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Angiotensin-(1-7) in Paraventricular Nucleus Contributes to the Enhanced Cardiac Sympathetic Afferent Reflex and Sympathetic Activity in Chronic Heart Failure Rats

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Abstract

Background/Aims—Cardiac sympathetic afferent reflex (CSAR) enhancement contributes to exaggerated sympathetic activation in chronic heart failure (CHF). The current study aimed to investigate the roles of angiotensin (Ang)-(1-7) in CSAR modulation and sympathetic activation and Ang-(1-7) signaling pathway in paraventricular nucleus of CHF rats.

Methods—CHF was induced by coronary artery ligation. Responses of renal sympathetic nerve activity (RSNA) and mean arterial pressure (MAP) to epicardial application of capsaicin were used to evaluate CSAR in rats with anesthesia.

Results—Ang-(1-7) increased RSNA, MAP, CSAR activity, cAMP level, NAD(P)H oxidase activity and superoxide anion level more significantly in CHF than in sham-operated rats, while Mas receptor antagonist A-779 had the opposite effects. Moreover, Ang-(1-7) augmented effects of Ang II in CHF rats. The effects of Ang-(1-7) were blocked by A-779, adenylyl cyclase inhibitor SQ22536, protein kinase A inhibitor Rp-cAMP, superoxide anion scavenger tempol and NAD(P)H oxidase inhibitor apocynin. Mas and AT₁ receptor protein expressions, Ang-(1-7) and Ang II levels in CHF increased.

Conclusions—These results indicate that Ang-(1-7) in paraventricular nucleus enhances CSAR and sympathetic output not only by exerting its own effects but also by augmenting the effects of

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Disclosure Statement

The authors declare no conflicts of interest.

Ang II through Mas receptor in CHF. Endogenous Ang-(1-7)/Mas receptor activity contributes to CSAR enhancement and sympathetic activation in CHF, and NAD(P)H oxidase-derived superoxide anions and the cAMP-PKA signaling pathway are involved in mediating the effects of Ang-(1-7) in CHF.

Keywords

Chronic heart failure; Angiotensin-(1-7); Paraventricular nucleus; Cardiac sympathetic afferent reflex; NAD(P)H oxidase-derived superoxide anions; CAMP-PKA signaling pathway

Introduction

Chronic heart failure (CHF) is a serious debilitating condition characterized by poor survival rates and an increasing level of prevalence. Exaggerated sympatho-excitation, a hallmark of CHF, contributes to arrhythmias [1], further hemodynamic deterioration and multiorgan damage and is a critical factor in the development and progression of the CHF state. The degree of sympatho-excitation is predictive of the likelihood of survival in CHF [2, 3]. The cardiac sympathetic afferent reflex (CSAR), a sympatho-excitatory reflex, can be induced by stimulating cardiac sympathetic afferents with exogenous chemicals or endogenous chemicals from the myocardium during myocardial ischemia in CHF [4, 5]. The CSAR is enhanced in CHF, and this enhancement at least partially contributes to increases in sympathetic outflow in the disease [6, 7]. Thus, CSAR suppression and, ultimately, sympatho-excitation inhibition may be good strategies for treating patients with CHF [2, 8].

The paraventricular nucleus (PVN) is an integrative site involved in regulating sympathetic outflow and cardiovascular activity [9] and is a component of the central neurocircuitry of the CSAR [10]. Angiotensin (Ang)-(1-7) is accepted as an important biologically active peptide in the renin-angiotensin system (RAS) family that adjusts sympathetic outflow and cardiovascular activity. Most of its effects are mediated by the Mas receptor [11, 12], which is abundantly expressed in the PVN [13, 14] and is selectively blocked by its specific antagonist, D-Alanine-Ang-(1-7) (A-779) [15, 16]. Our recent studies have shown that microinjection of Ang-(1-7) into either the PVN or the rostral ventrolateral medulla (RVLM), another important site for regulating sympathetic outflow, enhances the CSAR and increases arterial blood pressure and sympathetic activity in renovascular hypertensive rats [17–20]. These findings underline the important roles of Ang-(1-7) in the PVN or RVLM in modulating blood pressure, sympathetic activity and the CSAR in hypertension. However, it is not yet known whether Ang-(1-7) in the PVN contributes to CSAR enhancement and excessive sympathetic activation in CHF.

Previous studies have demonstrated that Ang-(1-7) inhibits vascular growth via prostacyclin-mediated cAMP production and protein kinase A (PKA) activation [21] and that inhibition of either adenylyl cyclase (AC) or PKA activity attenuates Ang-(1-7)-induced ERK1/2 activation in glomerular mesangial cells [22]. Our previous study also showed that the cAMP-PKA pathway participates in Ang-(1-7)-mediated effects in the RVLM [18] or PVN [17] in renovascular hypertension. In addition, superoxide anions scavengers have been shown to abolish both the effects of Ang II in the PVN [23, 24] and the effects of Ang-(1-7)

in the RVLM [25] on arterial blood pressure, sympathetic activity and CSAR in rats. However, whether the cAMP-PKA pathway and/or reactive oxygen species (ROS) are involved in mediating the effects of Ang-(1-7) in the PVN in CHF rats remains unclear. Thus, the present study was designed to determine whether Ang-(1-7) in the PVN contributes to the CSAR enhancement and sympathetic activation that occur in CHF, to elucidate its interactions with Ang II, and to determine whether the cAMP-PKA pathway and NAD(P)H oxidase-derived superoxide anions are involved in the effects of Ang-(1-7) on the CSAR and sympathetic outflow in CHF rats.

Materials and Methods

The experiments for this study were conducted using male Sprague–Dawley rats. The procedures were approved by the Nanjing Medical University Experimental Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). The rats were kept in a temperature-controlled room, maintained under a 12 h–12 h light–dark cycle and allowed free access to standard chow and tap water.

Model of CHF

CHF was induced by coronary artery ligation, as previous studies described [26–28]. Briefly, all the rats were anesthetized with sodium phenobarbital (50 mg/kg, i.p.) and handled using sterile techniques. The left coronary artery was ligated with a 6-0 suture near the point at which it branches off from the aorta. Approximately 30% of the animals used in the experiments died. Death occurred mainly during the first day after ligation. The sham-operated (Sham) rats were treated the same as the CHF rats, except their coronary arteries were not ligated. A final experiment was performed at 6–8 weeks after coronary ligation or sham surgery. The criteria used to define CHF were as follows: an elevated left ventricular end-diastolic pressure (LVEDP) (>13 mmHg) and a 40% decrease in the maximum of the first differentiation of left ventricular pressure ($LV +dp/dt_{max}$).

Echocardiography measurements

Rat transthoracic echocardiography was performed under light anesthesia (50 mg/kg sodium pentobarbital, i.p.) using an ultrasound system (Vevo 2100, VisualSonics, Canada) and a 21-MHz probe to assess left ventricular (LV) function, as described in our previous report [18]. The LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), interventricular septal thickness in diastole (IVSd), interventricular septal thickness in systole (IVSs), LV posterior wall thickness in diastole (LVPWd) and LV posterior wall thickness in systole (LVPWs) were measured, and the LV ejection fraction (EF), LV fractional shortening (FS), LV mass and LV mass/Body weight (BW) ratio were calculated. All measurements were averaged over three consecutive cardiac cycles.

General procedures of the acute experiments

The acute experiments were performed at 6–8 weeks after coronary ligation or sham surgery. Each rat was intraperitoneally anesthetized with urethane (800 mg/kg) and α -chloralose (40 mg/kg), and an appropriate level of anesthesia was maintained using supplemental doses of

anesthetic. A rodent ventilator (model 683, Harvard Apparatus Inc., USA) was used to mechanically ventilate the rats with room air. To minimize the confounding effects of the baroreflex on sympathetic activity and blood pressure, we performed and verified bilateral baroreceptor denervation and vagotomy, as previously described [18, 29]. The right carotid artery was cannulated and connected to a pressure transducer (MLT0380, ADInstruments, Australia) to record continuous arterial blood pressure (ABP), mean arterial pressure (MAP) and heart rate (HR), and a catheter entering the left ventricle from the right carotid artery was used to record LV pressure.

Renal sympathetic nerve activity (RSNA) recordings

The left renal sympathetic nerve was isolated after a retroperitoneal incision was made. To eliminate its afferent activity, we cut the renal nerve distally. The nerve was then placed on a pair of silver electrodes and immersed in warm mineral oil. Its signals were amplified and recorded by a 4-channel AC/DC differential amplifier (DP-304, Warner Instruments, Hamden, CT, USA) with a high-pass filter set at 10 Hz and a low-pass filter set at 3,000 Hz. RSNA was integrated at a time constant of 100 ms. At the end of each experiment, the background noise was determined after the central end of the nerve was sectioned and was subtracted from the integrated RSNA values. The raw and integrated RSNA, ABP, MAP and HR were simultaneously recorded with a PowerLab data acquisition system (8SP, ADInstruments, Australia). The change in RSNA was expressed as the percent change from the value recorded before chemical intervention.

Evaluation of the CSAR

The CSAR was evaluated as previously described [18, 29]. First, a limited left lateral thoracotomy was performed, and the pericardium was removed to expose the heart. Then, the CSAR was elicited by stimulating cardiac sympathetic afferent nerves via the epicardial application of a piece of filter paper (3 mm × 3 mm) containing capsaicin (1.0 nmol in 2.0 μ l) to the non-infarcted area of the LV anterior wall. One minute later, the filter paper was removed, and the ventricular surface was rinsed 3 times with 10 ml of normal saline (37 °C). The CSAR was evaluated based on the responses of RSNA and MAP to the epicardial application of capsaicin.

PVN microinjection

The rat was assembled in a stereotaxic frame (Stoelting, Chicago, USA). The stereotaxic coordinates for the PVN were 1.8 mm caudal from bregma, 0.4 mm lateral to the midline and 7.9 mm ventral to the dorsal surface, according to Paxinos & Watson's rat atlas. Bilateral PVN microinjections were performed with two glass micropipettes (50- μ m tip diameter), and the injection rate was controlled by a dual-channel microdialysis infusion syringe pump (53101V, Stoelting Co., Illinois, USA). The bilateral PVN microinjections were completed within 1 min, and the microinjection volume was 50 nl for each side of the PVN. At the end of the experiment, 50 nl of Evans Blue dye (2%) was injected into each microinjection site. The microinjection sites were subsequently histologically verified with a microscope, and the data pertaining to the microinjection sites outside the PVN were excluded from the data analysis.

PVN sample preparation

PVN tissue samples were collected and homogenized as previously reported [29]. Briefly, the brain was quickly removed from the skull of each rat and flash-frozen in liquid nitrogen before being stored at -70°C . The brain was subsequently coronal sectioned using a cryostat microtome (CM1900, Leica LTD), and the PVN area was punched out with a 15-gauge needle. The punched tissues were then homogenized and centrifuged, and the total protein in the homogenate supernatant was extracted and measured using a protein assay kit (BCA, Pierce).

Measurement of Ang II and Ang-(1-7) levels

Both Ang-(1-7) levels and Ang II levels in the PVN tissue homogenate supernatant were determined using commercial peptide enzyme-linked immunoassay kits (USCN Life Science Inc., USA), in accordance with the manufacturer's instructions.

Measurement of AT₁ receptor and Mas receptor protein expression levels

Mas and AT₁ receptor protein expression levels in the PVN were determined by western blotting, as described in previous reports [19, 20]. Briefly, the proteins in the PVN tissue homogenate supernatant were separated by a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane, which was subsequently probed with a rabbit polyclonal antibody against the AT₁ receptor (1:500; Abcam, Cambridge, MA, USA) or Mas receptor (1:200, Alomone Labs, Israel) before being incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000; Immunology Consultants Lab, Portland, OR, USA). The bands were visualized by enhanced chemiluminescence using an ECL system (Pierce Chemical, Rockford, IL, USA). GAPDH (1:5000; Bioworld Technology Inc., Louis, MN, USA) protein was used as a loading control. AT₁ receptor protein and Mas receptor protein levels were normalized to those of GAPDH protein.

Measurement of cAMP levels

cAMP levels in the PVN were determined by an enzyme-linked immunoassay kit (Cayman Chemical Co, USA), according to the manufacturer's instructions [18, 30].

Measurement of NAD(P)H oxidase activity

NAD(P)H oxidase activity in the PVN was measured by enhanced lucigenin chemiluminescence, as described in our previous reports [24, 31]. Briefly, NAD(P)H (100 μM) was added to the medium as a substrate to react with NAD(P)H oxidase to generate superoxide anions. The light emissions produced by the reactions between lucigenin (5 μM) and the superoxide anions were measured with a luminometer (20/20n, Turner, CA, USA) once every minute for ten minutes. The values represented NAD(P)H oxidase activity and were expressed as mean light unit (MLU) per minute per milligram of protein.

Measurement of superoxide anions

Lucigenin-derived chemiluminescence was used to determine superoxide anion levels in the PVN, as described in our previous reports [24, 26, 32]. Briefly, the reactions with superoxide anions were started by the addition of dark-adapted lucigenin (5 μM) to each tissue sample

to induce photon emissions, which were measured with a luminometer (20/20n, Turner, CA, USA) once every minute for ten minutes. The values represented superoxide anion levels and were expressed as a MLU per minute per milligram of protein.

Chemicals

Ang-(1-7) and A-779 were obtained from Bachem (Bubendorf, Switzerland). Ang II, capsaicin, tempol, diethyldithiocarbamic acid (DETC), apocynin, SQ22536, rp-adenosine-3',5'-cyclic monophosphothionate (Rp-cAMP), dibutyryl-cAMP (db-cAMP), N ω -nitro-l-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Ang-(1-7) and Ang II were made fresh before each experiment. All the chemicals were dissolved in normal saline, with the exception of apocynin, which was dissolved in normal saline containing 1% dimethyl sulfoxide (DMSO).

Protocols

The acute experiments were performed at 6–8 weeks after coronary ligation or sham surgery. Initially, the rats were kept in the supine position. The right carotid artery was cannulated first to detect LV pressure and then to continuously record ABP. Then, endotracheal intubation and sinoaortic denervation and vagotomy were performed. The rats were subsequently kept in a prone position, with their heads fixed in the stereotaxic frame for PVN positioning. The surgery mentioned above was completed in 30–35 min. Finally, the rats were kept in the right lateral position, with their heads fixed in the stereotaxic frame. A left lateral thoracotomy was performed to prepare for the application of chemicals to the epicardium. A left retroperitoneal incision was made, and the renal sympathetic nerve was isolated to place electrodes to record RSNA. The surgery and related operation were completed in another 20–25 min. After these surgeries, the rats were allowed to stabilize for 30 min before undergoing further treatments.

Protocol 1—The effects of PVN microinjections of saline, Ang-(1-7) (0.03 nmol), A-779 (3 nmol), A-779+Ang-(1-7), Ang II (0.03 nmol) and Ang-(1-7)+Ang II on baseline RSNA and MAP, as well as on the CSAR, were determined in 6 groups of Sham rats and 6 groups of CHF rats (n=6 for each group). The change in RSNA was expressed as the percent change from the value recorded before PVN microinjection. Baseline MAP and RSNA changes reached their maximum at the 8th min after Ang-(1-7) or A-779 microinjection or at the 3rd min after Ang II microinjection. At the time point at which the greatest response to Ang-(1-7), A-779 or Ang II occurred, the CSAR was evaluated by assessing the responses of RSNA and MAP to epicardial capsaicin application. The PVN pretreatment with A-779 or Ang-(1-7) was administered 8 min before Ang-(1-7) or Ang II treatment. The doses of treatment with Ang-(1-7), A-779 and Ang II in PVN were decided according to our preliminary studies and previous research reports [18, 19, 33, 34].

Protocol 2—Mas and AT₁ receptor protein expression levels in the PVN, as well as Ang-(1-7) and Ang II levels in the PVN, were determined in 2 groups of Sham rats and 2 groups of CHF rats (n=5 for each group).

Protocol 3—The effects of PVN microinjections of saline, SQ22536 (2 nmol), db-cAMP (1 nmol) or Rp-cAMP (1 nmol) on baseline RSNA and MAP and the CSAR, as well as on the responses of RSNA, MAP and the CSAR to Ang-(1-7) (0.03 nmol), were determined in 8 groups of Sham rats and 8 groups of CHF rats (n=6 for each group). The CSAR was evaluated 8 min after PVN microinjection. The PVN pretreatment was administered 8 min before Ang-(1-7) treatment.

Protocol 4—The effects of PVN microinjections of saline, DETC (10 nmol), tempol (20 nmol), 1% DMSO, apocynin (APO) (1 nmol) or L-NAME (15 nmol) on baseline RSNA and MAP and the CSAR, as well as on the responses of RSNA, MAP and the CSAR to Ang-(1-7) (0.03 nmol), were determined in 12 groups of Sham rats and 12 groups of CHF rats (n=6 for each group). CSAR was evaluated 8 min after PVN microinjection. The PVN pretreatment was administered 8 min before Ang-(1-7) treatment.

Protocol 5—The effects of PVN microinjections of saline, Ang-(1-7) (0.03 nmol), A-779 (3 nmol) and A-779+Ang-(1-7) on cAMP levels, NAD(P)H oxidase activity and superoxide anion levels in the PVN were detected in 4 groups of Sham rats and 4 groups of CHF rats (n=5 for each group). The A-779 pretreatment was administered 8 minutes before Ang-(1-7) treatment. Eight minutes after PVN microinjection, each rat was decapitated and prepared for measurements of cAMP levels, NAD(P)H oxidase activity and superoxide anion levels in the PVN.

Protocol 6—The effects of PVN pretreatment with saline and L-NAME (15 nmol) on the responses of RSNA, MAP and the CSAR to Ang-(1-7) (0.03 nmol) were determined in 2 groups of Sham rats and 2 groups of CHF rats (n=6 for each group). The CSAR was evaluated 8 min after PVN microinjection. The PVN pretreatment was administered 8 min before Ang-(1-7) treatment.

Statistical analysis

Data are expressed as the mean \pm S.E. One-way or two-way ANOVA followed by Bonferroni's post-hoc test was used analyze multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Anatomic and hemodynamic data pertaining to the CHF and Sham rats

BW, heart weights, infarct areas, and baseline hemodynamic parameters were measured at 6–8 weeks after coronary ligation or sham surgery (Table 1). The LV infarct area was 33.8% in CHF rats compared to 0% in Sham rats. The heart weight and heart-to-body weight ratio were significantly increased in CHF rats compared to Sham rats, a finding indicative of the occurrence of compensatory myocardial hypertrophy in the non-infarcted region of the myocardium. Systolic arterial pressure (SAP), pulse pressure (PP), LV peak systolic pressure (LVSP), LV developed pressure (LVDP) and LV $+dp/dt_{max}$ decreased, while LVEDP increased significantly in CHF rats compared to Sham rats. The combination of these

hemodynamic data and the abovementioned anatomic data were indicative of the presence of myocardial damage and suggestive of decreased contractile function in CHF rats.

Echocardiography

Echocardiography was used to evaluate LV function and geometry changes. At 6–8 weeks after coronary artery ligation, LV mass, LV mass/BW, LVEDD and LVESD were significantly enlarged, whereas the LV EF and LV FS were significantly decreased in CHF rats compared to Sham rats (Table 2). These results suggest that CHF rats displayed decreased contractile function and disease manifestations similar to those observed in dilated cardiomyopathy.

Effects of Ang-(1-7) on RSNA, MAP and the CSAR

The CSAR was enhanced in CHF rats, a finding consistent with those of our previous study [26, 35]. Microinjection of the Ang-(1-7) into PVN significantly increased the baseline RSNA and MAP, peaking at about 8th min and lasting at least 12 min, enhanced the CSAR in both Sham and CHF rats, and these effects were greater in CHF rats than that in Sham rats. PVN pretreatment with A-779, a Mas receptor antagonist, abolished the positive effects of Ang-(1-7) on RSNA, MAP and the CSAR. A-779 alone in the PVN decreased the baseline RSNA and MAP, peaking at about 8th min and lasting at least 30 min. Therefore PVN pretreatment with A-779 was administered 8 min before other treatments in this study. A-779 in PVN attenuated the CSAR in both CHF and Sham rats, and the inhibitory effects observed in CHF rats were much greater than those observed in Sham rats (Fig. 1). There is no significant difference in the period of these effects between Sham and CHF rats. Representative traces of the effects of PVN microinjections of Ang-(1-7), A779 and A779+Ang-(1-7) on baseline RSNA and MAP, and the CSAR in CHF rats are shown in Fig. 2 and 3.

Effects of Ang-(1-7) on the responses of RSNA, MAP and the CSAR to Ang II

There were no significant differences between Ang-(1-7) and Ang II with respect to the responses of RSNA, MAP and the CSAR. More importantly, PVN pretreatment with Ang-(1-7) augmented the effects of Ang II on RSNA, MAP and the CSAR in CHF rats. Interestingly, PVN pretreatment with Ang-(1-7) did not enhance the effects of Ang II on the above parameters in Sham rats (Fig. 4).

Mas receptor and AT₁ receptor protein expressions in the PVN

Both Mas receptor and AT₁ receptor protein expressions in the PVN were much higher in CHF rats than in Sham rats (Fig. 5).

Ang-(1-7) and Ang II levels in the PVN

Both Ang-(1-7) and Ang II levels in the PVN were increased in CHF rats compared to Sham rats (Fig. 5).

Effects of SQ22536, Rp-cAMP and db-cAMP

PVN pretreatment with either the AC inhibitor SQ22536 or the PKA inhibitor Rp-cAMP abolished the elevation-inducing effects of Ang-(1-7) on RSNA, MAP and the CSAR in both Sham and CHF rats, while PVN pretreatment with db-cAMP, a cAMP analogue, failed to augment the effects of Ang-(1-7) (Fig. 6). PVN microinjections of SQ22536 or Rp-cAMP alone attenuated the CSAR in both Sham and CHF rats. In addition, SQ22536 decreased baseline RSNA and MAP in CHF rats, but not in Sham rats. Db-cAMP caused greater increases in RSNA and MAP and greater enhancement of the CSAR in CHF rats than in Sham rats (Fig. 7).

Effects of tempol, DETC and apocynin

PVN pretreatment with the superoxide anion scavenger tempol or the NAD(P)H oxidase inhibitor apocynin markedly attenuated the effects of Ang-(1-7) on RSNA, MAP and the CSAR in both Sham and CHF rats, while DETC, an SOD inhibitor, had no potentiating effects when combined with Ang-(1-7) (Fig. 8). PVN microinjections of either tempol or apocynin alone decreased baseline RSNA and MAP while also inhibiting the CSAR in both sham and CHF rats, while PVN microinjections of DETC increased baseline RSNA and MAP as well as enhancing the CSAR. The magnitude of the response to each of these agents was more pronounced in CHF rats as compared with Sham rats (Fig. 9).

cAMP levels, NAD(P)H oxidase activity and superoxide anion levels in the PVN

There were no significant differences baseline cAMP levels in PVN between the CHF group and the Sham group. Ang-(1-7) caused greater increases in cAMP levels in PVN in CHF rats than in Sham rats, but A-779 had no significant effect on cAMP levels in either CHF or Sham rats. PVN pretreatment with A-779 blocked the abovementioned Ang-(1-7)-induced increases in cAMP levels (Fig. 10A). NAD(P)H oxidase activity and superoxide anion levels in the PVN in CHF rats increased compared to those in Sham rats. PVN microinjection of Ang-(1-7) increased NAD(P)H oxidase activity and superoxide anion levels within PVN in both CHF and Sham animals, an effect that was more pronounced in CHF rats. A-779 decreased basal NAD(P)H oxidase activity in both CHF and Sham rats, while superoxide anion level was significantly decreased in only the PVN from CHF rats, with a trend toward lower levels in sham animals. The effects of Ang-(1-7) in the PVN, with respect to NAD(P)H oxidase activity and superoxide anion level, were blocked by PVN pretreatment with A-779 (Fig. 10B & C).

Effects of L-NAME

PVN pretreatment with L-NAME, a nitric oxide (NO) synthase inhibitor, had no significant influence on Ang-(1-7)-induced RSNA, MAP and CSAR elevations in either Sham or CHF rats (Table 3).

Discussion

The findings of previous studies from our laboratory indicate that the CSAR is enhanced and contributes to sympathetic activation in CHF rats. PVN Ang II/AT₁ receptor activity plays a crucial role in enhancing the CSAR and inducing excessive sympathetic activation in CHF.

The findings of the present study show that Mas receptor activation by Ang-(1-7) in the PVN increases sympathetic output and enhances the CSAR in CHF rats at least in part through a ROS dependent cAMP-PKA pathway activation. Furthermore, Ang-(1-7) in the PVN enhances the effects of Ang II in CHF rats. Endogenous Ang-(1-7) and Mas receptor activity contribute to CSAR enhancement and excessive sympathetic activation in CHF rats.

It is well known that Ang II is an important biologically active peptide from the RAS family which is effected by some factors such as exercise [36, 37]. The discovery of Ang-(1-7) within the last decade added a new important component to the RAS family. Dense Mas receptor and AT₁ receptor expression occur in the PVN [13, 14, 19, 36]. Mas receptors in particular, have been shown to have unique and specific activity in response to Ang-(1-7) which is distinct from either Ang II or the angiotensin receptors, AT₁ and AT₂. Central Ang-(1-7) has also been found to play important roles in regulating sympathetic outflow and cardiovascular activity [38, 39]. Previous studies have shown that Ang II in the PVN enhances the CSAR and sympathetic output by activating the AT₁ receptor and contributes to the pathogenesis of CSAR enhancement in CHF [26, 40–42]. Some studies have reported that the Ang-(1-7)/Mas receptor axis plays hypotensive or sympathoinhibitory roles in some peripheral tissues and some parts of the brain [43–45], and opposes the effects of Ang II, presumably acting as a counter-regulator of classic Ang II/AT₁-mediated effects in heart failure [46], emotional stress [47] or inflammatory [48] conditions. However, interestingly, previous studies have also shown that this heptapeptide in the PVN uncovered an unusual role in the maintenance of sympathetic nerve activity. Indeed, microinjection of Ang-(1-7) into the PVN increases RSNA which can be blocked by its selective antagonist, A-779, indicating that this compound has an excitatory action on PVN neurons [34]. Numerous studies also have found that Ang-(1-7) plays a similar and possibly complimentary role to Ang II at various central sites, both mirroring the Ang II response and augmenting the effects of Ang II on cardiovascular activities [33, 49–52]. The findings of our recent studies indicate that microinjections of Ang-(1-7) into either the PVN or the RVLM increase RSNA and MAP and enhance the CSAR [17, 18]. Moreover, Ang-(1-7) augments the effects of Ang II in renovascular hypertensive rats [19, 20]. All of these reports above suggest that the overall effects of Ang-(1-7) on sympathetic nerve activity and blood pressure control are complex. Ang-(1-7) appears to have unique effects based on the central nervous system (CNS) area involved and, potentially, the experimental disease model utilized. In the present study, we found that the CSAR was enhanced in CHF rats, a finding consistent with those of our previous study [26] and that exogenous Ang-(1-7) administration in PVN increased RSNA and MAP and enhanced the CSAR to a greater extent in CHF rats than in Sham rats. The effects of Ang-(1-7) in the PVN are similar to the effects of Ang II, a consequence that is attributed to Mas receptor activation given that selective antagonism of this receptor with A-779 abrogated these effects. In addition, Ang-(1-7) in PVN potentiates the effects of Ang II on the CSAR, blood pressure and sympathetic outflow in CHF rats. These findings indicated that Ang-(1-7) enhances the CSAR and sympathetic output not only by exerting its own effects but also by enhancing the effects of Ang II in the CHF state, effects similar to those exerted by Ang-(1-7) in renovascular hypertension. These effects were facilitated by Mas receptor activation. We also found that treatment of the PVN with A-779 alone significantly decreased baseline RSNA and MAP, and attenuated the CSAR to a greater

extent in CHF rats than in Sham rats, indicating that there is a basal level of Ang-(1-7)/Mas receptor activity within the PVN that is more pronounced in CHF rats which contributes to CSAR enhancement and sympathetic activation in CHF rats and can be targeted to reduce both systemic blood pressure and renal sympathetic activity. The findings of the current study suggest that Ang-(1-7) in PVN may play an unusual or special role in regulating sympathetic outflow and blood pressure in CHF, a role that differs from its broader and well-established roles, which put forward a new and different cognition for the therapeutic potential of Ang-(1-7) for heart failure, as suggested in a previous report [46].

In the current study, we found that both Ang-(1-7) levels and Mas receptor protein expression in the PVN in CHF rats were increased compared with those in Sham rats, findings similar to those regarding the Ang II/AT₁ receptor, indicating that the enhanced effects of Ang-(1-7) in the PVN in CHF rats are probably the results of both Mas receptor expression upregulation and Ang-(1-7) level elevations. Both overexcited Ang-(1-7)/Mas receptor activity and Ang II/AT₁ receptor activity contribute to CSAR enhancement and sympathetic activity potentiation in the CHF state. Ang-(1-7)/Mas receptor activity seems to play a more important role in this process than Ang II/AT₁ receptor activity; thus, suppressing Ang-(1-7)/Mas receptor activity may slow the development and progression of the CHF state.

Our previous work identified that the cAMP-PKA pathway is involved in the excitatory effects of Ang-(1-7) on sympathetic activity, blood pressure and the CSAR in the PVN and RVLM; blockade of the cAMP-PKA pathway in the PVN or RVLM attenuates increased sympathetic activity induced by Ang-(1-7) in renovascular hypertension [17, 18]. Similarly, we were interested in this pathway for Ang-(1-7) mediated changes in the PVN from animals with CHF. It has been known that Ang-(1-7) elevates cAMP concentrations in primary cells, such as endothelial or mesangial cells. PKA inhibitors block the effect of Ang-(1-7) on the Ang II-stimulated proximal tubule Na⁺-ATPase, while cAMP mimics the effects of Ang-(1-7); Ang-(1-7) upregulates ATP-binding cassette transporter A1 expression through the cAMP signaling pathway in RAW 264.7 macrophages [22, 53–55]. In present study, reducing the production of cAMP, by using a specific membrane-permeable AC inhibitor, or blocking PKA activity, by a specific membrane-permeable PKA inhibitor, attenuated Ang-(1-7)-induced RSNA and MAP elevations and CSAR enhancement in both CHF and Sham rats. Microinjections of Ang-(1-7) into the PVN caused a greater increase in cAMP levels in CHF rats than in Sham rats. These effects were abolished by pretreatment with the Mas receptor antagonist A779. These results indicate that the cAMP-PKA pathway is involved in regulating the effects of Ang-(1-7) on RSNA, MAP and the CSAR in CHF rats. In addition, we also found that SQ22536 alone in the PVN decreased baseline RSNA and MAP in CHF rats, and that both SQ22536 and Rp-cAMP significantly attenuated the CSAR in both CHF and Sham rats, while db-cAMP, a cAMP analogue, caused greater increases in RSNA and MAP and greater enhancement of the CSAR in CHF rats than in Sham rats, which suggests that the cAMP-PKA pathway contributes to tonic control of the CSAR and sympathetic activation in CHF.

It has been reported that NAD(P)H oxidase-derived ROS in the PVN are novel molecules that contribute to the enhancement of the CSAR in CHF rats [26, 56, 57] and are involved in

the brain intracellular signaling pathways of Ang II [31, 58, 59]. The results of our previous study showed that NAD(P)H oxidase-derived superoxide anions in the RVLM also play roles in modulating the effects of Ang-(1-7) on RSNA and MAP and the CSAR in normal rats [25]. However, whether ROS are the intracellular signals that regulate the effects of Ang-(1-7) in the PVN in CHF rats remains unclear. In the present study, scavenging free radicals or inhibiting NAD(P)H oxidase inhibitor within the PVN abolished the effects of exogenous Ang-(1-7) on RSNA, MAP and CSAR in both CHF and Sham rats. By preventing the catalyzation of ROS species, using microinjection of a SOD inhibitor into the PVN, we show augmented RSNA, MAP and CSAR both in the presence and absence of exogenous Ang-(1-7). Again, the effects for each of these agents were more pronounced in CHF animals and even in the absence of Ang-(1-7) stimulation there was a prominent effect. NAD(P)H oxidase activity and superoxide anion levels were increased in CHF rats compared with Sham rats, and microinjections of Ang-(1-7) into the PVN caused additional increases in these levels, while A-779 treatment decreased NAD(P)H oxidase activity and superoxide anion levels to a greater extent in CHF rats than in Sham rats. Moreover, the effects of Ang-(1-7) were abolished by pretreatment with A779. These results suggested that the abovementioned NAD(P)H oxidase-derived superoxide anions in the PVN are important intracellular signaling molecules that mediate the positive effects of Ang-(1-7) on RSNA, MAP and the CSAR in CHF rats.

In the present study, we found that both the cAMP-PKA pathway and NAD(P)H oxidase-derived superoxide anions in the PVN are involved in mediating the effects of Ang-(1-7) on RSNA, MAP and the CSAR in CHF rats. These findings led us to question whether a relationship exists between these two intracellular signaling pathways, given that they mediate the effects of Ang-(1-7) together. Some studies have reported that a complicated relationship exists between the AC-cAMP-PKA signaling pathway and ROS production [60, 61]. Depletion of NAD(P)H oxidase 4 expression by siRNA decreased arginine vasopressin-induced cellular cAMP concentrations, PKA activity and aquaporin-2 mRNA expression induced by forskolin, a potent activator of AC, in mpkCCDcl4 cells [61]. Therefore, we speculated that the effects of Ang-(1-7) in the PVN in CHF rats is through Mas receptor activation followed by ROS-mediated activation of AC-cAMP-PKA pathway, a phenomenon that needs to be studied in the future.

It has been reported that NO mediates some effects of Ang-(1-7) via Mas receptors in some tissues [52, 62]. To determine whether NO is involved in mediating the effects of Ang-(1-7) on RSNA, MAP and the CSAR in CHF rats, we examined the influence of the NO synthase inhibitor L-NAME on the effects of Ang-(1-7). We found that L-NAME had no significant influence on Ang-(1-7)-induced increases in RSNA and MAP and enhancements of the CSAR in either Sham or CHF rats, which suggested that inhibiting NO synthase to decrease the NO generation had no effect on Ang-(1-7)-induced increases in RSNA and MAP or enhancement of the CSAR. NO is not involved in the sympatho-excitatory and CSAR-enhancing effects of Ang-(1-7) in the PVN in CHF.

In the present study, we found that Ang-(1-7) played roles similar to those of Ang II, moreover, Ang-(1-7) potentiated the effects of Ang II on the CSAR, blood pressure and sympathetic outflow in CHF rats. It is known that both AT₁ receptors and Mas receptors are

selective G protein-coupled receptors [16]. Furthermore, NAD(P)H oxidase-derived superoxide anions and cAMP-PKA signaling pathways implicated in the present study have also been shown to be important to the effects of Ang II [31, 59, 63]. Therefore, we speculate that Ang II and Ang-(1-7) participate in similar intracellular downstream signaling pathways in the PVN in CHF, which may explain the similarities in the effects exerted by these two agents and the abovementioned Ang-(1-7)-mediated enhancements of the effects of Ang II.

Conclusion

Ang-(1-7) in the PVN enhances the CSAR and sympathetic output in CHF rats not only by exerting its own effects but also by enhancing the effects of Ang II through Mas receptor activation, an effect that is further augmented in CHF rats. Endogenous Ang-(1-7) in the PVN contributes to CSAR enhancement and sympathetic activation in CHF. Both NAD(P)H oxidase-derived superoxide anions and the cAMP-PKA signaling pathway are important intracellular signaling mechanisms that mediate the positive effects of Ang-(1-7) on RSNA, MAP and the CSAR in CHF rats. Interventions targeting the Ang-(1-7)/Mas receptor activity in the PVN may be developed as therapeutic strategies for treating this cardiovascular diseases, CHF.

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Abbreviations

CHF	chronic heart failure
Ang-(1-7)	angiotensin-(1-7)
PVN	paraventricular nucleus
CSAR	cardiac sympathetic afferent reflex
RSNA	renal sympathetic nerve activity
ABP	arterial blood pressure
MAP	mean arterial pressure
LV	left ventricle
A-779	D-Alanine-Ang-(1-7)
ACE	angiotensin converting enzyme
Ang II	angiotensin II
AT₁	angiotensin type 1

SOD	superoxide dismutase
ROS	reactive oxygen species
PKA	protein kinase A
NO	nitric oxide

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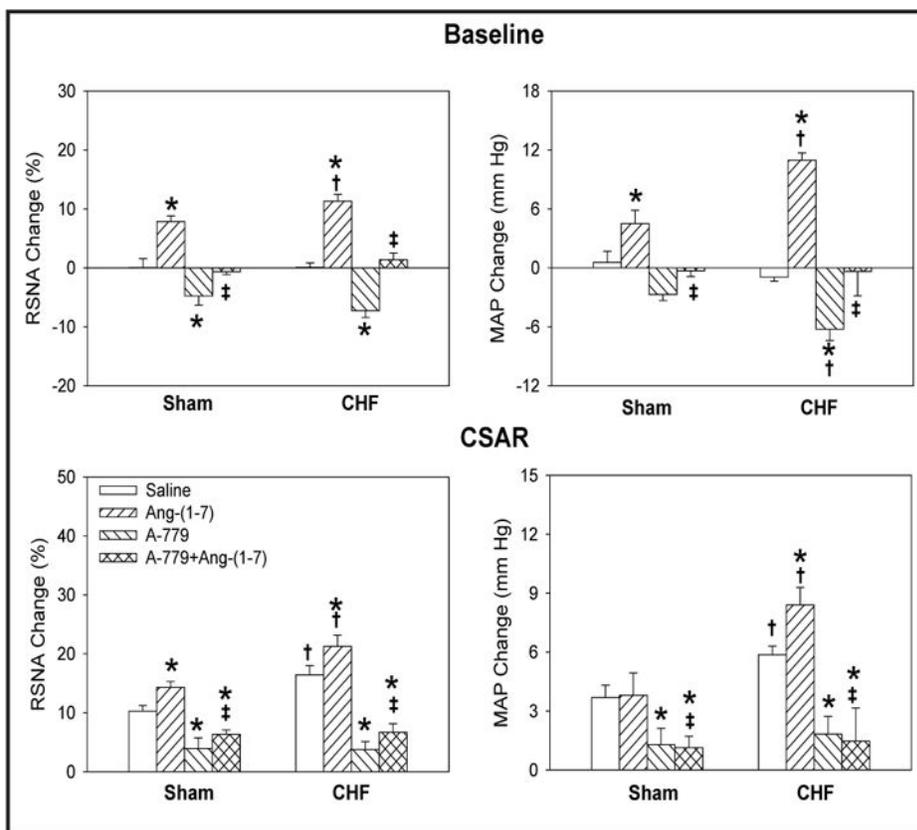


Fig. 1. Effects of PVN microinjection of saline, Ang-(1-7) (0.03 nmol), A-779 (3 nmol), A-779+Ang-(1-7) on the baseline RSNA and MAP and CSAR in Sham rats and CHF rats. The CSAR was evaluated by the RSNA and MAP response to epicardial application of capsaicin (1 nmol). Values are mean \pm SE. * $P < 0.05$ compared with saline. † $P < 0.05$ compared with Sham. ‡ $P < 0.05$ compared with Ang-(1-7) alone. $n = 6$ for each group.

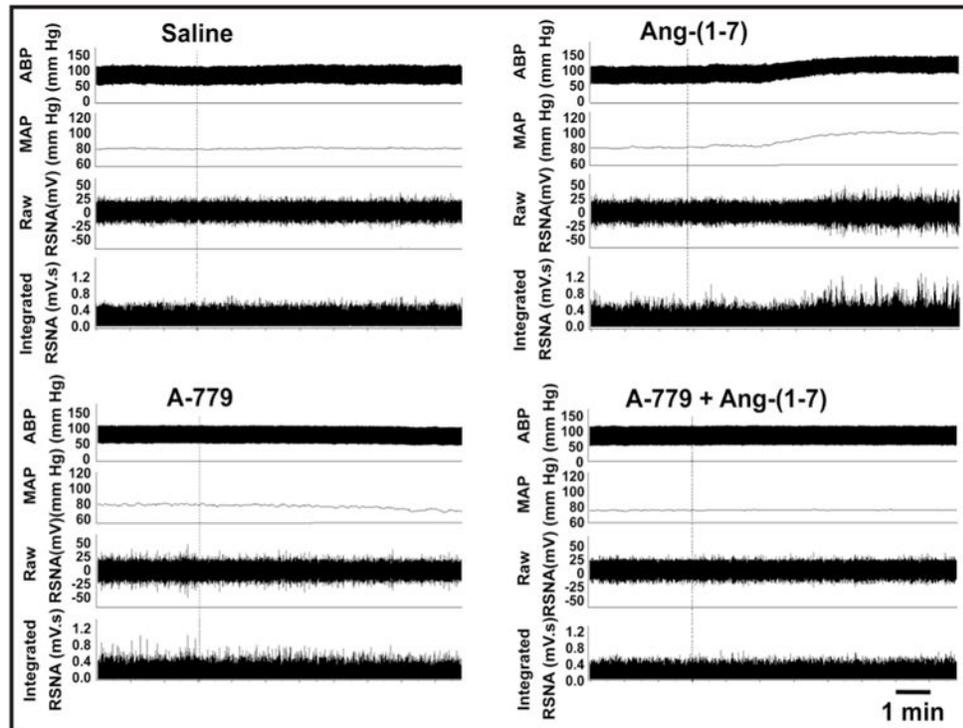


Fig. 2. Representative recordings showing the effects of PVN microinjection of saline, Ang-(1-7) (0.03 nmol), A-779 (3 nmol) and A-779+Ang-(1-7) on the baseline RSNA and MAP in CHF rats.

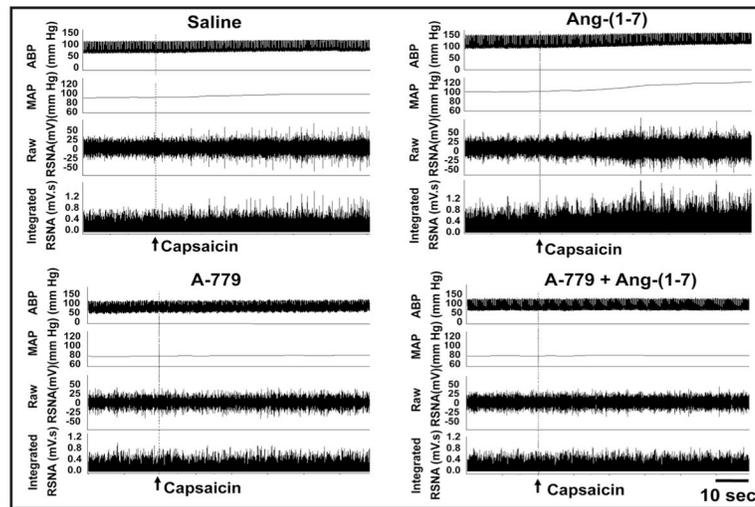


Fig. 3. Representative recordings showing the effects of PVN microinjection of saline, Ang-(1-7) (0.03 nmol), A-779 (3 nmol) and A-779+Ang-(1-7) on the CSAR in CHF rats. The CSAR was evaluated by the RSNA and MAP responses to epicardial application of capsaicin (1 nmol).

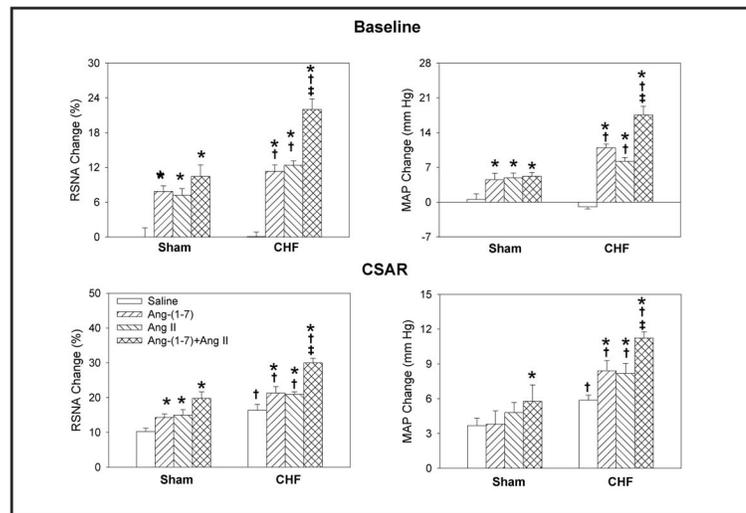


Fig. 4. Effects of PVN microinjection of saline, Ang-(1-7) (0.03 nmol), Ang II (0.03 nmol), Ang-(1-7)+Ang II on the baseline RSNA and MAP and CSAR in Sham rats and CHF rats. The CSAR was evaluated by the RSNA and MAP response to epicardial application of capsaicin (1 nmol). Values are mean \pm SE. * $P < 0.05$ compared with saline. † $P < 0.05$ compared with Sham. ‡ $P < 0.05$ compared with Ang II alone. $n = 6$ for each group.

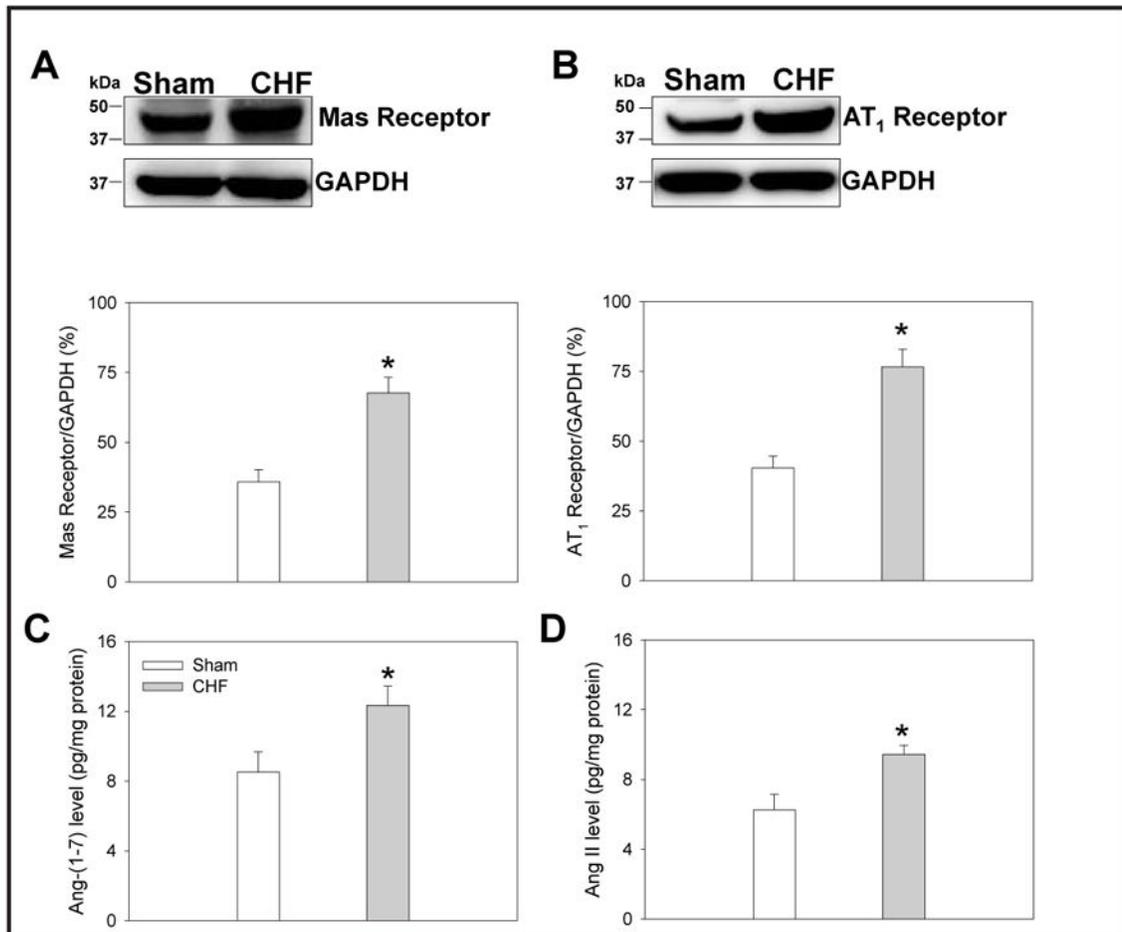


Fig. 5. Mas receptor protein expression (A), AT₁ receptor protein expression (B), Ang-(1-7) level (C) and Ang II level (D) in PVN in Sham rats and CHF rats. Values are mean ± SE. * P < 0.05 compared with Sham rats. n = 5 for each group.

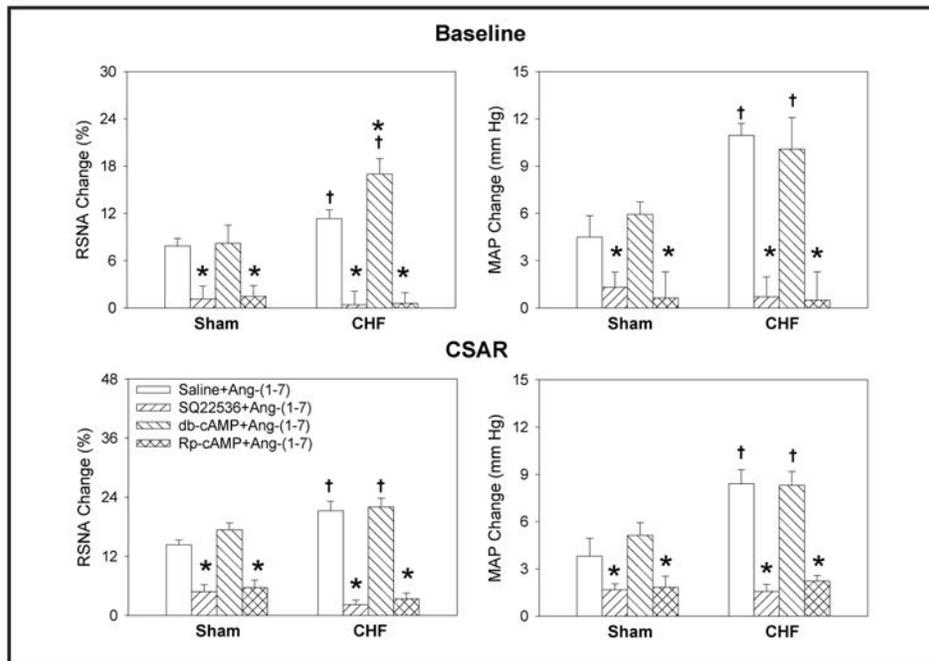


Fig. 6. Effects of the PVN pretreatment with saline, SQ22536 (2 nmol), db-cAMP (1 nmol) and Rp-cAMP (1 nmol) on the RSNA, MAP and CSAR responses to Ang-(1-7) (0.03 nmol) in Sham and CHF rats. The CSAR was evaluated by the RSNA and MAP response to epicardial application of capsaicin (1 nmol). Values are mean \pm SE. * $P < 0.05$ compared with saline. † $P < 0.05$ compared with Sham. $n = 6$ for each group.

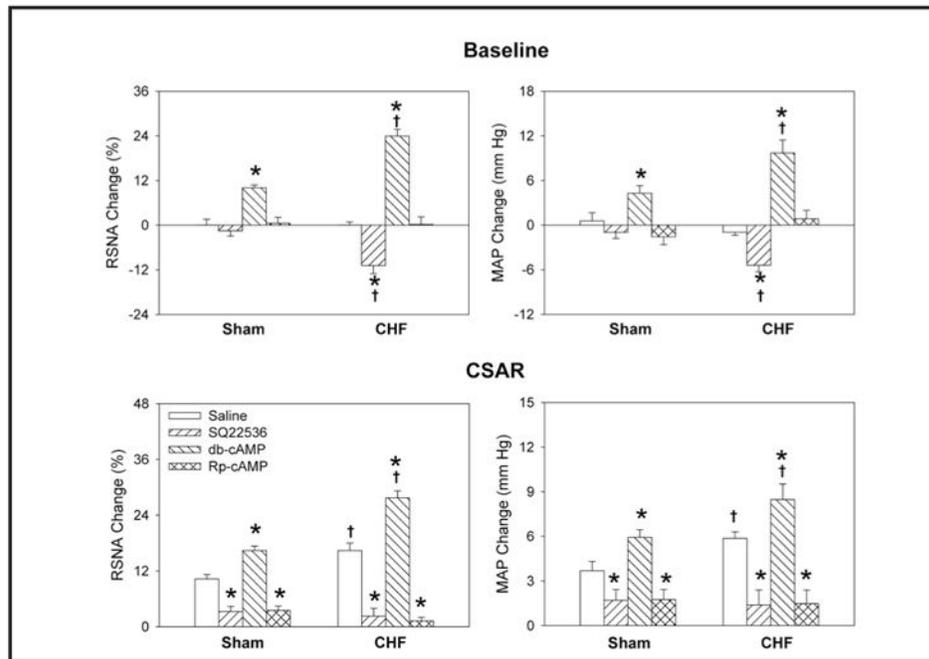


Fig. 7.

Effects of the PVN microinjection of saline, SQ22536 (2 nmol), db-cAMP (1 nmol) and Rp-cAMP (1 nmol) on the baseline RSNA and MAP and CSAR in Sham and CHF rats. The CSAR was evaluated by the RSNA and MAP response to epicardial application of capsaicin (1 nmol). Values are mean \pm SE. * $P < 0.05$ compared with saline. † $P < 0.05$ compared with Sham. $n = 6$ for each group.

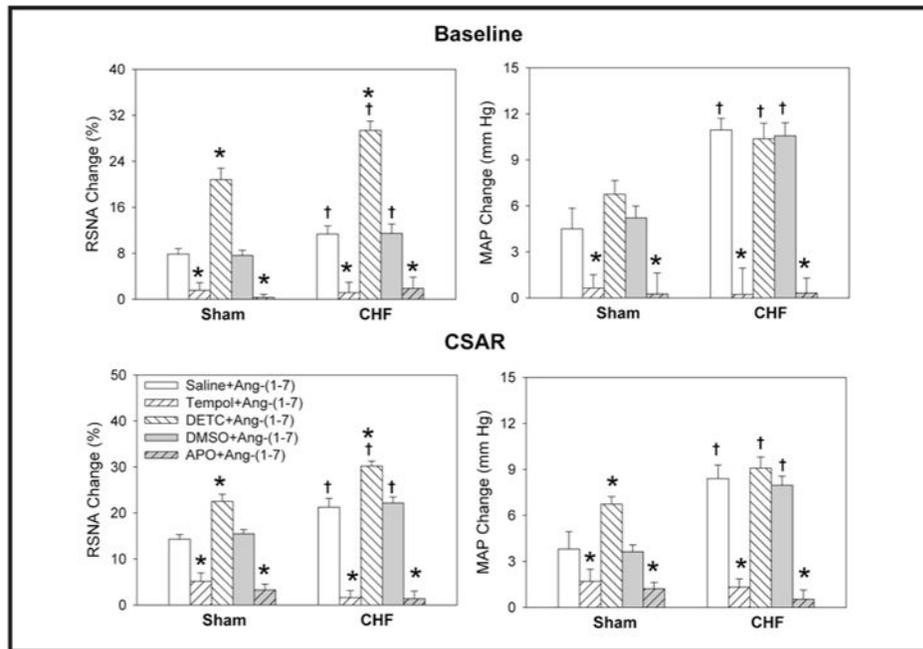


Fig. 8. Effects of the PVN pretreatment with saline, DETC (10 nmol), tempol (20 nmol), 1% DMSO or apocynin (APO) (1 nmol) on the RSNA, MAP and CSAR responses to Ang-(1-7) (0.03 nmol) in Sham rats and CHF rats. The CSAR was evaluated by the RSNA and MAP response to epicardial application of capsaicin (1 nmol). Values are mean \pm SE. * $P < 0.05$ compared with saline or DMSO. † $P < 0.05$ compared with Sham. $n = 6$ for each group.

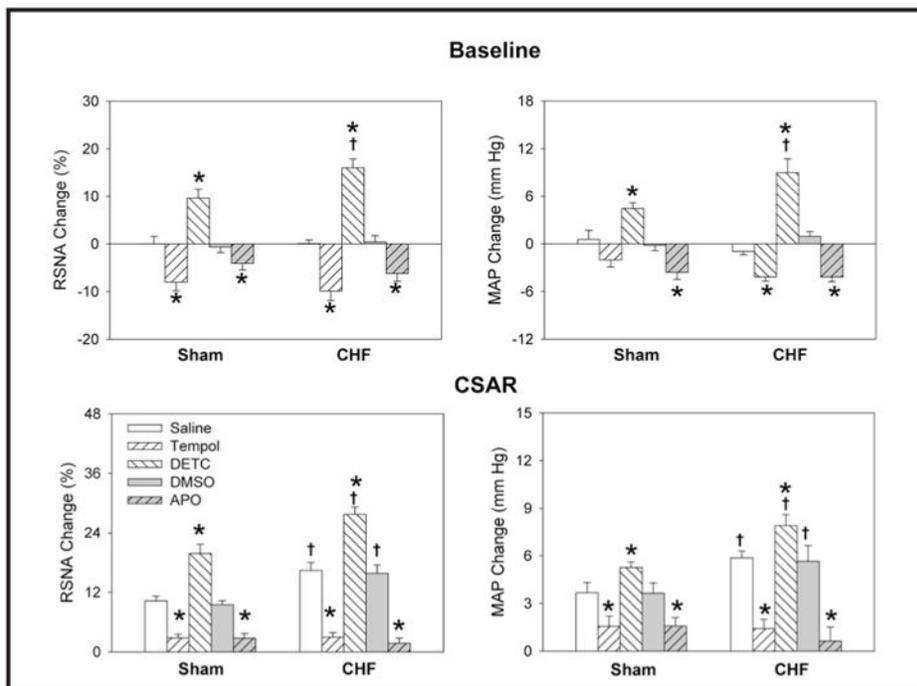
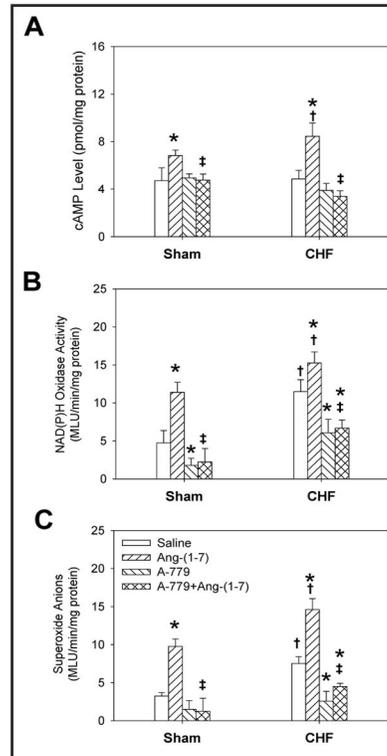


Fig. 9. Effects of the PVN microinjection of saline, DETC (10 nmol), tempol (20 nmol), 1% DMSO or apocynin (APO) (1 nmol) on the RSNA, MAP and CSAR in Sham rats and CHF rats. The CSAR was evaluated by the RSNA and MAP response to epicardial application of capsaicin (1 nmol). Values are mean \pm SE. * $P < 0.05$ compared with saline or DMSO. † $P < 0.05$ compared with Sham. $n = 6$ for each group.

**Fig. 10.**

Effects of the PVN microinjection of saline, Ang-(1-7) (0.03 nmol), A-779 (3 nmol) and A-779+Ang-(1-7) on the cAMP level, NAD(P)H oxidase activity and superoxide anions level in PVN in Sham and CHF rats. Values are mean \pm SE. * $P < 0.05$ compared with Saline. † $P < 0.05$ compared with Sham. ‡ $P < 0.05$ compared with Ang-(1-7). $n = 5$ for each group.

Table 1

Body weight, heart weight, infarcted area and baseline hemodynamics in a representative group of CHF and Sham rats. LV, left ventricle; LV +dP/dt_{max}, maximum of the first differentiation of LV pressure. Data are given as mean ± SE.

	Sham	CHF
Body weight (g)	333.7±4.8	322±8.9
Heart weight (g)	1.3±0.1	1.6±0.1 *
Heart weight/Body weight (g/kg)	3.8±0.1	4.9±0.2 *
Infarct size (% LV area)	0	33.8±2.2 *
Systolic arterial pressure (mm Hg)	128.7±3.8	105.7±5.6 *
Diastolic arterial pressure (mm Hg)	75.5±3.4	73.9±5.1
Pulse pressure (mm Hg)	53.2±3.1	31.8±2.6 *
Mean arterial pressure (mm Hg)	93.2±3.2	84.5±5.2
Heart rate (beats/min)	393.9±7.4	373.8±11.2
LV peak systolic pressure (mm Hg)	127.4±4.0	97.4±6.6 *
LV end-diastolic pressure (mm Hg)	-0.8±0.6	14.3±0.8 *
LV developed pressure (mm Hg)	128.2±4.2	83.2±6.9 *
LV +dP/dt _{max} (mm Hg/sec)	3580.6±83.5	1550.2±180.9 *

*P<0.05 compared with Sham rats.

n=6 for each group

Table 2

Echocardiographic data of the left ventricle in a representative group of Sham rats and CHF rats. LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; IVSd, interventricular septal thickness in diastole; IVSs, interventricular septal thickness in systole; LVPWd, left ventricular posterior wall thickness in diastole; LVPWs, left ventricular posterior wall thickness in systole; FS, fractional shortening; EF, ejection fraction; LV, left ventricular; BW, body weight. Values are mean \pm SE.

	Sham	CHF
LVEDD (mm)	7.12 \pm 0.23	9.56 \pm 0.58 *
LVESD (mm)	4.65 \pm 0.13	7.08 \pm 0.48 *
IVSd (mm)	1.79 \pm 0.05	1.83 \pm 0.19
IVSs (mm)	2.86 \pm 0.13	2.62 \pm 0.21
LVPWd (mm)	1.86 \pm 0.03	1.78 \pm 0.21
LVPWs (mm)	3.21 \pm 0.11	2.48 \pm 0.06 *
FS (%)	43.4 \pm 1.24	25.9 \pm 0.92 *
EF (%)	72.6 \pm 1.33	43.7 \pm 0.98 *
LV mass (g)	1.19 \pm 0.08	1.51 \pm 0.21 *
LV mass/BW (mg/s)	3.47 \pm 0.08	4.66 \pm 0.07 *

* P<0.05 compared with Sham.

n=6 for each group

Influence of pretreatment with saline or L-NAME on the effects of Ang-(1-7) in the PVN in Sham and CHF rats. The CSAR was evaluated by the RSNA and MAP responses to the epicardial application of capsaicin. Values are expressed as mean \pm SE.

Table 3

Pretreatment	Sham		CHF		
	Saline	L-NAME	Saline	L-NAME	
Baseline	RSNA change (%)	7.85 \pm 0.98	8.04 \pm 0.99	11.34 \pm 0.74 [†]	11.77 \pm 0.81 [†]
	MAP change (mm Hg)	4.5 \pm 1.35	5.2 \pm 0.85	11.0 \pm 0.75 [†]	10.2 \pm 0.99 [†]
CSAR	RSNA change (%)	14.31 \pm 1.01	14.85 \pm 1.11	21.26 \pm 1.92 [†]	20.05 \pm 0.94 [†]
	MAP change (mm Hg)	3.8 \pm 1.14	4.2 \pm 0.97	8.4 \pm 0.89 [†]	8.9 \pm 0.85 [†]

* P<0.05 vs. the Saline

[†] P<0.05 vs. Sham.

n=6 for each group