

Online Supplement

to

Angiotensin II Triggered p44/42 Mitogen-Activated Protein Kinase  
Mediates Sympathetic Excitation in Heart Failure Rats

Shun-Guang Wei,<sup>1,2</sup> Yang Yu,<sup>1</sup> Zhi-Hua Zhang,<sup>1</sup> Robert M. Weiss,<sup>1,3</sup> Robert B.  
Felder<sup>1,2,3</sup>

Department of Internal Medicine (1), Neuroscience Program (2)

University of Iowa Carver College of Medicine

and

Veterans Affairs Medical Center (3)

Iowa City, IA 52242

Running Title: MAPK and sympathetic excitation in heart failure

## **SPECIFIC 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.<sup>1</sup> Sham-operated (SHAM) rats underwent the same surgery but did not undergo coronary ligation.

### **Echocardiographic assessment of heart failure**

Approximately 24 hr after coronary artery ligation or sham surgery, rats were sedated with ketamine (50 mg/kg, IP) and left ventricular (LV) function was assessed by two-dimensional echocardiography as previously described.<sup>1</sup> The ischemic zone (IZ) as a percent of LV circumference (% IZ), LV ejection fraction, and LV end-diastolic volume were measured. Only animals with large infarctions (% IZ >35 %) were used in this study.

### **Baroreceptor denervation**

Sinoaortic baroreceptor denervation was performed in normal rats using a method described previously.<sup>2</sup> Briefly, baroreceptors were denervated by cutting the carotid sinus and aortic depressor nerves bilaterally. The aortic depressor nerves were identified by their characteristic anatomy and were cut near their junctions with the superior laryngeal nerves. The carotid sinus nerves were identified by their origin in the carotid bifurcation and insertion into the glossopharyngeal nerves, and were then sectioned near their insertion into the glossopharyngeal nerves. The adventitia was stripped from carotid

arteries and the arteries were swabbed with phenol. Baroreceptor denervation was confirmed by the lack of change in heart rate (HR) and renal sympathetic nerve activity (RSNA) when arterial pressure (AP) was increased or decreased by intravenous injection of phenylephrine (5 µg/kg) or sodium nitroprusside (10 µg/kg), respectively.

### **Acute electrophysiological and hemodynamic recording**

Details of the method have been described previously.<sup>2</sup> In brief, rats were anesthetized with urethane (1.5 g/kg, IP), supplemented as needed (0.1 g/kg). A femoral arterial catheter was implanted to measure arterial pressure. Body temperature was maintained at  $37 \pm 1^\circ\text{C}$  with a heating pad and heat lamp. A renal nerve was exposed via a left flank incision, and was dissected free from surrounding tissue and placed on bipolar silver wire recording electrodes to record RSNA. Alternatively, a lumbar nerve was exposed via a midline abdominal incision and was isolated and placed on bipolar silver wire recording electrodes to record lumbar sympathetic nerve activity (LSNA). Nerve and electrodes were stabilized with Kwik-Cast silicon sealant (WPI, INC, Sarasota, FL) and the incisions were closed.<sup>2</sup>

The animal was fixed in a stereotaxic frame and implanted with a 29-gauge stainless steel guide cannula with the tip of the cannula placed exactly 2 mm above the left lateral cerebral ventricle using the following coordinates: anteroposterior, -1.0 mm; dorsoventral, -2.5 mm; and mediolateral, -1.5 mm (left), with bregma as a reference.<sup>3</sup> To perform an intracerebroventricular (ICV) infusion, a 33-gauge injection cannula connected to 50 µl micro-syringe was inserted into the guide cannula. The 33-gauge

injection cannula was extended 2 mm past the tip of the guide cannula. The injections were performed with a micropump (Harvard Apparatus, Holliston, MA).

Data were acquired with a Cambridge Electronics Design laboratory interface (CED, model 1401; Cambridge, UK) linked to a personal computer. The RSNA or LSNA was initially processed with by Paynter filter (20-ms time constant, BAK Electronics; Germantown, MD) to rectify and integrate the raw multifiber signal. The AP signal was passed to the CED 1401 via a Gould TA240S chart recorder (Gould Instruments, Valley View, OH). HR was derived from the frequency of the AP pulses. Digitized data were stored for subsequent off-line analysis with Spike2 software (CED).

### **Drugs Infused**

Losartan (a gift from Du Pont/Merck) and angiotensin II (ANG II, Sigma, St Louis, MO) were dissolved in artificial cerebrospinal fluid (aCSF). PD98059, UO126, SP600125, SB203580 and LY 294002 were obtained from Tocris (Ellisville, MO). 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. Doses of the inhibitors were extrapolated from acute ICV injection studies<sup>4-6</sup>

### **Hemodynamic and anatomical assessment of heart failure**

At the conclusion of each experiment in the HF and SHAM rats, with the animal still under anesthesia, a Millar Mikro-tip catheter was advanced via the right carotid artery into the aorta to measure arterial pressure, and then into the LV to measure peak

systolic pressure (LVSP), end diastolic pressure (LVEDP) and the rate of change in LV systolic pressure over time (LV dp/dt). Finally, the rat was either perfused to collect the brain for immunohistochemical studies or euthanized with an overdose of anesthesia. The heart and lungs were collected, and the right ventricle and lungs were weighed.

### **Immunohistochemistry**

Brains were embedded with OCT and rapidly frozen in alcohol chilled dry ice. Coronal forebrain sections (12  $\mu\text{m}$ ) containing PVN were made using a cryostat and then stored at  $-80\text{ }^{\circ}\text{C}$ .

Immunohistochemical visualization of Fra-LI activity in PVN was performed using antibodies and avidin–biotin–peroxidase methods as previously described.<sup>7</sup> The primary antibody for detection of Fra-LI immunoreactivity was rabbit polyclonal antibody (K-25) to c-Fos (sc-253, Santa Cruz Biotechnology, CA) diluted 1:4000 in 0.01 M PBS with 0.3% Triton X-100. The secondary antibody was biotinylated goat anti-rabbit antibody solution (1:200). Finally, sections were treated with avidin–biotin–peroxidase complex (VECTASTAIN ABC Kit, PK6101) and peroxidase substrate solution (SK4100, Vector Laboratories, CA) until the desired stain intensity developed.

For immunohistochemical analysis, the numbers of Fra-LI positive neurons in a  $100\times 100\mu\text{m}$  window located over the dorsal parvocellular (PVN-dp), the ventrolateral parvocellular (PVN-vlp) or the posterior magnocellular subdivision (PVN-pm) of PVN<sup>8</sup> were counted manually. Data were represented as positive cells per  $10^4\mu\text{m}^2$

## **Immunofluorescence**

Immunofluorescent triple staining was used to examine the phosphorylated p44/42 MAPK expression and its co-localization with Fra-LI activity in PVN neurons in SHAM and HF rats. Double immunofluorescent staining was used to measure the Fra-LI activity in PVN neurons in rats treated with ICV ANG II combined with vehicle or PD98059. The sections were incubated with the primary antibodies, the rabbit monoclonal antibody to phospho-p44/42 MAPK (Thr202/Tyr204, #4376, Cell Signaling Technology) and/or the mouse monoclonal antibody to Fra-LI (sc-28310, 1:100, Santa Cruz), followed by secondary antibodies Alex Fluor 488 goat anti-rabbit IgG (A-11070, 1:200, Invitrogen) and Alex Fluor 546 goat anti-mouse IgG (A-11003, 1:200, Invitrogen). The sections were further incubated with To-Pro-3 (1:2000, Invitrogen) to counterstain cell nuclei. Immunofluorescent staining was visualized with a confocal laser-scanning microscope (Zeiss LSM 510, Carl Zeiss, Inc). The numbers of neurons with phosphorylated p44/42 MAPK and Fra-LI immunoreactivity, and the numbers of neurons with both, were counted manually in a 100x100 $\mu$ m window placed over PVN-dp, the PVN-vlp or the PVN-pm. Data were reported as positive cells per 10<sup>4</sup> $\mu$ m<sup>2</sup>.

## EXTENDED RESULTS

### Effects of ICV p44/42 MAPK inhibitors on LSNA

ICV administration of either PD98059 (**Figure S1 A and B**, n=7) or UO126 (**Figure S1 C and D**, n=7) did not significantly change LSNA ( $-4.5 \pm 3.2$ ;  $-3.0 \pm 3.1$  % change, respectively) in HF rats, even though MAP ( $-10.7 \pm 2.5$ ;  $-9.7 \pm 2.9$  mmHg, respectively) and HR ( $-17.8 \pm 4.3$ ;  $-15.1 \pm 4.1$  bpm, respectively) decreased. Since no effect on LSNA found in HF rats, no sham-operated rats were tested with p44/42 MAPK inhibitors.

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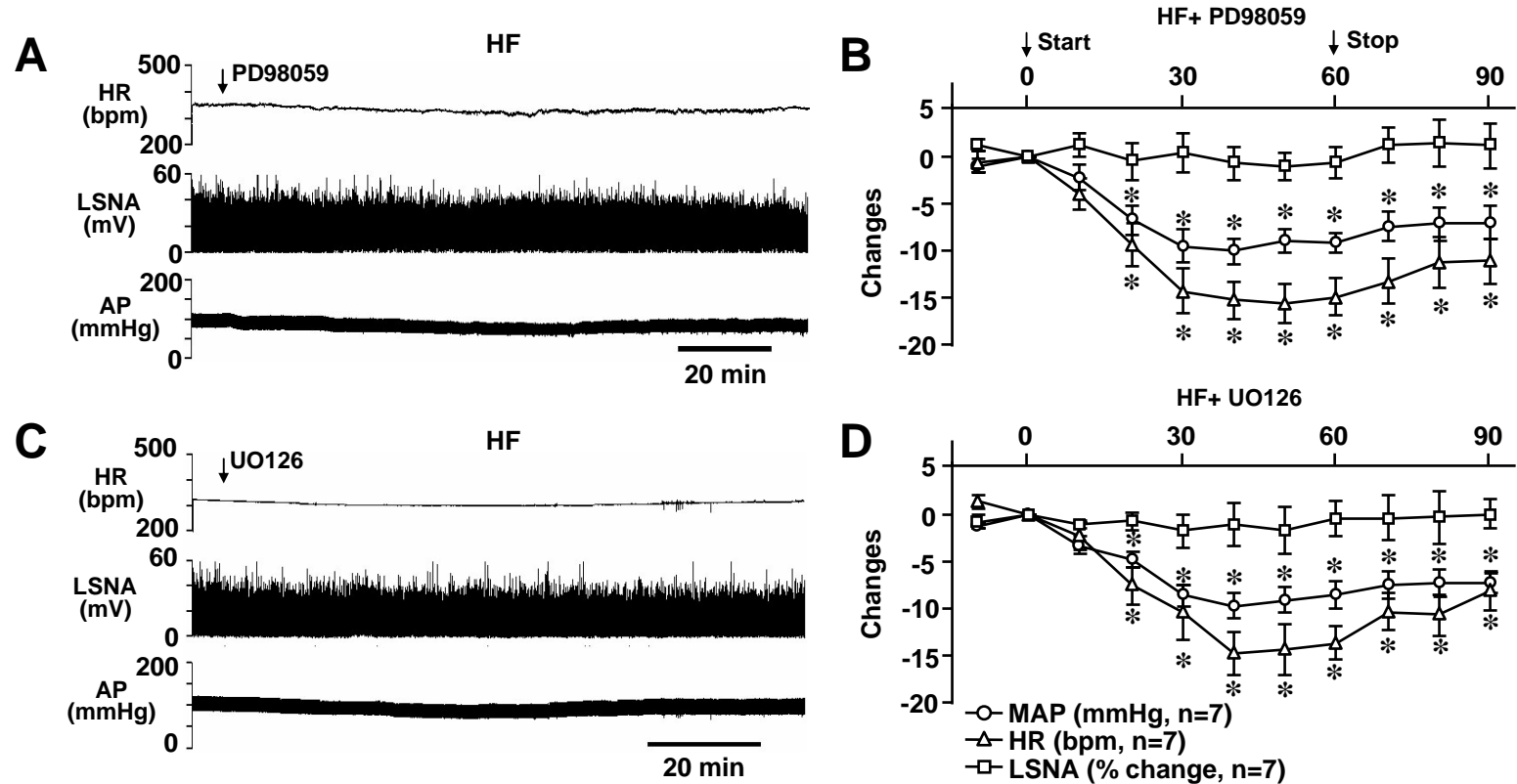
Table S1. Echocardiographic, Hemodynamic and Anatomical Measurements

<b>Variables at Baseline (~24 h)</b>	Sham (n=14)	HF+VEH (n=7)	HF+PD98059 (n=15)	HF+UO126 (n=15)	HF+SP600125 (n=7)	HF+SB203580 (n=7)	HF+LY294002 (n=8)	HF+PD98059+ Losartan (n=7)
LVEDV (ml)	0.30±0.03	0.78±0.10*	0.79±0.13*	0.74±0.15*	0.74±0.14*	0.71±0.13*	0.76±0.11*	0.73±11*
LVEF	0.85±0.05	0.32±0.04*	0.34±0.06*	0.38±0.05*	0.36±0.05*	0.35±0.04*	0.35±0.04*	0.33±.05*
%IZ	-----	46.1±3.7	48.0±4.3	46.7±3.8	45.4±3.9	47.4±3.4	45.9±4.0	45.8±3.6
<b>Variables at ~4 weeks</b>								
BW (g)	403 ± 6	395± 5	393 ± 6	394 ± 6	395 ± 5	392 ± 5	393 ± 6	397±6
RV/BW (mg/g)	0.66±0.03	1.56±0.13*	1.59 ± 0.16*	1.55±0.14*	1.53 ± 0.13*	1.61 ± 0.12*	1.58 ± 0.14*	1.54± 0.17*
Lung/BW((mg/g)	6.25±0.38	9.3±0.65*	9.02± 0.98*	9.20±0.98*	9.58 ±0.82*	9.76 ± 0.92*	9.49 ± 0.88*	9.38 ±0.79*
LVPSP (mmHg)	120 ± 4	104 ± 5*	96 ± 4*	97 ± 5 *	102 ± 6 *	101 ± 5 *	99 ± 5*	97 ± 4*
LVEDP (mmHg)	5.1±0.03	18.8±0.10*	18.6 ± 2.3*	19.2 ± 2.4*	18.5 ± 1.9*	17.8 ± 1.7*	18.4 ± 1.7*	19.0 ± 2.2*
LV dP/dt (mmHg/s)	8012±239	4025±246*	4097±274*	4053±272*	3950±223*	3984±249*	4038±261*	4111±234*

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: maximum rate of rise of LV pressure. Values are expressed as mean ± SEM. \**P*<0.05 versus Sham.

# Figure S1



Representative tracings showing the effects of ICV p44/42 MAPK inhibitor PD98059 (A) and UO126 (C) on AP, HR and lumbar sympathetic nerve activity (LSNA) in HF rats. Grouped data show no effect of ICV PD98059 (B) and UO126 (D) on LSNA in HF rats, even though MAP and HR are significantly decreased. \*  $P < 0.05$  compared with baseline. AP: arterial pressure; MAP: mean arterial pressure; HR: heart rate; RSNA: renal sympathetic nerve activity (integrated voltage).