Mitogen-Activated Protein Kinases Mediate Upregulation of Hypothalamic Angiotensin II Type 1 Receptors in Heart Failure Rats

Shun-Guang Wei, Yang Yu, Zhi-Hua Zhang, Robert M. Weiss, Robert B. Felder

Abstract—In heart failure (HF), angiotensin II type 1 receptor (AT1-R) expression is upregulated in brain regions regulating sympathetic drive, blood pressure, and body fluid homeostasis. However, the mechanism by which brain AT1-R are upregulated in HF remains unknown. The present study examined the hypothesis that the angiotensin II (Ang II)–triggered mitogen-activated protein kinases (MAPKs) p44/42, p38, and c-Jun N-terminal kinase contribute to upregulation of the AT1-R in the hypothalamus of rats with HF. AT1-R protein, AT1-R mRNA, and AT1-R immunoreactivity increased in the paraventricular nucleus of hypothalamus and the subfornical organ of rats with ischemia-induced HF compared with sham-operated controls. Phosphorylated p44/42 MAPK, c-Jun N-terminal kinase, and p38 MAPK also increased in paraventricular nucleus and subfornical organ. A 4-week ICV infusion of the AT1-R antagonist losartan decreased AT1-R protein and phosphorylation of p44/42 MAPK, c-Jun N-terminal kinase, and p38 MAPK in the HF rats. A 4-week ICV infusion of the p44/42 MAPK inhibitor PD98059 or the c-Jun N-terminal kinase inhibitor SP600125 significantly decreased AT1-R protein and AT1-R immunoreactivity in the paraventricular nucleus and subfornical organ, but the p38 MAPK inhibitor SB203580 did not. Treatment with ICV losartan, PD98059, and SP600125 had no effect on AT1-R expression by Western blot in sham-operated rats. In untreated HF rats 4 weeks after coronary ligation, a 3-hour ICV infusion of PD98059, SP600125, or losartan reduced AT1-R mRNA in paraventricular nucleus and subfornical organ. These data indicate that MAPK plays an important role in the upregulation of AT1-R in the rat forebrain in HF and suggest that Ang II upregulates its own receptor by this mechanism. (Hypertension. 2008;52:679-686.)

Key Words: MAPK ■ Ang II ■ AT1 receptor ■ heart failure ■ forebrain

The intrinsic brain renin-angiotensin system (RAS) is activated in heart failure (HF).1,2 Increased activity of the brain RAS is thought to play a pivotal role in the progression of HF by altering salt and water homeostasis, neurohormonal release, and sympathetic outflow.3,4 The fundamental functions of brain RAS are mediated by angiotensin II (Ang II), acting on the angiotensin II type 1 receptor (AT1-R), which is widely distributed in the central nervous system from the forebrain to the brain stem.5

In HF, AT1-R expression is upregulated in the subfornical organ (SFO) and the organum vasculosum of the laminae terminais, circumventricular organs lacking a blood–brain barrier, and in discrete nuclei inside the blood–brain barrier, including the paraventricular nucleus (PVN), the median preoptic nucleus, the nucleus tractus solitarius, and the rostral ventrolateral medulla.6,7 These are the key brain regions regulating blood pressure, body fluid homeostasis, and sympathetic drive. The intracellular mechanisms by which brain AT1-R are upregulated in these regions in HF remain unknown.

Chronic ICV infusion of Ang II increases the expression of AT1-R in the brain of rats8,9 and rabbits.6 These findings, along with the increase in brain AT1-R in high renin states like hypertension and HF, suggest that Ang II may upregulate its own receptor. Ang II binding to the AT1-R robustly activates the mitogen-activated protein kinase (MAPK) intracellular signaling pathways.10 Three major MAPK family members are p44/42 MAPK (also known as extracellular signal-regulated protein kinase 1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK). p44/42 MAPK is responsible for the transcriptional regulation of c-Fos,11 whereas JNK is responsible for the phosphorylation of c-Jun.12 c-Fos and c-Jun are primary components of the nuclear transcription factor activator protein 1 (AP-1).13 In rats, peripheral or central administration of Ang II induces strong expression of c-Fos and c-Jun in several cardiovascular autonomic regions of the forebrain, including the SFO and the PVN.14,15 Furthermore, AP-1 binding sequences have been identified in the upstream promoter of the cloned AT1-R gene of the rat.16

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suggesting that Ang II–induced MAPK signaling may lead to upregulation of the AT₁-R.

We tested the hypothesis that Ang II upregulates AT₁-R expression in the brain of HF rats by inducing MAPK activity. We examined the contribution of the MAPK signaling pathways to expression of AT₁-R in 2 representative regions: the SFO, which lies outside the blood–brain barrier and is exposed to circulating Ang II synthesized by the systemic RAS, and the PVN, which is protected by the blood–brain barrier but is exposed to Ang II synthesized by the intrinsic brain RAS.

Materials and Methods

Animals

Adult male Sprague-Dawley rats, weighing 275 to 300 g, were obtained from Harlan Sprague Dawley (Indianapolis). The animals were housed in temperature-controlled (23±2°C) rooms in the University of Iowa animal care facility and exposed to a normal 12-hour light/dark cycle. They were provided with rat chow ad libitum. These studies were performed in accordance with the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society.¹⁷ The experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee.

Experimental Protocols

Rats underwent coronary artery ligation to induce HF or a sham operation and were assigned to one of the following protocols.

Protocol I

Rats underwent a 4-week ICV infusion (0.25 µL/hour) of the AT₁-R antagonist losartan (10 mmol/L), the p44/42 MAPK inhibitor PD98059 (20 µmol/L), the JNK inhibitor SP600125 (100 µmol/L), or vehicle (VEH), beginning within 24 hours of coronary ligation or sham surgery. These rats were used for Western blot or immunohistochemistry studies measuring AT₁-R and MAPK protein expression in PVN and SFO. In this protocol, rats in the immunohistochemistry study groups underwent echocardiography and assessment of the effects of chronic ICV infusions of losartan and the MAPK inhibitors on hemodynamic and anatomic indicators of HF.

Protocol II

Rats underwent a 3-hour ICV infusion (40 µL/hour) of losartan (10 mmol/L), PD98059 (20 µmol/L), SP600125 (100 µmol/L), SB203580 (100 µmol/L), or vehicle 4 weeks after coronary artery ligation or sham surgery. These rats were used for real-time polymerase chain reaction studies measuring the contribution of MAPK to AT₁-R mRNA expression in PVN and SFO. All rats in this protocol underwent echocardiography and assessment of the effects of acute ICV infusions of losartan and the MAPK inhibitors on hemodynamic and anatomic indicators of HF.

Specific Materials and Methods

Specific materials and methods are available in the online data supplement (http://hyper.ahajournals.org).

Statistics

All values are expressed as the means±SEM. The significance of differences among groups was analyzed by 2-way repeated-measure
ANOVA followed by post hoc Fisher’s least significant difference test. Echocardiographic parameters were analyzed using 1-way ANOVA followed by post hoc Fisher’s least significant difference test. Differences between values were considered significant at \( P<0.05 \).

**Results**

**Protocol I: Chronic ICV infusion of Losartan and MAPK Inhibitors**

**Molecular Studies**

Western blot revealed significant increases in phosphorylated p44/42 MAPK (p-p44/42), phosphorylated INK (p-JNK), and phosphorylated p38 MAPK (p-p38) in PVN (Figure 1A) and SFO (Figure 1B) of VEH-treated HF rats compared with VEH-treated sham-operated rats. ICV infusion of losartan for 4 weeks markedly reduced the level of p-p44/42, p-JNK, and p-p38 in PVN (Figure 1A) and SFO (Figure 1B) in HF rats but had no effects on these variables in sham-operated rats.

AT1-R protein was also significantly increased in PVN (Figure 2A) and SFO (Figure 2B) of VEH-treated HF versus VEH-treated sham-operated rats. HF rats treated for 4 weeks with ICV losartan had a substantially lower level of AT1-R protein in the PVN and SFO than VEH-treated HF rats (Figure 2). HF rats treated ICV for 4 weeks with the p44/42 MAPK inhibitors PD98059 or the JNK inhibitor SP600125 also had lower AT1-R protein levels in PVN (Figure 2A) and SFO (Figure 2B) compared with VEH-treated HF rats. ICV treatment for 4 weeks with SB203580, a p38 MAPK inhibitor, had no effect on AT1-R protein levels (Figure 2). In sham-operated rats, ICV infusion of losartan, PD98059, and SP600125 for 4 weeks had no effect on AT1-R expression in the PVN or SFO (Figure 2).

**Immunohistochemical Studies**

Immunoreactivity for p-p44/42, p-p38, and p-JNK was increased in PVN (Figure 3) and SFO (Figure 4) in VEH-treated HF rats compared with VEH-treated sham-operated rats. The number of neurons containing phosphorylated MAPK in dorsal parvocellular (PVN-dp), medial parvocellular (PVN-mp), ventrolateral parvocellular (PVN-vlp), and posterior magnocellular (PVN-pm) subdivisions of the PVN18 as well as in central SFO (Figure 4) was significantly higher in VEH-treated HF rats than in VEH-treated sham-operated rats.

Immunoreactivity for AT1-R, identified with a rabbit polyclonal AT1-R antibody (ab18801; Abcam, Inc; Cambridge, MA), was also increased in all 4 regions (PVN-dp, PVN-mp, PVN-vlp, and PVN-pm) of the PVN (Figure 5) and in central SFO (Figure 6) in VEH-treated HF rats compared with VEH-treated sham-operated rats. In the sham-operated rats, AT1-R immunoreactivity was located predominantly in the PVN-mp. In HF rats, AT1-R immunoreactivity was more pronounced in PVN-mp, but the PVN-dp, PVN-vlp, and PVN-pm also exhibited distinct increases in AT1-R immunoreactivity. In the SFO, HF rats displayed increased AT1-R expression throughout compared with sham-operated rats.

**Indicators of HF**

Echocardiography at baseline demonstrated that HF animals in the immunohistochemistry study groups assigned to treatment with AT1-R antagonists and MAPK inhibitors versus VEH were well-matched with regard to left ventricular (LV)
systolic function (Table). Compared with sham-operated rats, HF rats had a significantly lower LV ejection fraction and a significantly higher LV end-diastolic volume.

Hemodynamic assessment of these rats at the completion of the treatment protocol revealed that VEH-treated HF rats had a lower LV peak systolic pressure and maximal rate of rise of LV systolic pressure (LV dP/dt max) and a higher LV end diastolic pressure than VEH-treated sham-operated rats. The right ventricular weight/body weight ratio was substantially higher in VEH-treated HF compared with VEH-treated sham-operated rats.

HF rats treated for 4 weeks with the AT1-R antagonist and the MAPK inhibitors had higher LV dP/dt max, lower LV end-diastolic pressure, and a lower right ventricular weight/body weight ratio than VEH-treated HF rats, but all of these values were still significantly different from VEH-treated sham-operated rats (Table). There were no differences in any of these variables related to the specific MAPK inhibitor infused. LV peak systolic pressure was not significantly different in drug-treated versus VEH-treated HF.

**Protocol II: Acute ICV Infusion of Losartan and MAPK Inhibitors**

**Molecular Studies**

Real-time polymerase chain reaction demonstrated higher AT1-R mRNA expression in both PVN and SFO (Figure 7) of VEH-treated HF rats 4 weeks after coronary ligation compared with VEH-treated sham-operated rats. A 3-hour ICV infusion of losartan in these rats with otherwise untreated HF resulted in a significantly lower AT1-R mRNA level in both PVN and SFO (Figure 7). A 3-hour ICV infusion of the p44/42 inhibitors PD98059 or the JNK inhibitor SP600125 also resulted in a lower AT1-R mRNA level in PVN and SFO. A 3-hour ICV infusion of the p38 MAPK inhibitor SB203580 had no effect on AT1-R mRNA level in PVN or SFO (Figure 7).

**Indicators of HF**

Baseline echocardiography revealed no differences in LV systolic function in sham-operated or HF rats assigned to acute ICV treatment with VEH, losartan, or the MAPK inhibitors (Table S1). A 3-hour ICV infusion of losartan or the MAPK inhibitors had no effect on LV variables of dP/dt max, LV end diastolic pressure, or right ventricular weight/body weight ratio.

**Figure 3.** Immunohistochemical analysis of p-p44/42, p-JNK, and p-p38 in the PVN in Sham and HF rats. A, Representative sections showing p-p44/42, p-p38, and p-JNK in the PVN of Sham (top panels) and HF (bottom panels) rats. Third ventricle is to the right. B, Grouped data showing numbers of p-p44/42, p-p38, and p-JNK-positive neurons counted in the PVN-dp, PVN-mp, PVN-vlp, and PVN-pm subdivisions of PVN. Values are expressed as means±SEM (n=6 for each group) *P<0.05; HF vs Sham.

**Figure 4.** Immunohistochemical analysis of p-p44/42, p-JNK, and p-p38 in the SFO in sham and HF rats. (A) Representative sections showing the expression of p-p44/42, p-p38, and p-JNK in central SFO of Sham (top panels) and HF rats (bottom panels). (B) Grouped data showing numbers of p-p44/42, p-p38, and p-JNK–positive neurons counted in central SFO of Sham and HF rats. Values are expressed as means±SEM (n=6 for each group). *P<0.05; HF vs Sham.
pressure, and LV peak systolic pressure or on right ventricular weight/body weight ratio compared with VEH-treated HF rats 4 weeks after coronary ligation (Table S1). For further details, please see the online supplement.

Discussion

Novel findings of this study are: (1) MAPK signaling pathways are activated in PVN and SFO of HF rats, concomitant with increases in AT1-R mRNA and AT1-R protein in these regions; (2) an AT1-R antagonist reduces the expression of p-p44/42 MAPK, JNK, and p38 MAPK in PVN and SFO in HF rats; and (3) either central inhibition of p44/42 MAPK and JNK activity or AT1-R blockade prevents upregulation of AT1-R mRNA and protein in HF rats at sites both inside (PVN) and outside (SFO) the blood–brain barrier.

In animal models of HF, upregulation of brain RAS activity and subsequent Ang II–dependent stimulation of reduced nicotinamide-adenine dinucleotide phosphate oxidase–dependent superoxide production contribute significantly to increased sympathetic nerve activity. An important and poorly understood aspect of this process is the upregulation of the AT1-R that mediates most of the known effects of Ang II. In normal animals and in animals with HF, an excess of Ang II appears to upregulate its own receptor in the brain. A putative explanation for this seemingly counterintuitive observation is that Ang II triggers MAPK signaling pathways that increase the expression of c-Fos and c-Jun, the primary components of the transcription factor AP-1. AP-1 binding sequences have been identified in the regulatory

Figure 5. Immunohistochemical analysis of AT1-R immunoreactivity in the PVN of VEH-treated Sham rats and HF rats treated for 4 weeks with ICV VEH, losartan, PD98059, SP600125, or SB203580. A, Representative sections of PVN from animals undergoing each treatment protocol. Third ventricle is to the right. B, Grouped data showing numbers of AT1-R-positive neurons counted in PVN-dp, PVN-mp, PVN-vlp, and PVN-pm. Values are expressed as means±SEM (n=6 to 7 for each group). *P<0.05 compared with Sham+VEH; †P<0.05 HF+ treatment compared with HF+VEH. ‡P<0.05, PVN-mp compared with other PVN regions, Sham+VEH.

Figure 6. Immunohistochemical analysis of AT1-R immunoreactivity in the SFO of VEH-treated Sham rats and HF rats treated for 4 weeks with ICV VEH, losartan, PD98059, SP600125, or SB203580. A, Representative sections of SFO from animals undergoing each treatment protocol. Third ventricle is at bottom of each image. B, Grouped data showing numbers of AT1-R positive neurons counted in central SFO. Values are expressed as means±SEM (n=6 to 7 for each group). *P<0.05 compared with Sham+VEH; †P<0.05 HF+ treatment compared with HF+VEH.
region of the AT1-R gene, and transactivation of AP-1 has been implicated recently in upregulation of the AT1-R in HF.

A recent study supported that hypothesis, demonstrating Ang II–dependent upregulation of AT1-R with concomitant c-Jun and JNK protein phosphorylation in the rostral ventrolateral medulla in rabbits with HF and normal rabbits infused with ICV Ang II and inhibition of Ang II–induced expression of AT1-R by losartan or a JNK inhibitor in neuronal cell cultures. Here, we expand on that observation, demonstrating in vivo that chronic ICV infusion of Ang II, subthreshold to raise arterial pressure or plasma aldosterone, upregulates AT1-R in PVN and SFO in rats with HF. Losartan has a similar effect of AT1-R by losartan or a JNK inhibitor in neuronal cell cultures. Here, we expand on that observation, demonstrating in vivo that chronic ICV infusion of Ang II, subthreshold to raise angiotensin-converting enzyme activity is increased in the SFO and the PVN, making local production of Ang II a likely possibility at both sites. Another potential source might be angiotensinergic projections from the circumventricular organs to PVN, activated by either circulating or locally produced Ang II or both. For example, microinjection of Ang II into SFO induces a 10-fold increase in Ang II release in PVN in the rat. Regardless of the source of Ang II, the Ang II–dependent signaling mechanisms upregulating AT1-R appear to be the same in both regions.

An intriguing finding of this study is the diffuse distribution of AT1-R throughout the PVN in HF rats. In normal unstressed rats, AT1-R are found primarily in PVN-mp, where they are associated with neuroendocrine (corticotropin releasing hormone) neurons. We observed a similar distribution in the SFO. In HF, angiotensin-converting enzyme activity is increased in the SFO and the PVN, making local production of Ang II a likely possibility at both sites. Another potential source might be angiotensinergic projections from the circumventricular organs to PVN, activated by either circulating or locally produced Ang II or both. For example, microinjection of Ang II into SFO induces a 10-fold increase in Ang II release in PVN in the rat. Regardless of the source of Ang II, the Ang II–dependent signaling mechanisms upregulating AT1-R appear to be the same in both regions.

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tion in sham-operated rats, in which AT₁-R were highly expressed in the PVN-mp subdivision, much less so in the PVN-dp, PVN-vlp, and PVN-pm subdivisions. In the HF rats, AT₁-R expression was pronounced not only in PVN-mp but also in the PVN-dp and PVN-vlp, and even in the magnocellular neurons in PVN-mp which do not normally express AT₁-R. A similar general broadening of the distribution of AT₁-R immunoreactivity was noted in the SFO in HF rats.

In the HF setting, in which the brain RAS is activated, these observations are not surprising. They are consistent with the known functions of the PVN in HF. Plasma levels of arginine vasopressin, a downstream product of the RAS, are increased in HF; the presence of Ang II has been shown to induce the expression of AT₁-R in magnocellular neurons of the PVN, a major source of arginine vasopressin. Local inhibition of AT₁-R in the PVN reduces sympathetic activation in HF rats, presumably by influencing presympathetic neurons in the PVN-dp and PVN-vlp. Clearly, the actions of Ang II in the PVN of HF rats extend beyond regulation of corticotropin releasing hormone. Upregulation of AT₁-R expression in regions in which they are not normally expressed may be a mechanism for recruiting autonomic and neuroendocrine neurons in response to the stress of HF. Increases in sympathetic nerve activity and plasma vasopressin, in addition to the increases in circulating corticosterone and epinephrine resulting from activation of the hypothalamic-pituitary-adrenal axis, are typical responses to stress. Of particular interest is the observation that the pattern of p-p44/42 and p-JNK expression matches the pattern of AT₁-R expression in the PVN and SFO in both sham-operated and HF rats, lending credence to their role in upregulating the AT₁-R.

The improvements in LV systolic function (LV dP/dt max) and volume regulation (LV end-diastolic pressure) observed in this study likely reflect a centrally mediated reduction in sympathetic drive. Similar improvements have been demonstrated in previous studies in which the RAS has been manipulated selectively at the central nervous system level. Supporting that interpretation are recent studies from our laboratory demonstrating that acute administration of the p44/42 MAPK inhibitors reduces renal sympathetic nerve activity in rats with HF but not in sham-operated controls. The opposite interpretation might be entertained, i.e., that the changes we observed in hypothalamic AT₁-R expression resulted from treatment-induced improvement in LV systolic function. Two observations counter that argument: (1) chronic infusion of the p38 MAPK inhibitor had similar salutary effects on LV dysfunction without affecting the hypothalamic expression of AT₁-R; and (2) acute ICV infusions of the inhibitors reduced AT₁-R mRNA in the absence of any change in LV function.

Finally, the mechanism by which the p38 MAPK inhibitor improves the peripheral dynamics of HF without affecting hypothalamic AT₁-R expression is not explained by these studies. However, p38 MAPK has been implicated in a variety of signaling pathways that may contribute to sympathetic activity in this setting.

A limitation of this study is that the indices of HF were measured in 2 subsets of the rats studied (those used for the immunohistochemical and the real-time polymerase chain reaction measurements) but not in those used for Western blot assessment of protein levels. However, in this and in previous studies, the method for induction of HF results in a reproducible degree of LV dysfunction.

Perspectives

The present study provides strong evidence from an in vivo model that the increased Ang II resulting from activation of the RAS in HF upregulates its own receptor in the brain by activating MAPK signaling pathways. The same feed-forward mechanism appears to affect central neurons inside and outside the blood–brain barrier, suggesting that Ang II produced by both the systemic RAS and the brain RAS may contribute. Other neurochemical mediators that are present in the HF brain (eg, aldosterone and proinflammatory cytokines) can activate these same intracellular signaling pathways and may contribute to upregulation of AT₁-R. Thus, MAPK signaling may provide a substrate for central interactions between other excitatory mediators and the RAS in HF.

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Disclosures

None.

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Mitogen-Activated Protein Kinases Mediate Upregulation of Hypothalamic AT$_1$ Receptors in Heart Failure Rats

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Running Title: MAPK and brain AT$_1$ receptors in heart failure
EXPANDED MATERIALS AND METHODS

Induction of heart failure

Heart failure was induced by ligation of the left anterior descending coronary artery in rats under ketamine plus xylazine anesthesia (90 mg/kg+10 mg/kg, IP), as described previously.\textsuperscript{1} Sham rats underwent the same surgery but did not undergo coronary ligation.

ICV infusions

Drug or vehicle was infused via a 26 ½ -gauge stainless steel cannula with the tip positioned in the left lateral cerebral ventricle (coordinates AP, −1.0 mm; DV, −4.5 mm; and ML, −1.5 mm, with bregma as a reference).\textsuperscript{2}

Protocol I: For the 4-week infusion protocol, the cannula was implanted under anesthesia with ketamine and xylazine (90+10mg/kg, IP) using sterile technique 1 week prior to coronary artery ligation or sham surgery. The cannula was secured in place with three protective screws and dental orthodontic resin was applied to the surface of the skull. In a separate surgery performed under anesthesia (ketamine plus xylazine: 90 mg/kg + 10 mg/kg, IP) the day after coronary ligation or sham surgery, an osmotic mini-pump (model 2004, Alza Corp., CA) containing drug or vehicle was implanted subcutaneously at the back of the neck and connected to the free end of the cannula.

Protocol II: For the 3-hour infusion protocol, the cannula was positioned under anesthesia (urethane: 1.5 g/kg, IP) and connected to a microsyringe for administration of drug or vehicle.
Proper location of the cannula tip in the lateral ventricle was verified at the end of the experiments by Pontamine sky blue administration. Data from animals whose cannula were found to be incorrectly placed were excluded from the analyses.

**Drugs infused**

The AT$_1$-R antagonist losartan (a gift from Du Pont/Merck) for ICV infusion was dissolved in artificial cerebrospinal fluid (aCSF). The selective p44/42 MAPK inhibitor PD98059, the JNK inhibitor SP600125 and the p38 MAPK inhibitor SB203580 were obtained from Tocris (Ellisville, MO). Doses of the MAPK inhibitors for ICV infusion were extrapolated from acute ICV injection studies$^{3-5}$ and previous experience of this laboratory with ICV infusion of other neuroactive substances. These inhibitors were dissolved in dimethyl sulfoxide (DMSO) first, and then diluted in aCSF to make a 0.1%-0.5% final DMSO concentration. The ICV vehicle (VEH) was aCSF containing 0.5% DMSO.

**Assessment of heart failure**

Approximately 24 hr after coronary artery ligation or sham surgery, rats used for the immunohistochemical studies in Protocol I and all studies in Protocol II were sedated with ketamine (25 mg/kg, IP) and underwent two-dimensional echocardiography to assess left ventricular (LV) systolic function, as previously described.$^1$ The ischemic zone (IZ) as a percent of LV circumference (\% IZ), LV ejection fraction (LVEF), and LV end-diastolic volume (LVEDV) were measured.

At the conclusion of the study protocols, these rats were anesthetized with urethane (1.5 g/kg, IP) and a Millar Mikro-tip catheter was advanced via the right carotid artery into the aorta and then into the LV to measure peak systolic pressure (LVPSP), end diastolic pressure
(LVEDP) and the rate of change in LV systolic pressure over time (LV dP/dt\text{max}). Finally, after the brain tissue was collected, the heart was removed and the right ventricle was weighed.

**Tissue preparation**

At the completion of each protocol, the animals were decapitated while still under anesthesia to obtain brain tissue for the designated studies. To obtain tissues for Western blot and real time PCR, the brains were immediately removed, frozen in liquid nitrogen and stored at −80 °C for subsequent use. The frozen brain was cut into 300-μm coronal sections and the target tissues were punched using a 15-gauge needle (inner diameter 1.5 mm). The punched tissues were homogenized in cell lysis buffer (Cell Signaling Technology, Beverly, MA) to extract protein for Western assay or in TRI reagent (Molecular Research Center, Cincinnati, OH) to extract RNA for real time PCR. In order to obtain a sufficient amount of tissue to detect AT\text{1}-R protein in each region, the samples from two different rats were combined. To collect tissues for immunostaining, rats were transcardially perfused with 4% paraformaldehyde. Brains were then embedded with OCT and rapidly frozen in alcohol chilled dry ice. Coronal forebrain sections (12 μm) of target tissues were made using a cryostat and then stored at −80 °C.

**Western blot**

AT\text{1}-R protein was detected using a polyclonal antibody to AT\text{1}-R (SC-1173, 1: 500, Santa Cruz, CA), and phospho-p44/42 MAPK, JNK and p38 were detected using monoclonal antibodies (1:500; Cell Signaling Technology, Inc., Beverly, MA) to phospho-p44/42 MAPK (#4377, 1: 250), phospho-JNK (#9251, 1:250) and phospho-p38 (#9215, 1:250), respectively. Immunoblots were visualized with an enhanced chemiluminescence reagent. Band intensities were quantified with NIH ImageJ software. The content of AT\text{1}-R protein was normalized by the
total β-actin. The p-p44/42, p-JNK and p-p38 level were normalized by the total p44/42 MAPK, JNK and p38 MAPK, respectively.

**Real-time PCR for quantification of AT1-R mRNA**

mRNA levels for AT1-R in PVN and SFO were measured with real-time PCR following reverse transcription of total RNA, as described previously.6,7 The sequences for primers and probe used were as follows: sense primer, 5’- GTA-GCC-AAA-GTC-ACC-TGC-ATC A -3’; antisense primer, 5’- GGT-AGA-TGA-CGG-CTG-GCA-AA-3’; probe, 5’- CAT-CTG-GCT-AAT-GGC-TGG-CTT-GGC-3’. TaqMan primer and probe for rat GAPDH were purchased from Applied Biosystems (Foster City, Calif.). Real-time PCR was performed using the ABI prism 7700 Sequence Detection System (Applied Biosystems). Rat GAPDH mRNA was quantified as an internal control for each sample and the final results of real-time PCR were expressed as the ratio of mRNA of interest to GAPDH mRNA.

**Immunohistochemistry**

Immunohistochemical visualization of AT1-R and phosphorylated MAPK expression in PVN and SFO was performed on frozen sections using antibodies and avidin–biotin-peroxidase methods as previously described.8 The primary antibodies for detection of immunoreactivity of AT1-R and phosphorylated MAPK were rabbit polyclonal antibody to AT1-R (#ab18801, Abcam, Inc, Cambridge, MA ), rabbit monoclonal antibody (Cell Signaling Technology, Beverly, MA) to phospho-p44/42 MAPK (#4376), phospho-p38 MAPK (#4631) and phospho-SAPK/JNK (#9251), respectively. For immunohistochemical analysis, the numbers of phosphorylated MAPK or AT1-R positive neurons in a 100x100 µm window located over the dorsal parvocellular (PVN-dp), the medial parvocellular (PVN-mp), ventrolateral parvocellular
(PVN-vlp) or the magnocellular subdivision (PVN-pm) of PVN, and a 200x200 μm window over the central SFO, were counted manually. In each rat, the number of positive neurons was counted in three sections from each subdivision of PVN, and from SFO, and averaged to obtain a single value for statistical analysis. The number of positive neurons was reported as positive cells per $10^4 \mu m^2$.

**Immunofluorescence**

Immunofluorescent staining was used to localize AT$_1$-R expression. The sections were incubated with rabbit polyclonal antibody to AT$_1$-R (sc-1173, 1:100, Santa Cruz) followed by secondary antibodies Alex Fluor 488 goat anti-rabbit IgG (A-11070, 1:200, Invitrogen) and further incubated with To-Pro-3 (1:2000, Invitrogen) to counterstain cell nuclei. AT$_1$-R staining was visualized with a confocal laser-scanning microscope (Zeiss LSM 510, Carl Zeiss, Inc). Fluorescent intensity of AT$_1$-R immunoreactivity in PVN and SFO, over the same areas used for immunohistochemistry, was quantified by NIH ImageJ software.

For triple staining with Fra-LI, the primary anti-Fra-1 antibody was the mouse monoclonal IgG (sc-28310, 1:100, Santa Cruz) and the secondary antibody was Alex Fluor 546 goat anti-mouse IgG (A-11003, 1:200, Invitrogen).
EXPANDED RESULTS

AT\textsubscript{1}-R immunoreactivity by immunofluorescent assay

In order to confirm the increased AT\textsubscript{1}-R expression in HF versus sham-operated rats, an immunofluorescent assay was carried out using a different rabbit polyclonal AT\textsubscript{1}-R antibody (sc-1173, 1:100, Santa Cruz). Confocal images revealed an increased AT\textsubscript{1}-R immunoreactivity in the PVN (Figure S1A) and SFO (Figure S1B) in HF (n=6) compared with sham-operated rats (n=6), in a general distribution very similar to that seen in the immunohistochemical studies. The semi-quantitive fluorescent intensities of AT\textsubscript{1}-R expression in PVN-dp, PVN-mp, PVN-vlp and PVN-pm, and central SFO are shown in Figure S1C.

Triple staining in 3 rats revealed that most of AT\textsubscript{1}-R was in Fra-like positive neurons in PVN in both sham-operated (Figure S2, top panel) and HF rats (Figure S2, bottom panel). The co-localization of AT\textsubscript{1}-R and Fra-like activity was also found in supraoptic nucleus (SON), another key cardiovascular area within the blood-brain barrier in the brain (n=3) in heart failure rat (Figure S3). In addition, the immunofluorescent studies revealed that AT\textsubscript{1}-R in PVN (Video S1A) and SFO (Video S1B) neurons were distributed throughout, in the cytoplasm as well as along the cell membrane. In contrast, AT\textsubscript{1}-R in brain cortex neurons appears to be localized mainly to the cell membrane (Video S1C).
EXPANDED DISCUSSION

Distribution of AT1-R by immunofluorescent staining

The immunofluorescent studies confirmed the distribution of AT1-R immunoreactivity observed in the immunohistochemical studies, using a different method and a different antibody. They also revealed two additional findings. The first is the appearance of AT1-R staining in the cytoplasm of PVN and SFO neurons (see Video S1). Most of the known effects of ANG II are mediated by AT1-R localized in cell membranes. The functional significance of the cytoplasmic AT1-R is still poorly understood, but it has been reported that intracellular AT1-R mediate intracellular ANG II–induced cytosolic Ca^{2+} concentration. The second finding is that, at least in the PVN, the upregulation of AT1-R in heart failure occurs predominantly in neurons that are also positive for Fra-LI activity. Whether these AT1-R contribute to or are products of the associated chronic neuronal excitation remains to be determined.

Specificity and efficacy of AT1-R antibodies and MAPK inhibitors

Controversy surrounds the issue of the specificity of antibodies used to identify the AT1-R in brain tissues. The specificity of the AT1-R antibody (sc-1173) used for the Western blot and immunofluorescence in this study was pre-tested (Figure S4). This antibody showed a single band at the appropriate molecular weight (43 KDa) for the AT1-R. Moreover, the anatomical distribution of AT1-R immunoreactivity identified by this antibody was consistent with known functions of the AT1-R in the brain. For example, AT1-R immunoreactivity was identified not only in the PVN and SFO, but also in the supraoptic nucleus (Figure S3), in which increased ANG II levels are associated with increased expression of AT1-R. A similar distribution of AT1-R was observed in immunohistochemical studies using a different antibody (#ab8801).
The specificity of the MAPK inhibitors was tested in a small number of animals using immunohistochemistry. In SB203580-treated HF rats (n=2), the expression of phosphorylated p44/42 MAPK and JNK were not inhibited compared with VEH-treated HF rats (n=2), but the activity of phosphorylated p38 MAPK was blocked (n=2). Similarly, the p44/42 MAPK inhibitor PD98059 did not inhibit p38 MAPK and JNK-like immunoreactivity in HF rats (n=2), and JNK inhibitor SP600125 did not affect the p44/42 and p38 MAPK immunoreactivity (n=2).

Finally, the p44/42 MAPK inhibitor PD98059 and JNK inhibitor SP600125 reduced the expression of AT₁-R protein and mRNA, as well as AT₁-R immunoreactivity, indicating that the upregulated expression of AT₁-R requires upstream activation of p44/42 MAPK and JNK. The p38 MAPK inhibitor SB203580, did not significantly block AT₁-R upregulation in the brain in HF rats, even with a dose 5-fold higher than the p44/42 inhibitors. The latter finding suggests but does not prove that p38 MAPK has no role in regulating AT₁-R in this setting.
REFERENCES


LEGENDS

Figure S1
Laser confocal immunofluorescent views of AT$_1$-R expression in the PVN (A) and SFO (B) in Sham (top panels) and HF rats (bottom panels). Bright green: AT$_1$-R staining; Red: Nuclear staining. Scale bar: 0.2 mm in PVN; 0.5 mm in SFO. (C) Quantification of AT$_1$-R immunoreactivity in the PVN-dp, PVN-vp, PVN-mp and SFO in Sham and HF rats. Values are expressed as means ± SEM of the immunofluorescent density units (n=6).

Figure S2
Immunofluorescent images of the PVN, triple-labeled for Fra-LI (red), AT$_1$-R (green) and nucleus (blue). Left panels, low power views from a sham (top) and a heart failure (bottom) rat, showing full expanse of PVN (unilateral, 3$^{rd}$ ventricle to left). Right panels, high power views taken from the ventrolateral PVN of the same sham (top) and heart failure (bottom) rat, in the regions indicated by the yellow rectangles in left panel. Fra-LI positive neurons have a pink to purple appearance, indicating the merge with blue nuclear marker. Note that AT$_1$-R labeling is localized predominantly to the Fra-LI positive neurons.

Figure S3
Detection of AT$_1$-R activity in supraoptic nucleus (SON), another key cardiovascular area within the blood-brain barrier in a heart failure rat. Triple-labeled images: AT$_1$-R (green); Fra-LI (red), and nucleus (blue). Similar to PVN, AT$_1$-R expression is
predominantly localized in the Fra-LI positive neurons. The concentrated expression of AT1-R in cardiovascular regions of the brain like SON, PVN and SFO is consistent with previous reports, and supports the argument for specificity of the AT1-R antibody used in this study. OC, optic chiasm.

**Figure S4**

Western gels illustrating the specificity of the Santa Cruz (SC)-1173 antibody for AT1-R. (A) Scattered bands detected in brain tissue with the (SC): SC-579 antibody. (B) A single band at 43 KDa, the molecular weight for the AT1-R, detected with SC-1173. The SC-1173 antibody was used for Western and immunofluorescent studies.

**Video S1**

Three-dimensional confocal images of AT1-R immunoreactivity in neurons of the PVN (A), SFO (B) and brain cortex (C). Green identifies AT1-R immunoreactivity; red identifies the nucleus. In the PVN and SFO neurons, most of the AT1-R immunoreactivity appears to be located in the cytoplasm. In cortical neurons, most of the AT1-R immunoreactivity appears to be located in the cell membrane. See attached Video S1A, S1B and S1C.
Table S1. Echocardiographic, Hemodynamic and Anatomical Measurements: Protocol II - Acute ICV Infusions

<table>
<thead>
<tr>
<th>Variables at Baseline</th>
<th>SHAM+VEH (n=6)</th>
<th>HF+VEH (n=7)</th>
<th>HF+PD98059 (n=7)</th>
<th>HF+SP600125 (n=7)</th>
<th>HF+SB203580 (n=6)</th>
<th>HF+Losartan (n=6)</th>
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<tbody>
<tr>
<td>LVEDV (ml)</td>
<td>0.31 ± 0.03</td>
<td>0.80 ± 0.12*</td>
<td>0.79 ± 0.13*</td>
<td>0.76 ± 0.14*</td>
<td>0.79 ± 0.14*</td>
<td>0.80 ± 0.13*</td>
</tr>
<tr>
<td>LVEF</td>
<td>0.79 ± 0.03</td>
<td>0.33 ± 0.04*</td>
<td>0.31 ± 0.03*</td>
<td>0.33 ± 0.04*</td>
<td>0.29 ± 0.04*</td>
<td>0.33 ± 0.04*</td>
</tr>
<tr>
<td>%IZ</td>
<td>------</td>
<td>47.6 ± 3.9</td>
<td>48.0 ± 4.3</td>
<td>46.8 ± 4.6</td>
<td>49.2 ± 3.9</td>
<td>47.3 ± 3.8</td>
</tr>
</tbody>
</table>

Variables at 4 weeks

| BW (g)                        | 387 ± 6        | 383 ± 8     | 383 ± 6          | 386 ± 6          | 388 ± 7          | 379 ± 6          |
| RV/BW (mg/g)                  | 0.68 ± 0.13    | 1.34 ± 0.17*| 1.29 ± 0.16*     | 1.33 ± 0.17*     | 1.35 ± 0.17*     | 1.36 ± 0.18*     |
| LVPSP (mmHg)                  | 120 ± 4        | 99 ± 5      | 102 ± 4*         | 100 ± 6*         | 101 ± 5*         | 104 ± 5*         |
| LVEDP (mmHg)                  | 4.8 ± 1.6      | 18.9 ± 1.9* | 18.6 ± 2.3*      | 19.2 ± 2.4*      | 18.5 ± 1.9*      | 17.8 ± 1.7*      |
| LV dP/dt_max (mmHg/s)         | 7911 ± 246     | 3934 ± 258* | 3930 ± 205*      | 3898 ± 223*      | 4104 ± 249*      | 3850 ± 252*      |

LVEDV: left ventricular (LV) end-diastolic volume; LVEF: LV ejection fraction; %IZ: ischemic zone as a percent of LV circumference; BW: body weight; RV: right ventricular; LVPSP: LV peak systolic pressure; LVEDP: LV end-diastolic pressure. LV dP/dt_max: maximum rate of rise of LV pressure. Values are expressed as mean ± SEM. * p<0.05 versus SHAM, † p<0.05 versus HF+VEH.
Figure S1

A

Sham

PVN

HF

PVN

0.2 mm

B

Sham

PVN

SFO

HF

PVN

SFO

0.2 mm

C

Fluorescent Units

Sham

HF

PVN

PVN

PVN

PVN

SFO

-dp

-mp

-vlp

-pm

-mp
Figure S2
Figure S3
Figure S4
Video S1

A

B

C

20 µm

20 µm

20 µm