Does Aldosterone Upregulate the Brain Renin-Angiotensin System in Rats With Heart Failure?

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

Abstract—The brain renin-angiotensin system (RAS) contributes to increased sympathetic drive in heart failure (HF). The factors upregulating the brain RAS in HF remain unknown. We hypothesized that aldosterone (ALDO), a downstream product of the systemic RAS that crosses the blood-brain barrier, signals the brain to increase RAS activity in HF. We examined the relationship between circulating and brain ALDO in normal intact rats, in adrenalectomized rats receiving subcutaneous infusions of ALDO, and in rats with ischemia-induced HF and sham-operated controls. Brain ALDO levels were proportional to plasma ALDO levels across the spectrum of rats studied. Compared with sham-operated controls rats, HF rats had higher plasma and hypothalamic tissue levels of ALDO. HF rats also had higher expression of mRNA and protein for angiotensin-converting enzyme and angiotensin type 1 receptors in the hypothalamus, increased reduced nicotinamide-adenine dinucleotide phosphate oxidase activity and superoxide generation in the paraventricular nucleus of the hypothalamus, increased excitation of paraventricular nucleus neurons, and increased plasma norepinephrine. HF rats treated for 4 weeks with intracerebroventricular RU28318 (1 µg/h), a selective mineralocorticoid receptor antagonist, had less hypothalamic angiotensin-converting enzyme and angiotensin type 1 receptor mRNA and protein, less reduced nicotinamide-adenine dinucleotide phosphate–induced superoxide in the paraventricular nucleus, fewer excited paraventricular nucleus neurons, and lower plasma norepinephrine. RU28318 had no effect on plasma ALDO or on angiotensin-converting enzyme or angiotensin type 1 receptor expression in brain cortex. The data demonstrate that ALDO of adrenal origin enters the hypothalamus in direct proportion to plasma levels and suggest that ALDO contributes to the upregulation of hypothalamic RAS activity and sympathetic drive in heart failure. (Hypertension. 2008;51:727-733.)

Key Words: hypothalamus ■ sympathetic nerve activity ■ superoxide ■ angiotensin-converting enzyme ■ angiotensin type 1 receptor

The intrinsic brain renin-angiotensin system (RAS) is activated in heart failure (HF). Both angiotensin-converting enzyme (ACE), the final step in the production of angiotensin II (Ang II), and the angiotensin type 1 receptors (AT1-R) that mediate the central effects of Ang II are upregulated in cardiovascular regulatory centers of the brain.1,2 It is generally agreed that Ang II generated by the systemic RAS at the brain RAS regulatory centers of the brain (AT1-R) that mediate the central effects of Ang II are upregulated in cardiovascular regulatory centers of the brain.3,4 Ang II in the brain may activate the sympathetic nervous system by stimulating reduced nicotinamide-adenine dinucleotide phosphate (NAD[P]H) oxidase–dependent superoxide production5,6 or by increasing ion channel activity.5 Selective inhibition of brain ACE activity7 or of brain AT1-R8 has been shown to substantially reduce sympathetic activity in HF.

Surprisingly little is known about the factors that increase brain RAS activity in HF. In the present study, we explored the possibility that circulating aldosterone (ALDO) is one such factor. ALDO is released from the zona glomerulosa of the adrenal glands in response to Ang II. Unlike Ang II, ALDO penetrates the blood-brain barrier,9-13 and ALDO levels measured in whole-brain tissue reliably reflect plasma levels.14 In peripheral tissues, ALDO acts on mineralocorticoid receptors (MR) to increase the synthesis of key components of the RAS both in vitro15-17 and in vivo.18,19 In the present study, we explored the hypothesis that ALDO acts similarly in the brain, upregulating RAS activity in a forebrain region that contributes to sympathetic drive in HF.

We posed 3 questions. First, does the ALDO concentration in hypothalamic tissue reflect the ALDO level in plasma? Second, does ALDO increase in the hypothalamus of rats with HF? Third, does activation of brain MR upregulate the synthesis of key components of the brain RAS in the hypothalamus of rats with HF? We also examined the effects of blocking brain MR on NAD(P)H-mediated superoxide production and neuronal excitation in the paraventricular...
nucleus (PVN) of hypothalamus, a critical cardiovascular and autonomic center that regulates sympathetic drive in HF. The PVN was selected for study because ACE and AT1-R are upregulated in the PVN in rats with HF, and inhibition of AT1-R in the PVN reduces sympathetic drive in rats with HF.

Methods

Animals

Intact and adrenalectomized (ADX) adult male Sprague-Dawley rats weighing 250 to 300 g were obtained from Harlan Inc (Indianapolis, IN). ADX rats were provided 0.9% saline to replace sodium losses. All of the rats were housed in temperature- (23±2°C) and light-controlled animal quarters and were fed rat chow ad libitum. These studies were performed in accordance with the “Guiding Principles for Research Involving Animals and Human Beings.” The experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee.

Experimental Protocols

Study 1: Relationship Between Plasma and Hypothalamic ALDO in ADX Rats

Sixteen ADX rats were divided into 4 treatment groups (n=4 for each group) that received no treatment (ADX); subcutaneous ALDO (ADX+ALDO) in doses of 0.1 µg/h or 0.5 µg/h; or 0.1 mg/mL of corticosterone (CORT) in drinking water (ADX+CORT). The average daily dose of CORT was 2.5 mg/d, determined by measuring CORT. The average plasma ALDO level was 2.5 mg/d, determined by measuring water intake. After 2 weeks of treatment, animals were euthanized with an overdose of pentobarbital. Plasma and hypothalamic tissue were collected for measurement of ALDO level by ELISA. Results were compared with data obtained from normal control rats (n=4).

Study 2: Relationship Between Plasma and Brain ALDO in HF Rats

Ten rats underwent coronary artery ligation to induce HF (n=5) or a sham surgical procedure (SHAM; n=5) and echocardiography to confirm left ventricular (LV) function. They were euthanized 4 weeks later with an overdose of pentobarbital. Plasma, hypothalamus and cortex were collected for measurement of ALDO level by ELISA. Results were compared with data obtained from normal control rats (n=4).

Study 3: Effects of Blocking Brain MR on the Brain RAS in HF Rats

Eighty rats underwent coronary artery ligation to induce HF (n=54) or SHAM (n=26) and echocardiography to assess LV function. They were divided into 3 treatment groups: SHAM rats that received no treatment (SHAM; n=26); HF rats that received intracerebroventricular (ICV) infusion of the selective MR antagonist RU28318 at 1 µg/h (HF+RU28318; n=26); and HF rats that received ICV vehicle (artificial cerebrospinal fluid; HF+VEH; n=28). Cannulas for ICV infusion were implanted 1 week before coronary ligation, and osmotic minipumps to infuse RU28318 or VEH were implanted within 24 hours after coronary ligation. RU28318 and VEH were infused for 4 weeks. In some rats, a second echocardiogram was obtained near the end of the treatment protocol. After 4 weeks of treatment, rats were anesthetized with pentobarbital to obtain hemodynamic measurements. Approximately 30 minutes later, while still under anesthesia, they were euthanized with an overdose of pentobarbital to collect blood and brain tissues for molecular studies or perfused with fixative for immunohistochemical studies.

Specific Methods

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plasma and the hypothalamus in the ADX rats infused subcutaneously with ALDO 0.5 µg/h simulated the values in the plasma and hypothalamus of control normal rats.

**HF Rats**

HF rats had significantly higher plasma (Figure 1B and 1C) and hypothalamic tissue (Figure 1B) ALDO levels than SHAM rats. The plasma and hypothalamic ALDO levels in the SHAM rats were similar to those observed in the normal control rats and the ADX rats infused with ALDO at 0.5 µg/h.

**Correlational Analysis**

The relationship between plasma and hypothalamic ALDO was examined across a variety of experimental conditions: normal (control) rats, ADX rats receiving CORT or varying levels of ALDO supplementation, and HF and SHAM rats (Figure 1D). Hypothalamic ALDO correlated closely with plasma ALDO over a wide range of plasma ALDO levels, whether circulating ALDO levels were normal (control and SHAM rats), controlled by chronic ALDO infusion, or increased in response to induction of HF.

**Effects of MR Blockade on the Brain RAS**

Real-time PCR revealed that ACE and AT1-R mRNA expression in the hypothalamus were increased 2.7-fold and 2.5-fold, respectively, in HF+VEH rats compared with SHAM rats (Figure 2A and 2B). Compared with HF+VEH rats, HF+RU28318 rats had significantly lower levels of ACE mRNA and AT1-R mRNA in the hypothalamus (by 40% for both). There were no statistically significant changes of ACE and AT1-R mRNA expression in the brain cortex among 3 groups (Figure 2A and 2B).

Western blotting analysis confirmed that protein levels for ACE and AT1-R paralleled mRNA induction (Figure 2C and 2D). ACE and AT1-R proteins were markedly upregulated in the hypothalampus of HF+VEH rats compared with SHAM rats. There was significantly less ACE and AT1-R protein in the hypothalamus of HF+RU28318 rats compared with HF+VEH rats. ACE and AT1-R protein levels in brain cortex did not differ among the 3 groups. The RU28318 treatment had no effect on circulating ALDO in HF rats (Figure 1C).

**Effects of MR Blockade on Hypothalamic Superoxide Production**

Compared with SHAM rats, HF+VEH rats exhibited a significant increase in hypothalamic mRNA for p47phox and gp91phox, 2 subunits of NAD(P)H oxidase. These increases were markedly inhibited by ICV infusion of the MR blocker RU28318 (Figure 3A). Superoxide production was enhanced in HF rats, and this was also attenuated by ICV infusion with RU28318 (Figure 3B). The NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI) (at a final concentration of 100 µmol/L) totally blocked the superoxide anion production in the hypothalamic homogenates from both groups (Figure 3B), identifying NAD(P)H oxidase as the predominant source of superoxide formation. Finally, intracellular superoxide production was detected using dihydroethidium (DHE). DHE fluorescence was abundant throughout the PVN in HF+VEH rats, including both presympathetic and neuroendocrine regions, compared with SHAM rats (Figure 4). ICV infusion of RU28318 in HF rats significantly reduced DHE fluorescence in posterior magnocellular and dorsal parvocellular regions of PVN (Figure 4B). There was no difference across treatment groups in DHE staining in hypothalamic regions surrounding PVN (data not shown).
Effect of MR Blockade on Sympathetic Excitation

Central Neuronal Excitation
The expression of Fra-LI activity was increased diffusely throughout the PVN in HF/VEH rats 4 weeks after coronary ligation compared with SHAM rats (Figure 5A and 5B). HF/RU28318 rats had fewer Fra-LI–positive PVN neurons than HF/VEH rats but more than the SHAM rats.

Plasma Norepinephrine
Plasma norepinephrine (NE), a marker of sympathetic nerve activity, was higher in HF/VEH rats compared with SHAM rats (Figure 5C). Plasma NE levels were lower in HF/RU28318 than HF/VEH rats but still higher than SHAM rats.

Characteristics of the HF Rats
HF rats assigned to treatment with RU28318 or VEH were well-matched with regard to echocardiographically defined LV function. Echocardiography performed within 24 hours of coronary ligation revealed that LV ejection fraction was reduced and LV end-diastolic volume was increased in the rats subjected to coronary artery ligation (HF rats) compared with the sham-operated rats (SHAM rats). Four weeks after coronary artery ligation, echocardiography showed that HF rats treated with RU28318 or VEH still had significant increases in LV end-diastolic volume and decreases in LV ejection fraction compared with SHAM rats. Treatment with RU28318 had no effect on LV end-diastolic volume, LV ejection fraction, or the ischemic zone as a percentage of LV circumference in HF rats. The echocardiographic data are shown in Table S2.

Systolic blood pressure, LV peak systolic pressure, and the maximum rate of rise of LV pressure were lower and LV end-diastolic pressure was higher in HF/VEH rats than in SHAM rats. The right ventricle/body weight (BW) and wet lung/BW ratios were substantially higher in HF/VEH rats compared with SHAM rats. HF/RU28318 rats had higher maximum rates of rise of LV pressure, lower LV end-diastolic pressure, and lower right ventricle/BW and wet lung/BW ratios than HF/VEH rats, but all of these values were still significantly different from SHAM rats. Systolic blood pressure and LV peak systolic pressure were not affected. There were no significant differences in diastolic blood pressures or heart rates across the experimental groups. The hemodynamic and anatomic data are shown in Table S3.

Discussion
Novel findings of this study are as follows: (1) hypothalamic tissue concentrations of ALDO correlate closely with plasma concentrations; (2) ALDO increases in the hypothalamus in rats with ischemia-induced HF; (3) inhibition of brain MR reduces hypothalamic expression of ACE and AT1-R, 2 key components of the brain RAS system, in rats with ischemia induced HF; and (4) inhibition of brain MR reduces the generation of reactive oxygen species in the PVN, an important hypothalamic nucleus that regulates sympathetic drive, in rats with HF. Concomitantly, as expected, chronic excitation of neurons in the PVN and plasma NE levels decrease. Taken together, these findings strongly suggest that ALDO of
adrenal origin, circulating in parallel to Ang II, signals the brain to increase RAS activity and, thus, sympathetic drive in HF.

Particularly striking in this study is the relationship between activation of brain MR and the brain RAS. Upregulation of brain RAS activity, with increased ACE and AT1-R binding in the PVN, has been reported previously in this model of HF. Here we demonstrate that ACE and AT1-R mRNA and protein are increased in the hypothalamus of HF rats and that chronic inhibition of brain MR significantly reduces hypothalamic RAS activity in HF. Concomitantly, NAD(P)H oxidase–dependent production of superoxide, a putative downstream mediator of the angiotensin message, is decreased in the PVN, along with excitation of PVN neurons and peripheral NE release. Previous studies in animal models of HF have found that central interventions that block MR or AT1-R or quench superoxide all reduce sympathetic nerve activity, but the interplay among these systems is still poorly understood. The present results suggest that activation of MR occurs early in the sequence of central events, facilitating the activity of the brain RAS and ultimately leading to sympathoexcitation. This interpretation is consistent with previous studies demonstrating that ALDO increases the binding of Ang II to its receptors in the PVN and that subcutaneously administered ALDO has a synergistic interaction with centrally administered Ang II on sodium consumption, arterial pressure, and other central effects of Ang II.

ALDO is not the only natural ligand for brain MR. Corticosterone (in rats) or cortisol (in humans) binds to brain MR with equal affinity. However, subsets of MR in peripheral tissues and in the brain are protected from activation by corticosterone and, thus, preserved for activation by ALDO, by colocalization with the enzyme 11β-hydroxysteroid dehydrogenase type 2. The genomic influences of ALDO-sensitive MR are inhibited by classical MR antagonists like RU28318. The present study demonstrates that tissue levels of ALDO are high in the hypothalamus of HF rats, mirroring the high levels in plasma. In peripheral tissues, such increases in ALDO lead to upregulation of tissue RAS activity. For example, in rats infused with ALDO, ACE mRNA and protein and ACE activity increase in aortic tissue, along with tissue content of Ang II and NAD(P)H oxidase subunits, and all of these effects are prevented with an MR antagonist. In rats with ischemia-induced HF, an MR antagonist prevents increases in ACE, NAD(P)H oxidase subunit p22^phox, and reactive oxygen species in aortic tissue. In the present study, an MR antagonist prevents the increases in ACE and NAD(P)H oxidase activity and superoxide in the hypothalamus—similar results, suggesting that the actions of ALDO in the brain closely resemble those in the periphery.

The extent to which these influences of ALDO on local tissue RAS indicate ALDO-induced gene transcription versus downstream responses to more limited genomic effects of ALDO cannot be fully addressed in these in vivo studies. In vitro studies suggest that ALDO induces gene expression of ACE and renin, and so may simply facilitate the synthesis of Ang II. In vivo, an ALDO-induced increase in Ang II might then account for the observed increases in NAD(P)H oxidase activity and upregulation of AT1-R. Thus, whereas ALDO may activate NAD(P)H oxidase independently, it may also increase NAD(P)H oxidase activity by increasing the Ang II available for binding to AT1-R. Similarly, ALDO may increase the expression of AT1-R by upregulating components of the mitogen-activated protein kinase/activator protein-1 signaling pathway or simply by generating more Ang II to activate this same pathway via the AT1-R. The precise mechanisms accounting for upregulation of brain RAS in HF remain to be determined, but the binding of ALDO to the MR seems to be an important contributing factor.

The present study confirms the previous observation that ALDO in brain tissues of normal rats is almost entirely of adrenal origin, fluctuating in parallel with plasma levels. It extends that observation by demonstrating that the close correlation between plasma and brain ALDO concentrations exists in the hypothalamus but not in the cortex. We can only speculate regarding the reason(s) for the apparent predilection of ALDO for hypothalamic tissue in the HF rats. Early work demonstrated a preferential distribution of labeled ALDO in hypothalamic tissue soon after acute systemic administration, but the relevance of that observation to a persistent high ALDO state like HF is not readily apparent. There may be a greater density of ALDO-sensitive MR in the hypothalamus. In a previous study, we found a greater expression of mRNA for 11β-hydroxysteroid dehydrogenase type 2 in PVN than in the cortex. Another factor may be the dense microvascular network in the PVN region of the hypothalamus, facilitating access of circulating ALDO to ALDO-sensitive MR. Receptor density and facilitated access to receptors may assume greater importance when circulating levels of ALDO are high. However, further study will be required to determine the reason(s) for this differential distribution of ALDO in hypothalamic and cortical tissues.

Whatever the mechanism, the association between increased ALDO in hypothalamic tissues, varying in direct proportion to circulating ALDO levels, and increased ACE and AT1-R expression in the hypothalamus suggests an important function for blood-borne ALDO in cardiovascular and autonomic regulation. HF rats exhibited increased superoxide (DHE staining) and increased chronic neuronal excitation (Fra-LI activity) diffusely throughout the PVN, involving neurons in both presympathetic and neuroendocrine regions of the PVN. Treatment with the MR antagonist reduced superoxide production and neuronal excitability diffusely throughout the PVN but with greater effect in parvocellular regions. One may surmise that at least some of the parvocellular PVN neurons influenced by RU28318 were presympathetic, because plasma NE levels also declined with treatment.

A caveat to be considered is that the measurements of LV hemodynamics in this study were made under pentobarbital anesthesia, which is known to reduce sympathetic drive. Because sympathetic responses to stress (eg, air jet stress) may be exaggerated in HF, the overall effect of pentobarbital may have been to minimize the responses of the HF rats and, thus, the differences between the HF and sham-operated groups. Nevertheless, mild but significant improvements in
LV end-diastolic pressure, maximum rate of rise of LV pressure, and right ventricle/BW and lung/BW ratios were observed in HF rats treated with RU28318 compared with vehicle treated HF rats, suggesting some improvement in LV function. However, these parameters are preload dependent and do not necessarily reflect differences in LV remodeling. Echocardiography, performed under ketamine sedation, revealed no differences in LV end-diastolic volume, LV ejection fraction, or percentage of ischemic zone between HF rats treated with RU28318 or vehicle. In a previous study from this laboratory, chronic oral administration of another MR antagonist had similar effects, improving volume-dependent measures of HF without affecting echocardiographic indices of LV remodeling.

**Perspectives**

The realization that neurochemical changes in the brain lead to autonomic dysfunction in HF presents new opportunities and new challenges. In experimental models, central interventions that inhibit ACE activity, the binding of Ang II to AT1-R, the binding of ALDO to MR, and NAD(P)H-dependent superoxide production, or that quench reactive oxygen species all reduce sympathetic nerve activity. Some of these interventions reduce volume accumulation and LV remodeling, likely as a consequence of their effect on sympathetic discharge. Central interventions can be as effective as peripheral interventions with the same agents but without the undesirable adverse effects. A challenge for the future is how to apply this knowledge. In clinical practice, the brain RAS is not readily accessible to therapeutic intervention. ACE inhibitors, AT1-R blockers, and MR antagonists may all cross the blood-brain barrier to a greater or lesser extent, but doses sufficient to block central neurochemical mechanisms are unlikely to be achieved without incurring serious adverse effects, eg, hypotension or hyperkalemia. A more reasonable approach to the central nervous system abnormalities in HF might be to target modifiable peripheral signals, like circulating ALDO, that stimulate the brain RAS. Clinical trials have already demonstrated a beneficial influence of ALDO antagonists in HF, likely via their effects on peripheral tissues. Agents that modify adrenal synthesis and release of ALDO may confer additional benefit by minimizing the stimulus to central neural activation.

**Sources of Funding**

This work was supported by a Grant-In-Aid award (0750164Z) from the American Heart Association Heartland Affiliate (to R.B.F.), a Merit Review award (to R.B.F.) from the Department of Veterans’ Affairs, an RO1HL073986 (to R.B.F.) from the National Institutes of Health, and institutional funds from the University of Iowa.

**Disclosures**

None.

**References**

24. Huang BS, Leenen FH. Blockade of brain mineralocorticoid receptors or Na+ channels prevents sympathetic hyperactivity and improves cardiac function.


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Hypertension. 2008;51:727-733; originally published online January 28, 2008;
doi: 10.1161/HYPERTENSIONAHA.107.099796

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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to

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Running Title: Aldosterone and the brain renin-angiotensin system
SPECIFIC METHODS

Induction of heart failure

Rats underwent sterile surgery under anesthesia (ketamine 90 mg/kg + xylazine 10 mg/kg, i.p.) to induce heart failure (HF), as previously described.1 In brief, the rats were intubated and mechanically ventilated. The heart was exposed via a left thoracotomy, the pericardium was opened and the heart was exteriorized. The left anterior descending coronary artery was ligated between the pulmonary outflow tract and the left atrium with a 6-0 silk suture passed through the superficial layers of myocardium. The heart was returned to the chest cavity, the lungs reinflated, and the chest closed. Sham-operated rats (SHAM) were prepared in the same manner but the coronary artery was not ligated.

Echocardiography

Echocardiography was performed under sedation (ketamine 25 mg/kg, i.p.) within 24 hours of coronary ligation to assess left ventricular (LV) function and assign rats to treatment groups, as previously described.2 In some rats, echocardiography was repeated at 4 week to evaluate the effect of treatment on LV function. Measurements of the ischemic zone as a percent of LV circumference (%IZ), LV ejection fraction (LVEF) and LV end-diastolic volume (LVEDV) were made. Only animals with large infarctions (IZ ≥ 40%) were used in the study.

Implantation of intracerebroventricular cannula and osmotic mini-pumps

Rats underwent sterile surgery under anesthesia (ketamine 90 mg/kg + xylazine 10 mg/kg, i.p.) to implant a cannula into a lateral cerebral ventricle (stereotaxic coordinates: 1.5 mm lateral to
midline, 1.0 mm caudal to bregma, and 3.5 mm ventral of dura). The cannula was fixed to the cranium using small screws and dental cement. An osmotic mini-pump (Alzet Osmotic Pump, Model# 2002, 0.5 µl/hr) was implanted subcutaneously behind the neck and connected to the cannula with silastic tubing.

**Hemodynamic measurements**

Rats were anesthetized with pentobarbital (50 mg/kg IP). Measurements of systolic blood pressure, diastolic blood pressure, LV end-diastolic pressure, LV peak systolic pressure, maximum rate of rise of LV pressure and heart rate were obtained with a Millar catheter, as described previously.²,³

**Anatomical measurements**

Wet lung weight and right ventricular weight, with respect to body weight, were measured as indices of pulmonary congestion and right ventricular remodeling, respectively.²

**Measurement of aldosterone in the brain and plasma**

Aldosterone (ALDO) levels in brain tissue and plasma were measured using a sensitive ELISA method.⁴ Briefly, the whole hypothalamus or a part of cortex were removed, weighed, and then homogenized in 5 ml water. The homogenate was extracted with 25 ml dichloromethane. Steroids in the dichloromethane extract were adsorbed on to a silica gel column (silica gel grade 62; Sigma-Aldrich, St. Louis, MO), pre-washed with 5 ml of dichloromethane and then eluted with 5 ml of dichloromethane containing 7% methanol to clean the sample. The organic extract was evaporated and reconstituted in 250 µl of ELISA buffer. Triplicate samples of 50 µl each
were used in for the ALDO assay. For estimation of recovery, an additional 10 homogenates from hypothalamus or cortex were loaded with 2,000 counts/min of tritiated aldosterone and processed as above. Recovery was measured by a scintillation counter and a mean recovery parameter (93 ± 3 %, mean ± SD) determined from the radioactive controls was used to correct each tissue sample. Results were expressed as picograms per gram.

For ALDO assay in plasma, 0.5 ml plasma was extracted in 5 ml of dichloromethane and reconstituted in 200–250 µl of ELISA buffer, and 50 µl were used to measure ALDO by ELISA as described. Results were expressed as picograms per milliliter.

**Measurement of norepinephrine in plasma**

The norepinephrine level in plasma was measured using a high sensitivity ELISA kit (Rocky Mountain Diagnostics, Inc, Colorado Springs), as previously described.

**Quantification of mRNA expression**

The total RNA was extracted from the brain hypothalamus or cortex using TRI Reagent (Molecular Research Center, Inc). mRNA levels for components of the renin-angiotensin system (ACE and AT1-R) and NADPH oxidase subunits (p47\textsuperscript{phox} and gp91\textsuperscript{phox}) were measured with real-time PCR following reverse transcription of total RNA. The sequences for primers and probe used are summarized in *Table S1*. Primers and probes for GAPDH were purchased from Applied Biosystems (Foster City, CA). Real-time PCR was performed using the ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The final results of real-time
PCR, expressed as the ratio of mRNA of interest to GAPDH, are displayed as a percent of the SHAM value in hypothalamic tissue.

**Western blotting analysis**

Protein was extracted from hypothalamus or brain cortex using cell lysis buffer (Cell Signaling Technology Inc, Beverly, Mass). Protein levels for ACE and AT1-R were measured with Western blotting analysis, as previously described,\(^2,5\) using polyclonal primary antibodies to ACE and AT1-R (Santa Cruz Biotechnology Inc, Santa Cruz, Calif). The density of the bands was quantified with NIH image software.

**Measurement of superoxide production**

Superoxide production in the hypothalamus was measured using lucigenin-enhanced chemiluminescence.\(^6-8\) Briefly, hypothalamic tissue was homogenized in cold Krebs/HEPES buffer (PH 7.4) for protein extraction. 30 µg protein was added to preheated Krebs/HEPES buffer (37°C) containing 5 µmol/L of lucigenin and then read in a Sirius luminometer at 30-s interval for 10 minutes. The value was subtracted from background. NADPH (100 µM) was used to stimulate NADPH oxidase. To confirm the source of superoxide, samples were preincubated with diphenylene iodonium (DPI, 10, 50 and 100 µM), a flavoprotein inhibitor of NADPH oxidase. Chemiluminescence was reported as relative light units per second normalized to protein concentration.
**Dihydroethidium staining**

Dihydroethidium (DHE), an oxidative fluorescent dye, was used to detect superoxide in situ in the paraventricular nucleus of hypothalamus (PVN) by laser scanning confocal microscope as described by others.\(^8\) Briefly, the brain was removed and immediately frozen at \(-80^\circ\text{C}\) for 1 hour, blocked in the coronal plane, and sectioned into 30-\(\mu\text{m}\) slices with a cryostat. The sections at the level of the PVN were mounted on microscope slides and incubated with DHE (5 \(\mu\text{mol/L},\) Molecular Probes) for 30 minutes at 37\(^\circ\text{C}\) in a light-protected humidified chamber. Images were visualized and fluorescence in posterior magnocellular, ventrolateral parvocellular, medial parvocellular and dorsal parvocellular of PVN were analyzed with NIH image software. The subregions of PVN were defined as described in a previous study by others.\(^9\)

**Detection of Fra-like activity**

The expression of the Fra-like (Fra-LI, fos family gene) activity has been used as a marker for chronic neuronal activation, including in studies of rats or mice with ischemia-induced HF.\(^{10-12}\) The expression of the Fra-LI activity was detected using a rabbit polyclonal antibody (c-fos K-25, Santa Cruz Biotechnology, Santa Cruz, CA). Fra-LI-positive cells in PVN were counted and analyzed as described before.\(^{10}\)
LITERATURE CITED


Table S1. Sequences for primers and probes

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Table S2. Echocardiographic measurements

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<tr>
<td>LVEDV (ml)</td>
<td>0.34 ± 0.02</td>
<td>0.79 ± 0.12*</td>
<td>0.71 ± 0.06*</td>
<td>0.73 ± 0.08*</td>
</tr>
<tr>
<td>LVEF</td>
<td>0.78 ± 0.03</td>
<td>0.31 ± 0.05*</td>
<td>0.32 ± 0.03*</td>
<td>0.34 ± 0.02*</td>
</tr>
<tr>
<td>%IZ</td>
<td>----</td>
<td>45 ± 3*</td>
<td>47 ± 2*</td>
<td>46 ± 2*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables at 4 weeks</th>
<th>SHAM (n=8)</th>
<th>HF (---)</th>
<th>HF+VEH (n=8)</th>
<th>HF+RU28318 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDV (ml)</td>
<td>0.32 ± 0.03</td>
<td>----</td>
<td>1.19 ± 0.10*</td>
<td>1.14 ± 0.07*</td>
</tr>
<tr>
<td>LVEF</td>
<td>0.73 ± 0.03</td>
<td>----</td>
<td>0.29 ± 0.02*</td>
<td>0.32 ± 0.02*</td>
</tr>
<tr>
<td>%IZ</td>
<td>----</td>
<td>----</td>
<td>44 ± 2*</td>
<td>40 ± 1*</td>
</tr>
</tbody>
</table>

SHAM: sham-operated control; HF: heart failure; HF+VEH: vehicle-treated HF; HF+RU28318: RU28318-treated HF. LVEDV: left ventricular end-diastolic volume; LVEF: left ventricular ejection fraction; %IZ: ischemic zone as a percent of left ventricular circumference. Values are expressed as mean ± SEM. *P<0.05 versus SHAM at same week.
**Table S3.** Anatomical and hemodynamic measurements

<table>
<thead>
<tr>
<th>Variables at 4 Weeks</th>
<th>SHAM (n=15)</th>
<th>HF+VEH (n=16)</th>
<th>HF+RU28318 (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>399 ± 5</td>
<td>394 ± 5</td>
<td>396 ± 10</td>
</tr>
<tr>
<td>RV/BW (mg/g)</td>
<td>0.55 ± 0.04</td>
<td>1.04 ± 0.07*</td>
<td>0.80 ± 0.04* †</td>
</tr>
<tr>
<td>Lung/BW (mg/g)</td>
<td>3.55 ± 0.08</td>
<td>8.68 ± 0.53*</td>
<td>6.77 ± 0.59* †</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>321 ± 9</td>
<td>319 ± 7</td>
<td>325 ± 11</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>122 ± 4</td>
<td>106 ± 3*</td>
<td>105 ± 4*</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>89 ± 3</td>
<td>87 ± 4</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>LVPSP (mmHg)</td>
<td>116 ± 2</td>
<td>99 ± 2*</td>
<td>104 ± 3*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>4 ± 1</td>
<td>19 ± 2*</td>
<td>14 ± 2* †</td>
</tr>
<tr>
<td>LV dP/dt (mmHg/s)</td>
<td>8278 ± 356</td>
<td>4381 ± 141*</td>
<td>6027 ± 121* †</td>
</tr>
</tbody>
</table>

BW: body weight; RV: right ventricular; HR: heart rate; SBP: systolic blood pressure; DBP: diastolic blood pressure; LVPSP: left ventricular peak systolic pressure; LVEDP: left ventricular end-diastolic pressure; dP/dt: maximum rate of rise of left ventricular pressure. Values are expressed as mean ± SEM. *P<0.05 versus SHAM, †P<0.05 versus HF+VEH.