Neuronal Angiotensin II Type 1 Receptor Upregulation in Heart Failure

Activation of Activator Protein 1 and Jun N-Terminal Kinase


Abstract—Chronic heart failure (CHF) is a leading cause of mortality in developed countries. Angiotensin II (Ang II) plays an important role in the development and progression of CHF. Many of the important functions of Ang II are mediated by the Ang II type 1 receptor (AT,R), including the increase in sympathetic nerve activity in CHF. However, the central regulation of the AT,R in the setting of CHF is not well understood. This study investigated the AT,R in the rostral ventrolateral medulla (RVLM) of rabbits with CHF, its downstream pathway, and its gene regulation by the transcription factor activator protein 1 (AP-1). Studies were performed in 5 groups of rabbits: sham (n=5), pacing-induced (3 to 4 weeks) CHF (n=5), CHF with intracerebroventricular (ICV) losartan treatment (n=5), normal with ICV Ang II treatment (n=5), and normal with ICV Ang II plus losartan treatment (n=5). AT,R mRNA and protein expressions, plasma Ang II, and AP-1–DNA binding activity were significantly higher in RVLM of CHF compared with Sham rabbits (240.4±30.2%, P<0.01; 206.6±25.8%, P<0.01; 280±36.5%, P<0.05; 207±16.4%, P<0.01, respectively). Analysis of the stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) pathway showed that phosphorylated c-Jun proteins, phosphorylated JNK proteins, and JNK activity increased significantly in RVLM of CHF compared with sham (262.9±48.1%, 213.8±27.7%, 148.2±10.1% of control, respectively). Importantly, ICV losartan in CHF rabbits attenuated these increases. ICV Ang II in normal rabbits simulated the molecular changes seen in CHF. This effect was blocked by concomitant ICV losartan. In addition, Ang II–induced AT,R expression was blocked by losartan and a JNK inhibitor, but not by extracellular signal-regulated kinase or p38 MAP kinase inhibitors in a neuronal cell culture. These data suggest that central Ang II activates the AT,R, SAPK/JNK pathway. AP-1 may further regulate gene expression in RVLM in the CHF state. (Circ Res. 2006;99:1004-1011.)

Key Words: angiotensin II □ sympathetic nerve activity □ chronic tachycardia

Chronic heart failure (CHF) is a leading cause of mortality in developed countries. CHF is characterized, in part, by activation of the renin/angiotensin/aldosterone system and the sympathetic nervous system. Excessive activity of the sympathetic nervous system contributes to the development and progression to CHF in patients.1,2 In experimental animals, it has been well established that the renin/angiotensin system is markedly activated and angiotensin II (Ang II) is elevated in moderate to severe CHF.3–5

Ang II is considered to be a prime candidate in the regulation of sympathetic outflow in the CHF state, because Ang II can alter sympathetic function at several sites from the central nervous system to the periphery.6,7 Our laboratory has clearly demonstrated that rabbits with pacing-induced CHF exhibit elevated plasma Ang II compared with sham rabbits.8,9 We have also shown that central blockade of the Ang II type 1 receptor (AT,R) reduces sympathetic nerve activity and increases baroreflex function in the CHF state.10–12 DiBona and colleagues reported similar results in rats with chronic myocardial infarction induced CHF.13–15 Suppression of the AT,R gene in the brain using antisense techniques reduces resting sympathetic nerve activity in rats with CHF but not in sham rats.16

Complementary to the above evidence, in previous studies, we observed that the AT,R was upregulated in the rostral ventrolateral medulla (RVLM) of rabbits with CHF.9 Intracerebroventricular (ICV) Ang II given to normal rabbits exhibits an upregulation of AT,R expression and an increase in sympathetic outflow. Losartan, however, normalized these changes.9,17

It is well known that Ang II activates p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated protein kinase (ERK), and stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK), which are critical protein kinases for cell growth, cell death, and gene expression.18 Ang II has been shown to activate both p38 MAPK19 and JNK20 in cultured vascular smooth muscle cells (VSMCs). p38 MAPK positively regulates VSMC growth induced by
Ang II, whereas JNK was activated in a balloon-injured artery and could be inhibited by an AT1R antagonist. It has also been well established in other studies that JNK is involved in the activation of the transcription factor AP-1. Although these pathways have been relatively well studied in VSMC and cardiac myocytes, there has been little investigation of this important Ang II signaling pathway in neurons that control sympathetic outflow, especially in the CHF state.

We hypothesized that based on data in the peripheral circulation, AT1R expression in central cardiovascular neurons would be regulated by the JNK pathway and that AP-1 plays a major role in AT1R gene transcription. The current study examined the relationship between plasma Ang II and the expression of AT1R in CHF. We further investigated the intracellular mechanism for the upregulation of the AT1R by Ang II.

### Materials and Methods

#### Animals

Experiments were performed on 25 male New Zealand White rabbits weighing between 3.2 and 4.1 kg. These experiments were reviewed and approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and conformed to the Guidelines for the Care and Use of Experimental Animals of the American Physiological Society and the NIH. Rabbits were assigned to 5 different groups (n=5 per group). These groups were sham, pacing-induced CHF, CHF plus intracerebroventricular (ICV) infusion of losartan, sham plus ICV Ang II, and sham plus ICV Ang II and losartan.

#### Cell Culture

A neuronal cell line (CATH.a) was purchased from American Type Culture Collection and grown in RPMI 1640 containing 8% horse serum, 4% FBS, 100 IU/L penicillin, at 37°C in 95% air and 5% CO2 in a humidified atmosphere. For experiments, cells were plated on polystyrene tissue culture dishes at a density of 1×10⁵ cells/100-mm plate, or 1.5×10⁶ cells/well in 6-well culture plates. After subculture, cells were allowed to grow for 2 days and then treated with Ang II (100 nmol/L), an AT1 receptor blocker (losartan, 10 μmol/L), a JNK inhibitor (SP600125, 10 μmol/L), a p38 inhibitor (SB202190, 10 μmol/L), or an ERK inhibitor (PD98059, 10 μmol/L).

#### Induction of CHF

CHF was induced by chronic ventricular tachycardia, as previously described. Sham animals were prepared identically to CHF animals but were not paced.

#### Chronic ICV Infusion

In the ICV infusion groups, a 19-gauge cannula was implanted into a lateral cerebral ventricle as previously described. An osmotic minipump (Model 2001, Durect Corp) filled with Ang II 100 ng/mL per hour or losartan 30 μg/μL per hour in artificial cerebrospinal fluid was implanted subcutaneously in the back of the neck and connected to the ICV cannula. The infusion was continued for 6 days.

#### Cardiac Function, Arterial Pressure, Heart Rate, Left Ventricular Pressure, and Ejection Fraction

Cardiac function was measured by echocardiography (Acuson Sequoia 512C), with the rabbits hand-held in the conscious state. Arterial pressure was measured with a radiotelemetry unit (Data Sciences International). Left ventricular (LV) pressure was measured with a Millar transducer (Model SPR-524, Millar Instruments Inc). Details of the procedure can be found in the online data supplement at http://circres.ahajournals.org.

#### Preparation of RVLM Tissue

At the end of the experiment, the rabbits were killed with pentobarbital sodium. The brain was removed and immediately frozen on dry ice, blocked in the coronal plane, and sectioned at 300-μm thickness in a cryostat. The RVLM was punched according to the method of Palkovits and Brownstein for analysis of mRNA and protein of the AT1R receptor and other molecular studies.

#### Plasma Ang II Radioimmunoassay

Ang II peptide in rabbit plasma was measured using a radioimmunoassay (RIA). The protocol for Ang II RIA was modified from that described by Raff et al to increase the recovery rate and sensitivity. Details of the procedure can be found in the online data supplement.

#### RNA Extraction, cDNA Synthesis, and Real-Time PCR

Total RNA from 3-mm punches of RVLM or CATH.a cells was isolated using the RNeasy kit (Qiagen). cDNA was generated using the iScript cDNA Synthesis Kit (Bio-Rad). Gene-specific primers and probes are listed in the Table 1 and were synthesized in the University of Nebraska Medical Center Eppeley DNA Synthesis Core Facility. Gene-specific probes were labeled with carboxyfluorescein (FAM) at the 5’ site with Black Hole Quenchers at the 3’ site to add specificity and sensitivity (Glen Research). β-Actin was used as an internal control for calculation of relative expression levels of target genes in the rabbit studies. GAPDH was used as an internal control for calculation of relative expression levels of target genes in CATH.a cells. Both β-actin and GAPDH are commonly used as internal controls in Ang II studies by others. Real-time PCR was performed by using HotStarTaq DNA polymerase (Qiagen) on CHROMO4 Continuous Fluorescence Detector (Bio-Rad). Analysis of relative gene expression was based on the method described by Livak and Schmittgen.

#### Western Blot Analysis

Homogenates were prepared from RVLM. Protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, II). Details of the procedure can be found in the online data supplement.

#### SAPK/JNK Activity Assays

SAPK/JNK activity were measured by using the ASPK/JNK assay kit from Cell Signaling Technology. Details of the procedure can be found in the online data supplement.

### Table 1. Gene-Specific Primers and Probes

<table>
<thead>
<tr>
<th>Forward Primers</th>
<th>Reverse Primers</th>
<th>Probes</th>
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<tr>
<td>Rabbit α-actin (AF309819)</td>
<td>CTCAGGTTACCCACGAC</td>
<td>GGAACGTGGTCTTTCCTCTC</td>
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<tr>
<td>Rabbit AT1R (S59041)</td>
<td>CATCAGAAGACCAAAATATC</td>
<td>GATCGAGAAAGGGATTAGGAAG</td>
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<tr>
<td>Rabbit c-Jun (AJ291306)</td>
<td>GTTTTTCACTTTTTCCTCTA</td>
<td>AGTCTCAGGAAGGGATCA</td>
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<tr>
<td>Mouse AT1R</td>
<td>GTTCTTCTGCTCATGGTCT</td>
<td>GATGATGCAGGTGACTTTS</td>
</tr>
<tr>
<td>Mouse GAPDH</td>
<td>ACCAATTTTGGCATTGGCAA</td>
<td>GTGACAGGGATGATGTCTG</td>
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Electrophoretic Mobility-Shift Assay

Nuclear extracts of rabbit brain tissues were prepared with the NE-PER nuclear extraction reagent (Pierce). Details of the procedure can be found in the online data supplement.

Statistics

Data are expressed as mean ± SEM. All statistical analyses were performed by a 1-way analysis of variance (ANOVA) for repeated measurements using SigmaStat (SPSS, Chicago, Ill). If significance was found between groups post hoc analyses were performed using the Bonferroni correction. P < 0.05 was considered significantly different.

Results

Body Weight, Heart Weight, Hemodynamics, and Echocardiographic Data

Table 2 shows the values for body weight, ratio of organ weight to body weight, hemodynamics, and echocardiographic data in the rabbits from the 5 groups studied. The CHF group exhibited a significantly higher ratio of wet lung weight to body weight, heart rate, LV systolic diameter, LV end-diastolic diameter, plasma Ang II, and a significantly lower ejection fraction and cardiac output compared with the normal group. No significant differences in these parameters were found between CHF and CHF plus ICV losartan. There was a trend for MAP to increase in response to ICV Ang II infusion in sham animals, but this did not reach statistical significance.

<table>
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<th>TABLE 2. Baseline Hemodynamic Parameters in CHF and Sham Rabbits</th>
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<td>BW, kg</td>
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<tr>
<td>LVSD, mm</td>
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<tr>
<td>Ejection fraction</td>
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<td>Ang II, pg/mL</td>
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</table>

Values are mean ± SEM. BW indicates body weight; HW, heart weight; WLW, wet lung weight; DLW, dry lung weight; MAP, mean arterial pressure; LVSP, LV systolic pressure; LVEDP, LV end-diastolic pressure; LVSD, LV systolic diameter; LVEDD, LV end-diastolic diameter. *P < 0.05 vs sham group.

AT₁R Expression

AT₁R protein and mRNA expression in the RVLM of the CHF group were significantly higher compared with sham (Figure 1). Animals with CHF exhibited a 240.4 ± 50.2% increase in AT₁R mRNA (P < 0.01) and a 206.6 ± 25.8% (P < 0.01) in AT₁R protein. Following 6 days of ICV losartan infusion to CHF rabbits, AT₁R protein and mRNA expressions were normalized. A similar increase in AT₁R protein and mRNA expressions was observed when normal rabbits were infused with ICV Ang II for 6 days. In Ang II–infused normal rabbits that were given losartan the increase in AT₁R expression was inhibited (Figure 1). These data strongly suggest that Ang II plays an important positive feedback role in AT₁R upregulation in CHF.

Increases of AP-1–Binding Activity in CHF and in Response to ICV Ang II Infusion

Binding activity of the transcription factor AP-1 was increased significantly in CHF rabbits and in sham rabbits subjected to ICV infusion of Ang II compared with sham rabbits (Figure 2A). Administration of ICV losartan to CHF and Ang II–infused sham rabbits inhibited AP-1 activity compared with their respective controls but were not different compared with sham rabbits (Figure 2A).

The specificity of the DNA/protein complex was characterized by competitive and supershift assays. The binding to the AP-1–specific oligonucleotide was inhibited in the presence of unlabeled oligonucleotides with the same sequence. The addition of anti–c-Jun antibody to the binding reaction produced a supershift complex as shown in Figure 2B. These data suggest that more active c-Jun transcription factors are available in CHF and Ang II–infused rabbits to bind to the AP-1 sequence.

Increases of c-Jun mRNA and Phosphorylated c-Jun in CHF and ICV Ang II infusion

To determine the role of c-Jun in transcriptional regulation of the AT₁R, we measured c-Jun activation in punches of RVLM. The AP-1 transcription factor is a dimer of Fos and Jun or a dimer of the members of the Jun family. To activate AP-1 transcription, both components of AP-1 have to be phosphorylated. We measured phosphorylated c-Jun as an indicator of activation of AP-1. As shown in Figure 3, c-Jun mRNA expressions and phosphorylated c-Jun level increased significantly in CHF and Ang II–infused groups compared with sham. Losartan inhibited c-Jun activation in both CHF...
and Ang II–infused groups (Figure 3). These data strongly suggest that c-Jun as a monomer of the AP-1 dimer is activated in CHF and that Ang II and its binding to the AT1R may be responsible for AP-1 activation.

**Activation of JNK in CHF and in Response to ICV Ang II Infusion**

We measured phosphorylated JNK to determine whether the JNK pathway was necessary for AP-1 activation and AT1R upregulation. As shown in Figure 4, phosphorylated JNK and JNK activities increased significantly in CHF and Ang II–infused groups compared with sham rabbits. Again, losartan reduced this response. These data suggest that increased phospho-JNK may be responsible for increased levels of phospho-c-Jun and thereby increased AP-1 activity in CHF.

**Ang II–Induced Stimulation of AT1R mRNA Through JNK but Not p38 or ERK**

To further explore the mechanism of AT1R upregulation in CHF, we used a neuronal cell line to investigate the specific pathway for AT1R mRNA transcriptional regulation. Treatment of CATH.a cells with Ang II (100 nmol/L) for 0 to 24 hours at 37°C produced a time-dependent increase in AT1R mRNA expression (Figure 5A), which peaked at 6 hours. The effect of various agents on AT1R mRNA expression in response to Ang II (100 nmol/L) was examined after a 3-hour incubation (Figure 5B). Pretreatment with the AT1R antagonist losartan (10 μmol/L, 1 hour) inhibited the response to Ang II. Pretreatment with the JNK inhibitor, SP 600125 (10 μmol/L, 1 hour) also inhibited AT1R expression. The increase in AT1R mRNA expression in Ang II–treated cells was not blocked by pretreatment with the p38 inhibitor (SB 202190, 10 μmol/L), or the ERK inhibitor (PD 98059, 10 μmol/L). These data suggest that AT1R transcription in CATH.a cells occurs downstream of JNK activation but not downstream of p38 or ERK activation.
The main finding in this study is that the AT1R is upregulated in the RVLM of rabbits with CHF and that the upregulation depends on the increase in the transcription factor AP-1, which is activated by the SAPK/JNK pathway but not by other MAPK pathways. This conclusion is drawn from the following observations. (1) AT1R protein and mRNA expressions were increased in the RVLM of CHF rabbits. ICV losartan normalized AT1R up regulation. ICV infusion of Ang II in normal rabbits mimicked the change in AT1R expression in the CHF state, even though there were no changes in hemodynamic parameters. (2) An increase in AP-1–DNA binding indicated that AP-1 activity increases in the regulation of AT1R gene transcription in CHF. (3) The AP-1 upstream signaling pathway consisting of phosphorylated JNK and phosphorylated c-Jun expression which were also increased in CHF and ICV Ang II–infused rabbits. (4) In a neuronal cell culture, Ang II–stimulated AT1R mRNA expression. Losartan and a JNK inhibitor blocked Ang II–induced AT1R expression; however, inhibition of ERK or p38 had no effect.
Increasing evidence indicates that the activation of the AT1R occurs in diverse tissues and mediates the pathogenesis of various diseases. Ang II has been reported to rapidly cause downregulation of its receptor. Binding studies indicate that Ang II receptor density in the glomerulus and peripheral vasculature is decreased under conditions of elevated circulating Ang II and is increased when plasma Ang II is low. However, chronic increases in peripheral or central Ang II are associated with a gradually developing hypertension, which would require continued expression of the AT1R. Porter found that 1 week of ICV Ang II infusion produced a significant increase in brain AT1R in young rats, which was thought to play a role in the development of cardiovascular control mechanisms. Moellenhoff et al showed an increase in AT1R receptor number in the hypothalamus of rats after repetitive stimulation with Ang II. Cheng et al found that Ang II upregulated AT1R in the renal proximal tubule which had a significant impact on sodium reabsorption.

In this study, we observed that AT1R expression in the RVLM was significantly higher in CHF than in sham rabbits. Zhu et al observed AT1R expression increased in the paraventricular nucleus (PVN) of CHF rats. Previous data from this laboratory showed that an increase in AT1R expression in RVLM correlated with an increase in renal sympathetic activity and that chronic ICV losartan reduced sympathetic nerve activity and normalized the AT1R increase in CHF. In the present study, ICV losartan reversed the changes in AT1R expression and its signaling pathways in CHF. The changes in AT1R expression along with c-Jun did not reflect normalization in hemodynamic parameters following losartan treatment. There may be several reasons for this discrepancy. First, it may suggest that early molecular changes may not be translated into functional changes for a longer period of time. Second, abnormalities in cardiac and peripheral function are multifactorial. Central sympathetic excitation is just one of many factors responsible for the progression of heart failure. Ribeiro reviewed several losartan clinical trials over the past 15 years and concluded that losartan confers its cardiovascular and renal protective effects beyond its ability to lower blood pressure.

In a previous study from our laboratory, we observed an increase in renal sympathetic nerve activity in rabbits subjected to chronic ICV infusion of Ang II. This study also showed a central upregulation of AT1R similar to that observed here. Furthermore, chronic central stimulation with Ang II evoked a profound increase in reactive oxidant stress in the RVLM. These data, along with that reported here, suggest a novel pathway by which Ang II exerts its deleterious effects on the central regulation of sympathetic outflow. Since Shibakuma et al reported that the treatment of cells with H2O2 induced the transcription of c-fos and c-jun, redox regulation of transcription factor function has emerged as a potentially important and widespread mechanism of gene regulation. The growing list of redox-regulated transcription factors currently includes such well-known factors as AP-1, Egr-1, nuclear factor κB, and p53. It has also been reported that Ang-II stimulates the binding activity of AP-1 via a reactive oxygen species (ROS)-signaling pathway in cultured neonatal rat ventricular myocytes. A role for ROS in AP-1 activation in the central nervous system following Ang II infusion or in CHF remains to be determined.

This study provides evidence that AP-1–DNA binding activity is significantly increased in the RVLM of CHF rabbits and of rabbits exposed to ICV Ang II. AP-1 is redox sensitive through the conserved cysteine residues located in the DNA-binding domain. AP-1 consists of a dimer of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and...
Fra2) family members. Jun family members form homo- and heterodimers that recognize a TGAGTCA consensus DNA sequence. Fos family members, which are unable to dimerize with each other, augment transcriptional activation by the association with Jun family members. ROS generation in response to various external stimuli has been shown to be related to changes in AP-1. AP-1 is involved in the expression of numerous genes responsible for cell proliferation and tissue remodeling by binding to the AP-1 consensus sequence present in their promoter regions. The promoter region of the AT1R gene contains an AP-1 consensus sequence. We analyzed the promoter regions of human, rat, and mouse AT1R genes for the putative transcription factor binding sites by using Transcription Element Search Software (TESS) (http://www.cbil.upenn.edu/cgi-bin/tess/tess). Human, mouse, and rat AT1R genes harbor the consensus transcription factor binding sites for AP-1. The increased binding properties of these AP-1 complexes can result in persistent gene transcription. Thus, an increased expression of the AT1R gene is under positive control by AP-1 activity.

We next determined the signal transduction pathway for the Ang II–induced AP-1 transactivation for AT1R upregulation. Increasing evidence suggests that JNK plays an important role in mediating neuronal injury and apoptosis by oxidative stress. Fleegal and Sumners have reported that Ang II acts via AT1R-stimulated AP-1–DNA binding in neurons of newborn Sprague–Dawley rats and the stimulatory effects of Ang II on AP-1–DNA binding require activation of JNK. To this end, we measured phosphorylated JNK and its activity in CHF and Ang II–infused normal rabbits. Phosphorylated JNK and its activity in the RVLM were increased in both groups. Losartan blocked the activation of JNK. The effects of Ang II on neuronal c-Jun mRNA and phosphorylated c-Jun protein have also been evaluated. The increased level of phospho–c-Jun in CHF rabbits further suggests the activation of AP-1.

Because ICV infusion of Ang II stimulates a variety of tissue types in the intact animal, we examined the effect of Ang II in a neuronal cell line (CATH.a). These results confirmed an Ang II–mediated upregulation of AT1R expression, which was totally blocked by the AT1R blocker losartan. In CATH.a cells, we also tested the effects of specific blockers for JNK, ERK, and p38 on AT1R expression. Ang II–induced AT1R expression was blocked by the JNK inhibitor but not by ERK or p38 blockers. These results suggest that AT1R transcription occurs downstream of JNK activation and does not involve p38 or ERK activation.

Overall, this study provides insight into the mechanisms that may contribute to AT1R upregulation in RVLM neurons of CHF rabbits. We recognize that other areas in the mid- and hindbrain may be important Ang II–dependent regulators of sympathetic nerve activity; however, because the RVLM is the final common pathway for premotor sympathetic neurons projecting to the spinal cord, this mechanism may be important in setting the level of sympathetic outflow in CHF and other hyperadrenergic states. Figure 6 illustrates a possible mechanism for AP-1 activation and AT1R upregulation, consistent with the data presented here. Further investigations will be necessary to elucidate additional complex mechanisms such as posttranscriptional modification of the AT1R (e.g., mRNA stability and/or alternative splicing).

Acknowledgments
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Disclosures
L.H.Z. is a member of the scientific advisory board of CVRx Inc (Maple Grove, Minn).

References
Neuronal Angiotensin II Type 1 Receptor Upregulation in Heart Failure: Activation of Activator Protein 1 and Jun N-Terminal Kinase
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Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2006/09/28/01.RES.0000247066.19878.93.DC1
**Angiotensin II Radioimmunoassay**

At the end of each experiment arterial blood was taken from the central ear artery in the conscious animal. Blood was added to EDTA plus aprotinin tubes and kept on ice until centrifuged. Plasma was stored at -80°C until assayed. Ang II peptide in rabbit plasma was measured using a radioimmunoassay (RIA) and a highly specific antibody provided to us by Dr. Ian Reid (University of California at San Francisco). After clarification of plasma by centrifugation, samples were applied to a phenylsilylsilica extraction column (ALPCO Diagnostics). After elution from the column with 100% methanol, samples were dried on a Speed-Vac and reconstituted for radioimmunoassay. The sensitivity of the assay was 2.5 pg/tube.

**Cardiac Function, Arterial Pressure, Heart Rate, LV Pressure, Ejection Fraction**

A 2-dimensional, short-axis view of the LV was obtained at the level of the papillary muscles. M-mode tracings were recorded through the anterior and posterior LV walls, and anterior and posterior wall thicknesses (end-diastolic and end-systolic) and LV internal dimensions were measured.

A catheter connected to a radiotelemetry unit (Data Sciences International) was inserted into the descending aorta via a branch of the right femoral artery under general anesthesia for direct measurement of arterial pressure (AP). HR was derived from the AP pulse with the use of a PowerLab (Model 8S, AD Instruments, Inc.) data acquisition system.
At the end of the experiment, the rabbits were anesthetized with ketamine (100 mg/kg ip). Through a midline incision in the neck, a common carotid artery was exposed and catheterized with a Millar transducer (Model SPR-524, Millar Instruments, Inc.) for measurement of LV pressure.

**Western Blot Analysis**

Equal amounts of homogenate were loaded and then separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The proteins of these samples were electrophoretically transferred at 100 V for 1.5 h onto nitrocellulose membranes. The membrane were blocked with 5% non-fat milk in tris-buffered saline-tween 20, and probed at room temperature with primary antibodies against the AT1 receptor, c-Jun, Phospho-c-Jun, JNK1/2, phospho-JNK1/2, ERK, phospho-ERk, P38, phospho-P38 (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. After washing, the membranes were incubated for 30 min with peroxidase-conjugated secondary antibody. The membranes were then washed and incubated for 5 min with Supersignal West Pico Chemiluminescent detection reagents (Pierce; Rockford, IL). The bands on the membrane were digitized and analyzed using UVP BioImaging Systems (UVP, Upland, CA).

**SAPK/JNK Activity Assays**

Homogenates were prepared from RVLM in Lysis Buffer. The samples were sonicated four times for 5 seconds each at an amplitude of 40 watts and centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was saved and protein concentration was measured. 20 µl of the immobilized c-Jun fusion protein bead slurry was added to 200 µl
lyaste (total 200 µg protein), incubated overnight at 4°C, then centrifuged at 14,000 x G for 30 seconds at 4°C. The pellets were washed twice with 500 µl of Lysis Buffer, twice with 500 µl of Kinase Buffer. The pellets were suspended in 50 µl of Kinase Buffer. After incubation for 30 minutes at 30°C, the reaction was terminated with 25 µl of 3X SDS buffer. 20 µl of the mixture was by Western blot analysis. The bands of phosphoralted c-Jun fusion protein on the membrane were visualized and analyzed using UVP BioImaging Systems.

**Electrophoretic Mobility Shift Assay (EMSA)**

Two complementary oligos with the AP-1 consensus sequence, 5'-CGC TTG ATG ACT CAG CCG GAA-3', were synthesized in the UNMC Eppley DNA Synthesis Core Facility at the University of Nebraska Medical Center. The oligomers were labeled at the 3' end with biotin using the 3' End DNA Labeling Kit (Pierce), then annealed at room temperature to obtain double-stranded DNA. The binding reactions contained 10 µg of nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 1 µg of poly(dI-dC), and 2 nM of biotin-labeled DNA. The reactions were incubated at 23 °C for 20 min. The competition reactions were performed by adding 100-fold excess unlabeled double-stranded AP-1 consensus oligonucleotide to the reaction mixture. In antibody supershift assays, incubation was performed in the absence or presence of 2 µg of anti-c-Jun antibody (Santa Cruz Biotechnology) added to the reaction mixture. The reactions were then electrophoresed on a 5% native polyacrylamide gel in 0.5X TBE at 100 V for 1 h in 0.5X TBE buffer. The reactions were transferred to a nylon membrane. The biotin-labeled
DNA was detected with a LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce). The bands on the membrane were digitized and analyzed using UVP BioImaging Systems.