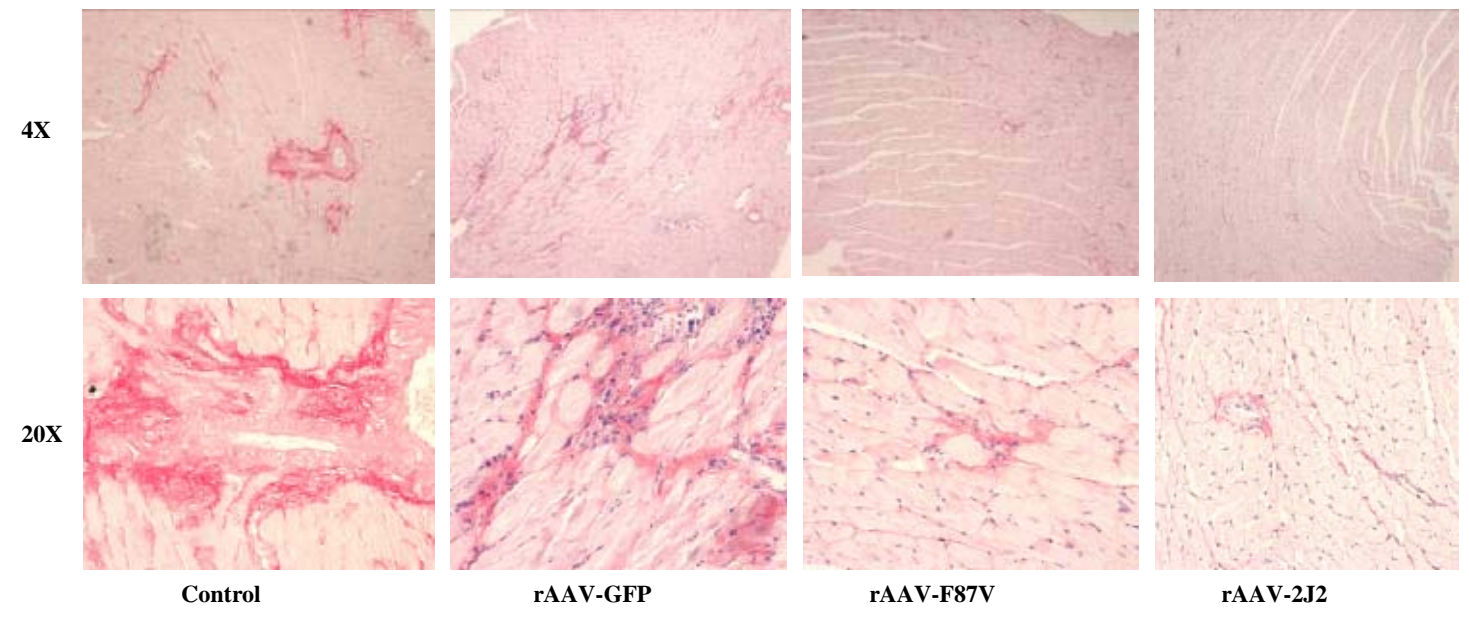
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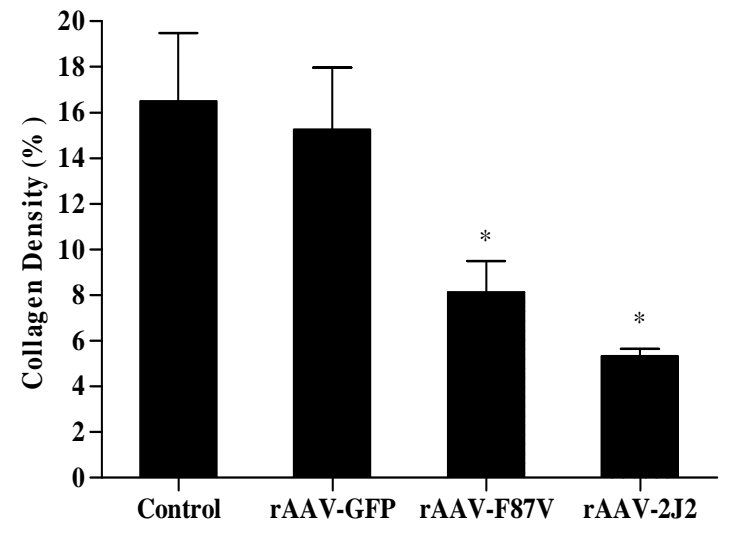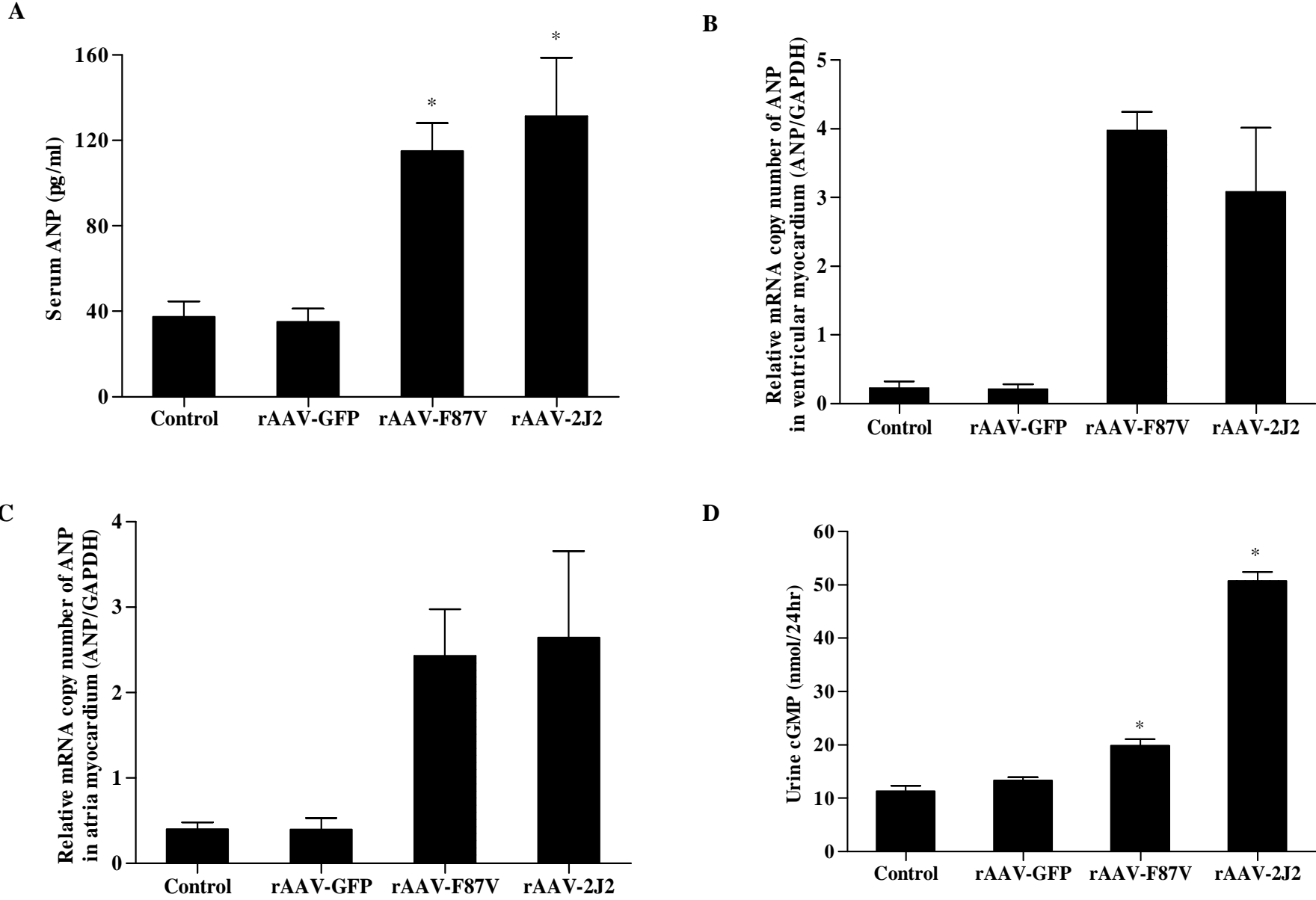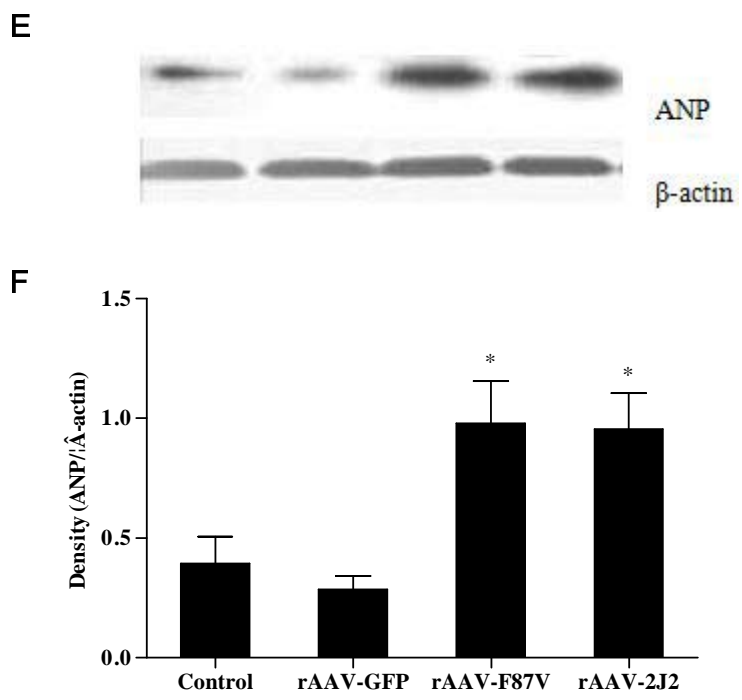
Figure 5

Figure 5

Figure 6

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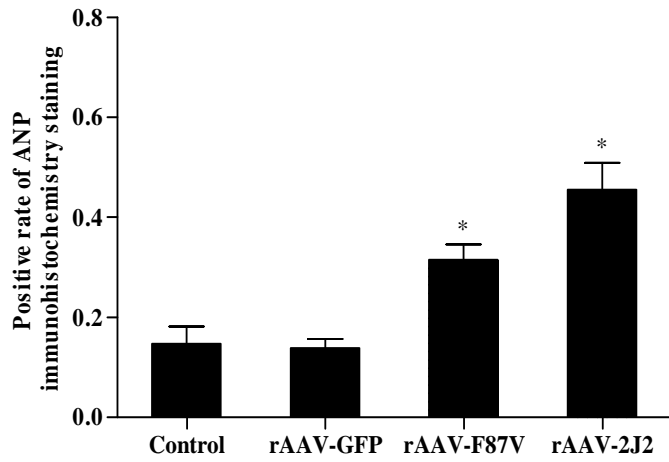
Figure 6

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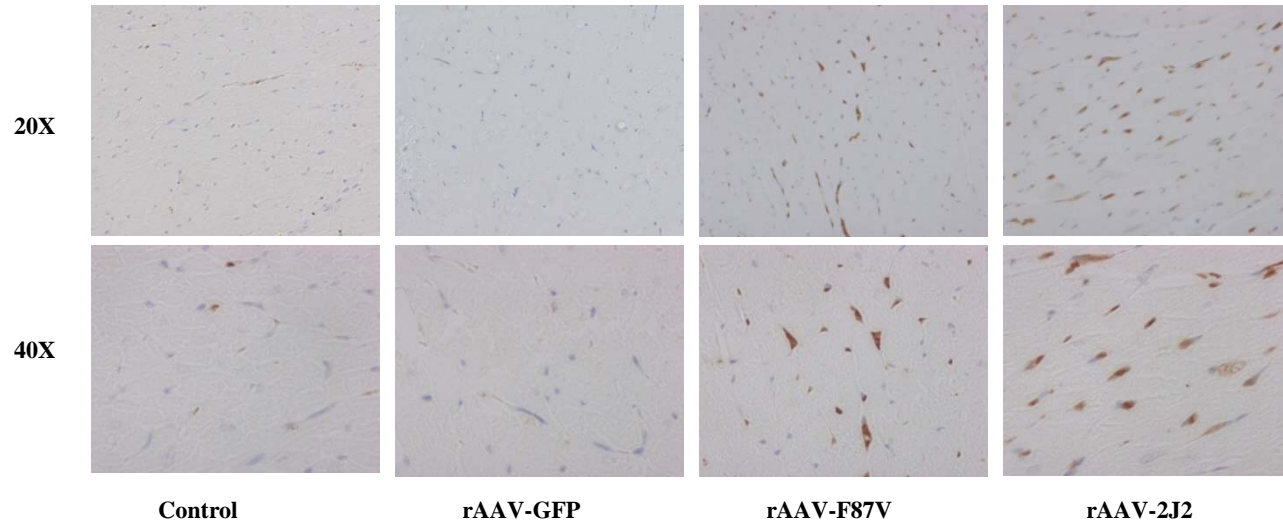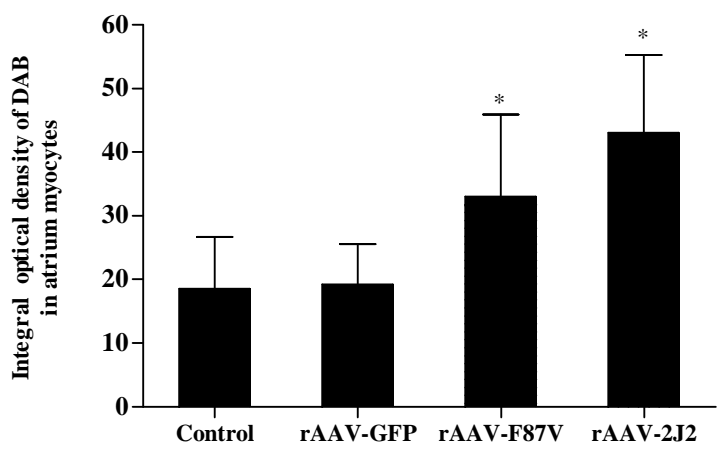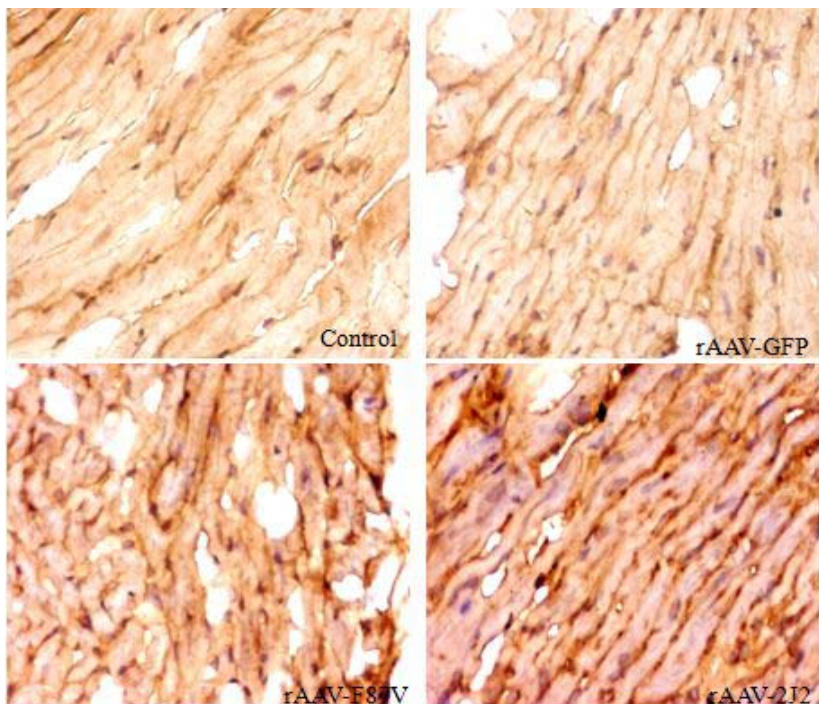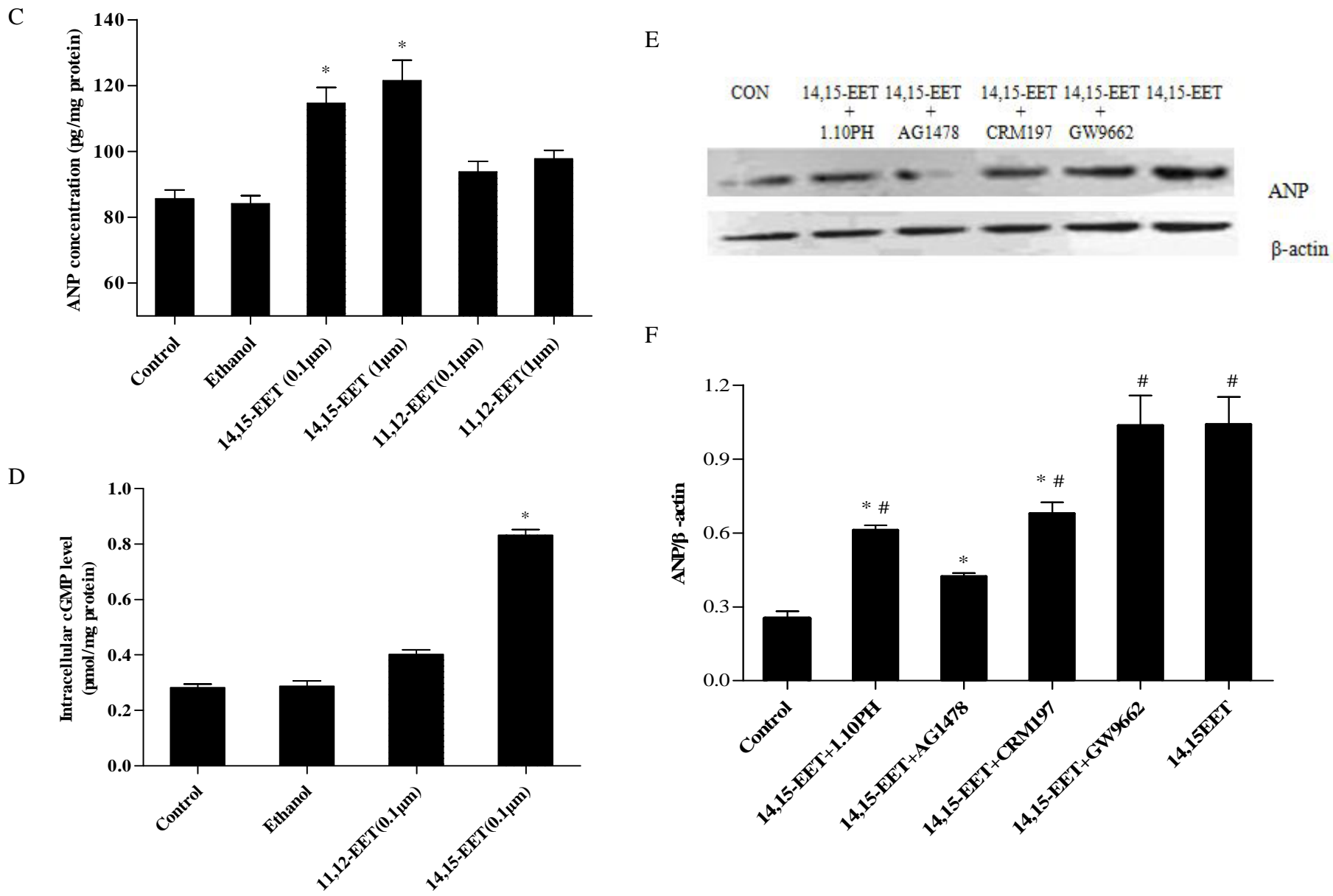**Figure 6**

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

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

Figure S1
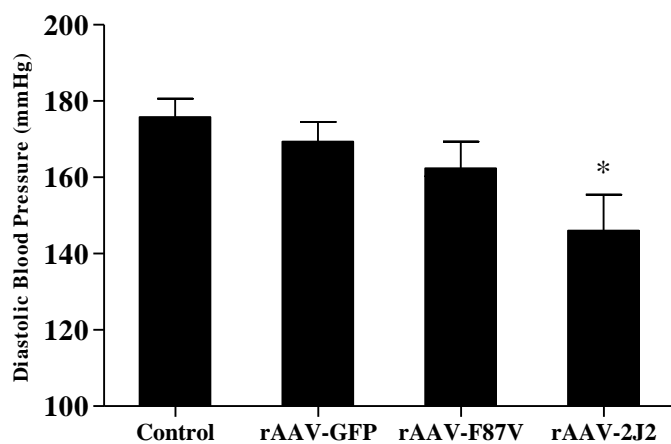
Figure S2. The effects of rAAV-CYP2J2 and rAAV-CYP102 F87V treatments on diastolic blood pressure recorded using intraarterial catheterization in SHR before sacrificing animals. * $p < 0.05$, compared with control.

Figure S2
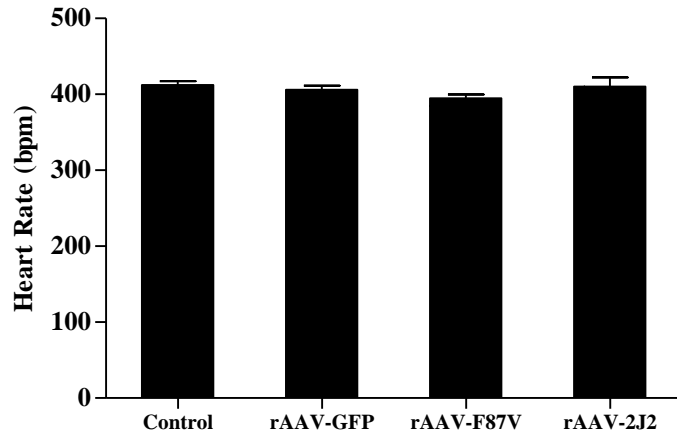
Figure S3. The effects of rAAV-CYP2J2 and rAAV-CYP102 F87V treatments on heart rate in SHR. The figure shows there is no significantly difference between groups.
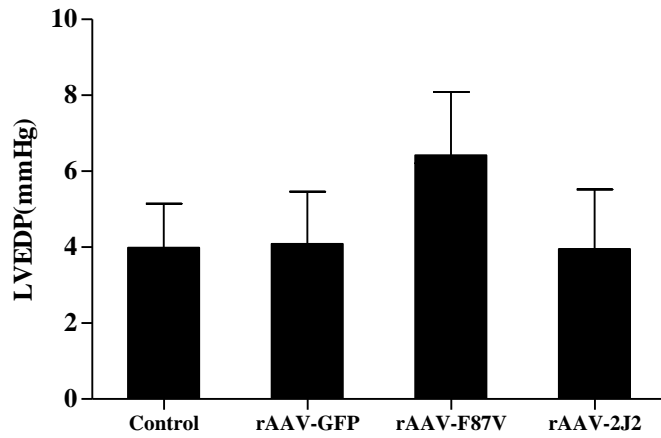
Figure S3

Figure S4. The effects of rAAV-CYP2J2 and rAAV-CYP102 F87V treatments on LVEDP of each group. There is no significantly difference between groups.
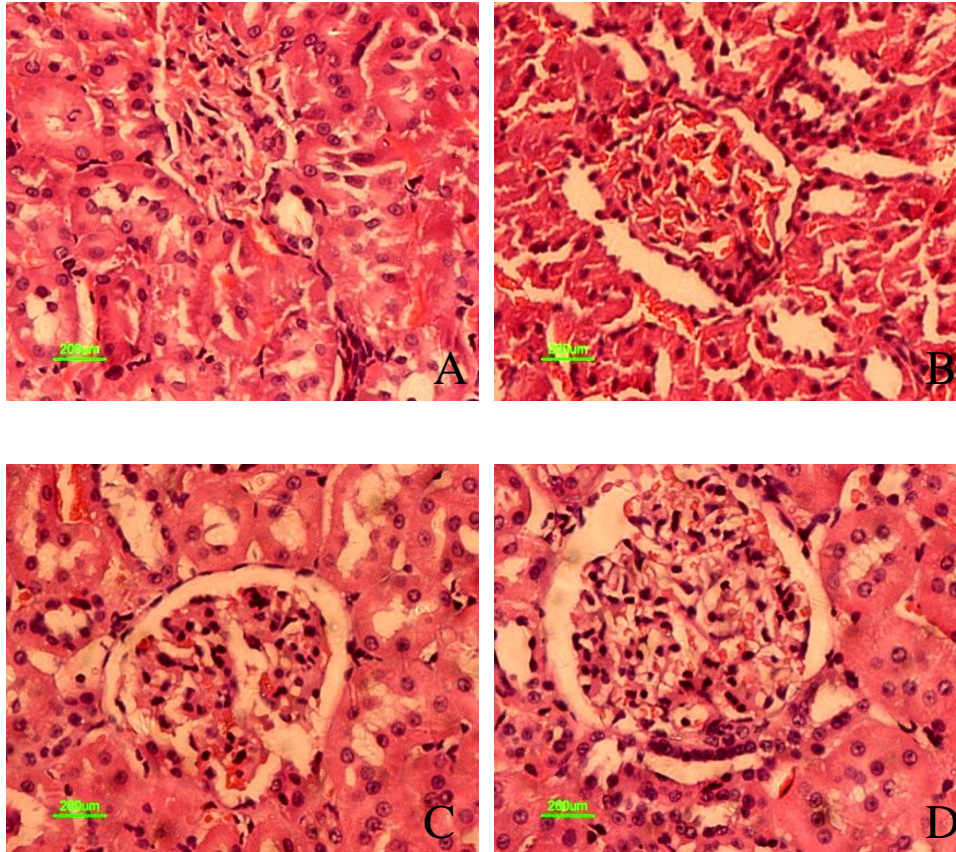
Figure S4

Figure S4. A lot of glomerulars and tubular are atrophied in control and GFP groups, but overexpreesion of epoxygenase reverse that in the 2J2 and F87V groups. A, Control; B, AAV-GFP; C, AAV-F87V; D, AAV-2J2
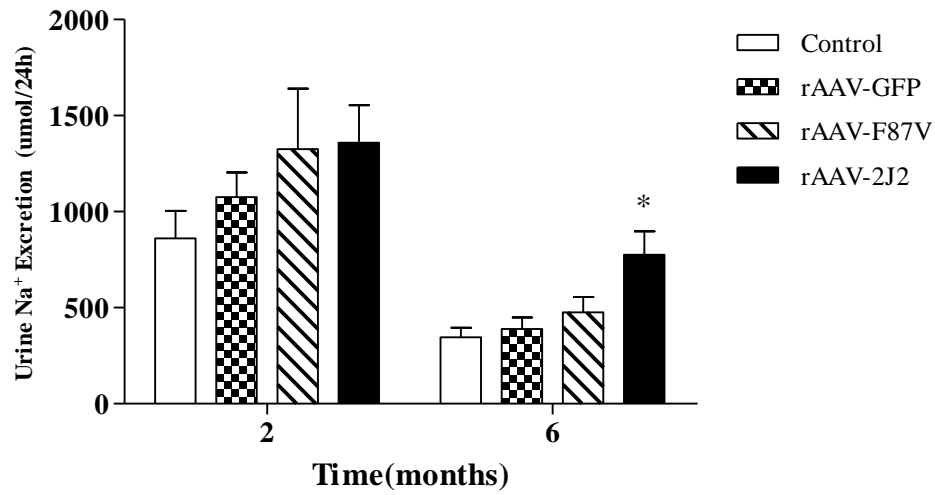
Figure S5

Figure S5. Effects of gene therapies on urinary Na$^+$ excretion. * indicates $p < 0.05$ compared with control and rAAV-GFP groups.
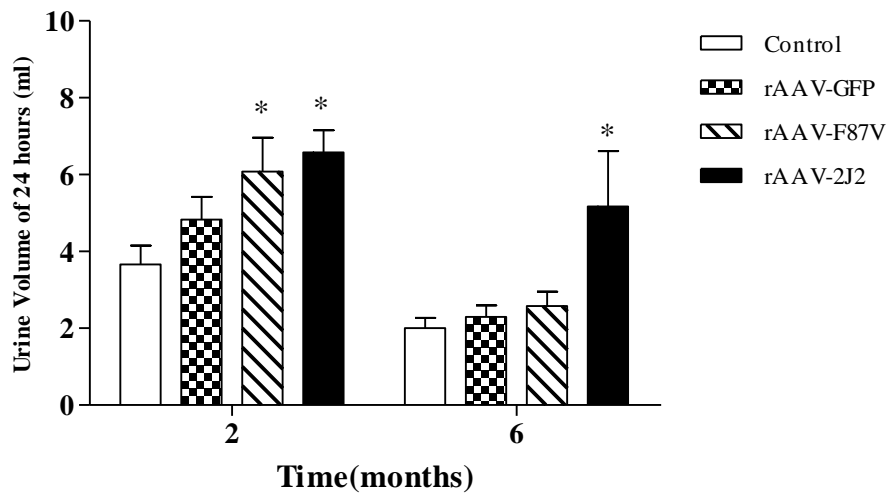
Figure S6

Figure S6. Effects of rAAV-CYPF87V and rAAV-CYP2J2 treatments on 24 hours urine volue. * indicates $p < 0.05$ compared with control and rAAV-GFP groups.
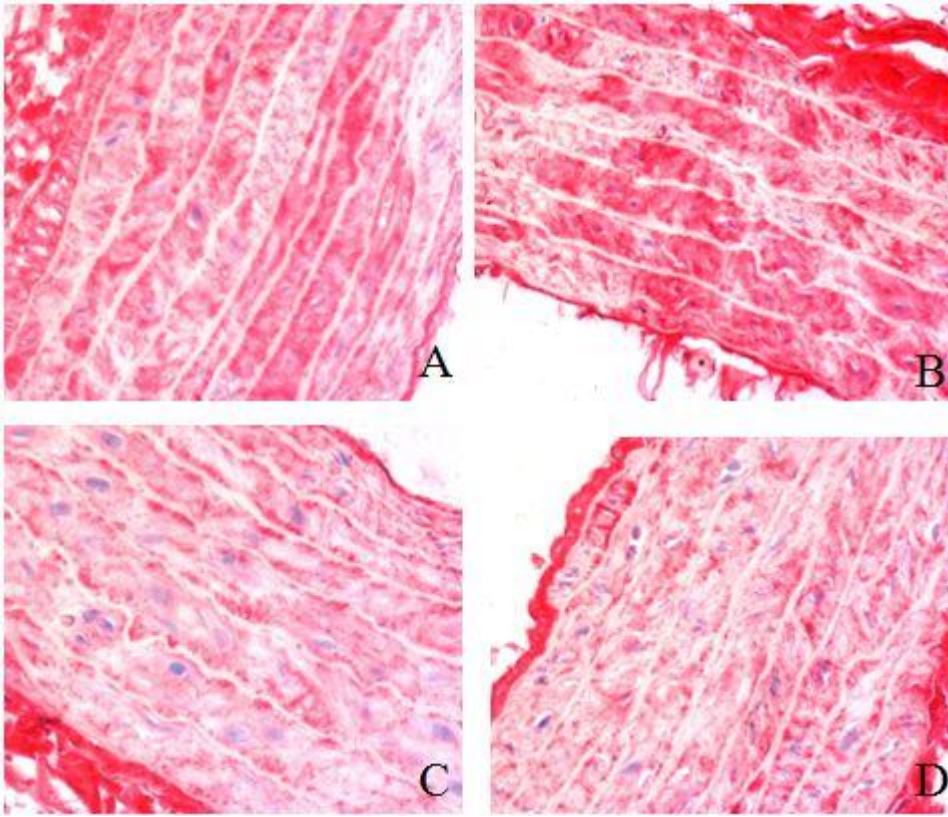
Figure S7

Figure S7. Artery collagen content assessed by Sirius red staining. A, Control; B, AAV-GFP; C, AAV-F87V; D, AAV-2J2.

Table S1

Primers		
ANP	forward 5'-CAAGGGCTTCTTCCTCTTCC-3'	
	reverse 5'-TCCAGGTGGTCTAGCAGGTTC-3'	
GAPDH	forward 5'-GGCAAGTTCAACGGCACAGTCA-3'	
	reverse 5'-CTCAGCACCAGCATCACCCCAT-3'	

Real-time PCR was carried out for 40 cycles each at 95°C for 5 sec, 60 °C for 5 sec and 72 °C for 20 sec. The relative values for ANP were calculated by dividing by the mean GAPDH value for each sample.

Table S2. The effects of rAAV-CYP2J2 and rAAV-CYP102 F87V treatments on cardiac hemodynamics 6 month after AAV injection.

Groups	Maximum Cardiac Pressure (mmHg)	End-Systolic pressure (mmHg)	dP/dt max (mmHg/s)	dP/dt min (mmHg/s)	Stroke Volume (µL)	Cardiac Output (µL /min)	Preload adjusted maximal power (mWatts/ µL^2)
Control	245.9 ±7.3	237.6 ±6.3	17208.3±1719.8	-18015.7 ±3564.3	96.3 ±6.7	19826.1 ±1299.6	18.4 ±2.9
AAV-GFP	236.5 ±5.3	228.1 ±4.3	17005.5±1850.8	-16930.8±2080.1	96.7 ±5.4	19644.5 ±1173.2	19.2 ±2.3
rAAV-F87V	214.9 ±7.0*	212.5 ±6.8*	13129.0±1625.4 *	-12788.5±3528.1 *	139.8 ±12.4*	27369.5 ±2394.6*	7.5 ±0.8*
rAAV-2J2	201.8 ±14.7*	198.9 ±14.9*	13223.4±1792.4 *	-12258.1±4155.6 *	137.9 ±9.4*	26870.7 ±2876.2*	11.0 ±2.1*

* $p < 0.05$ compared to controls.