Nervous System

Inhibition of Brain Mitogen-Activated Protein Kinase Signaling Reduces Central Endoplasmic Reticulum Stress and Inflammation and Sympathetic Nerve Activity in Heart Failure Rats

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

Abstract—Mitogen-activated protein kinase (MAPK) signaling and endoplasmic reticulum (ER) stress in the brain have been implicated in the pathophysiology of hypertension. This study determined whether ER stress occurs in subfornical organ and hypothalamic paraventricular nucleus in heart failure (HF) and how MAPK signaling interacts with ER stress and other inflammatory mediators. HF rats had significantly higher levels of the ER stress biomarkers (glucose-regulated protein 78, activating transcription factor 6, activating transcription factor 4, X-box binding protein 1, P58IPK, and C/EBP homologous protein) in subfornical organ and paraventricular nucleus, which were attenuated by a 4-week intracerebroventricular infusion of inhibitors selective for p44/42 MAPK (PD98059), p38 MAPK (SB203580), or c-Jun N-terminal kinase (SP600125). HF rats also had higher mRNA levels of tumor necrosis factor-α, interleukin-1β, cyclooxygenase-2, and nuclear factor-κB p65, and a lower mRNA level of IkB-α, in subfornical organ and paraventricular nucleus, compared with SHAM rats, and these indicators of increased inflammation were attenuated in the HF rats treated with the MAPK inhibitors. Plasma norepinephrine level was higher in HF rats than in SHAM rats but was reduced in the HF rats treated with PD98059 and SB203580. A 4-week intracerebroventricular infusion of PD98059 also improved some hemodynamic and anatomic indicators of left ventricular function in HF rats. These data demonstrate that ER stress increases in the subfornical organ and paraventricular nucleus of rats with ischemia-induced HF and that inhibition of brain MAPK signaling reduces brain ER stress and inflammation and decreases sympathetic excitation in HF. An interaction between MAPK signaling and ER stress in cardiovascular regions of the brain may contribute to the development of HF.

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Key Words: brain ■ endoplasmic reticulum stress ■ heart failure ■ hypothalamic paraventricular nucleus ■ subfornical organ ■ sympathetic nerve activity

Heart failure (HF) is characterized by increased sympathetic nerve activity, driven in part by increases in brain inflammation and renin–angiotensin system (RAS) activity in cardiovascular regulatory regions of the brain, including the subfornical organ (SFO) and the hypothalamic paraventricular nucleus (PVN). Activity of the mitogen-activated protein kinase (MAPK) signaling cascade that results in the phosphorylation of 3 major terminal effector kinases—p44/42 MAPK (also called extracellular signal–regulated protein kinases 1/2), p38 MAPK, and c-Jun N-terminal kinases—also increases in the SFO and PVN in HF. Angiotensin II (Ang II) and proinflammatory cytokines (PICs) both activate MAPK signaling, which has been implicated in upregulation of Ang II type 1 receptor expression in the SFO and PVN and sympathetic excitation in HF. However, the mechanisms by which MAPK activates sympathetic nerve activity remain poorly defined.

The endoplasmic reticulum (ER) is an intracellular organelle that plays an important role in the synthesis, folding, and translocation of proteins. ER stress is caused by an excessive accumulation of unfolded proteins in the ER lumen, which evokes the unfolded protein response with activation of a series of downstream signal transduction pathways, including MAPK signaling. In peripheral tissues, Ang II and PICs can also induce ER stress and the unfolded protein response. ER stress has been implicated in the pathophysiology of a variety of chronic disease states, including diabetes mellitus, obesity, and cardiovascular diseases. More recently, ER stress in the brain has been found to be involved in the regulation of sympathetic drive and cardiovascular function in Ang II–induced hypertension and in obesity.

Because RAS activity and inflammation are both upregulated in the brain of HF rats and both can induce ER stress and MAPK signaling in peripheral tissues, we investigated whether...

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indicators of ER stress are upregulated in SFO and PVN in HF rats and whether MAPK signaling has a role in that process. We also examined the effects of inhibiting brain MAPK signaling on gene expression of the nuclear factor-κB (NF-κB) and the inflammatory mediators tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), cyclooxygenase (COX)-1, COX-2, and circulating norepinephrine as an indicator of sympathetic nerve activity. Because brain p44/42 MAPK is known to contribute to sympathetic activation in HF and hypertension, and the p44/42 MAPK inhibitor proved particularly effective in reducing indicators of ER stress in this study, we further examined its effects on anatomic and physiological indicators of HF.

Methods

Animals

Experiments were carried out using adult male Sprague–Dawley rats, weighing 275 to 325 g, which were purchased from Harlan Sprague–Dawley (Indianapolis). The rats were housed in temperature-controlled (23±2°C) and light-controlled rooms in the University of Iowa Animal Care Facility and fed rat chow ad libitum. All experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee. The studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Protocols

Rats underwent coronary artery ligation to induce HF or a sham operation (SHAM). All rats underwent echocardiography within 24 hours of surgery to determine left ventricular (LV) function. Only animals with large infarctions (ischemic zone >35% of LV circumference) were assigned to HF treatment groups.

HF rats underwent a 4-week intracerebroventricular infusion (0.25 µL/h; 0.6 mmol/L) of the p44/42 MAPK inhibitor PD98059 (HF+PD), the p38 MAPK inhibitor SB203580 (HF+SB), the c-Jun N-terminal kinase inhibitor SP600125 (HF+SP), or vehicle (VEH; HF+VEH) via osmotic micropumps (Alzet, Cupertino, CA), beginning within 24 hours of coronary ligation or sham surgery. SHAM rats receiving intracerebroventricular VEH (SHAM+VEH) served as control. Four weeks after induction of HF, some rats underwent a second echocardiogram to assess the effects of the treatment protocols on LV function.

At the conclusion of the treatment protocols, some animals were anesthetized with urethane, and a Millar catheter (Millar, Houston, TX) was inserted in the right carotid artery and advanced into the LV to measure LV end-diastolic pressure and the maximal rate of rise of LV systolic pressure (LV dP/dt max). These animals were then euthanized to collect heart and lungs for anatomic studies. Others were decapitated or transcardially perfused while deeply anesthetized to collect brain tissues for molecular and immunofluorescent studies.

Real-Time Polymerase Chain Reaction and ELISA Studies

Real-time polymerase chain reaction was performed on SFO, PVN, and cortical tissues from all 5 groups (n=6 in each group) to measure gene expression of the ER stress biomarkers glucose-regulated protein 78 (GRP78), activating transcription factor 6 (ATF6), RNA-activated protein kinase p58IPK, and C/EBP homologous protein, the PICs TNF-α, IL-1β, COX-1, and COX-2, and the NF-κB components p65 and IκB-α.

Trunk blood from rats in all 5 groups (n=6 in each group) was assayed by ELISA for plasma concentration of norepinephrine, a general indicator of the level of sympathetic nerve activity.

Figure 1. Quantitative analysis by real-time polymerase chain reaction showing the mRNA expression of endoplasmic reticulum stress biomarkers glucose-regulated protein 78 (GRP78), activating transcription factor 6 (ATF6), RNA-activated protein kinase p58IPK, and C/EBP homologous protein (CHOP) in subfornical organ (SFO), hypothalamic paraventricular nucleus (PVN), and cerebral cortex (Cortex) in heart failure (HF) rats treated for 4 weeks with intracerebroventricular infusion of the p44/42 mitogen-activated protein kinase (MAPK) inhibitor PD98059 (HF+PD), the p38 MAPK inhibitor SB203580 (HF+SB), the c-Jun N-terminal kinase inhibitor SP600125 (HF+SP) or vehicle (VEH; HF+VEH), and VEH-treated SHAM (SHAM+VEH) rats. Values are mean±SEM (n=6 for each group) and expressed as a fold change relative to SHAM+VEH control. *P<0.05, vs SHAM+VEH; †P<0.05, HF+PD, HF+SB, and HF+SP vs HF+VEH.
Western Blot and Immunofluorescent Studies

Further studies were conducted to determine the effect of inhibiting p44/42 MAPK signaling on ER stress-related proteins in SFO and PVN. Western blot was used to measure the protein levels of GRP78, X-box binding protein 1 (XBP-1), and ATF4 in the SHAM+VEH, HF+VEH, and HF+PD rats (n=6 in each group). Immunofluorescent staining was used to examine the expression of GRP78, XBP-1, and ATF4 in the HF+VEH and HF+PD rats (n=6 in each group).

Hemodynamic and Anatomic Assessment

The presence of myocardial scar was confirmed visually in all HF groups. In the SHAM+VEH, HF+VEH, and HF+PD groups, the heart weight/body weight (BW), right ventricle/BW, and wet lung weight/BW ratios were determined.

Specific Materials and Methods

Further description of Materials and Methods is available in the online-only Data Supplement.

Statistical Analysis

The significance of differences among groups was analyzed by 2-way repeated-measure ANOVA followed by post hoc Fisher test. For other unpaired data, a Student t test was used for comparison between groups. P<0.05 was considered to indicate statistical significance.

Results

Echocardiographic Assessment of HF

Echocardiographic assessments within 24 hours after coronary artery ligation demonstrated that LV ejection fraction (LVEF) was reduced and LV end-diastolic volume (LVEDV) was increased in rats with HF, compared with SHAM (Table S1 in the online-only Data Supplement). The HF animals assigned to each treatment group 24 hours after coronary artery ligation were well matched with regard to ischemic zone as a percentage of LV circumference and LVEDV. Repeat echocardiograms obtained in some HF+VEH rats at 4 weeks revealed that LVEF did not change significantly but LV volume/mass ratio increased as HF progressed (Table S1).

Effects of MAPK Inhibitors on Gene Expression of ER Stress Markers

HF+VEH rats had significantly higher mRNA levels for the ER stress biomarkers GRP78, ATF6, p58IPK, and C/EBP homologous protein in SFO and PVN 4 weeks after coronary ligation, compared with SHAM+VEH rats (Figure 1).

![Figure 2](http://hyper.ahajournals.org/)

Figure 2. Quantitative analysis by real-time polymerase chain reaction showing the mRNA expression of the inflammatory mediators tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), cyclooxygenase (COX)-1, COX-2, nuclear factor-κB (NF-κB) p65, and IκB-α in subfornical organ (SFO), hypothalamic paraventricular nucleus (PVN), and cerebral cortex in heart failure (HF) rats treated for 4 weeks with intracerebroventricular infusion of the p38 MAPK inhibitor SB203580 (HF+SB), the c-Jun N-terminal kinase inhibitor SP600125 (HF+SP) or vehicle (VEH; HF+VEH), and VEH-treated SHAM rats (SHAM+VEH). Values are mean±SEM (n=6 for each group) and expressed as a fold change relative to SHAM+VEH control. *P<0.05, vs SHAM+VEH; and †P<0.05, HF+PD, HF+SB, and HF+SP vs HF+VEH.
HF+PD and HF+SB rats had significantly lower mRNA levels of these ER stress biomarkers in both nuclei, with SB203580 being most effective in SFO and PD98059 most effective in PVN. HF+SP rats had lower mRNA levels of GRP78, ATF6, and C/EBP homologous protein, but not p58IPK, in SFO and PVN. There were no differences across treatment groups in the gene expression of these ER stress biomarkers in cerebral cortex.

Effects of MAPK Inhibitors on Gene Expression of Inflammatory Mediators

The HF+VEH rats had higher mRNA levels of TNF-α, IL-1β, and COX-2 in SFO and PVN, compared with the SHAM-VEH rats (Figure 2). HF+PD, HF+SB, and HF+SP rats all had lower mRNA levels of TNF-α, IL-1β, and COX-2 in SFO and PVN than HF+VEH rats. There was no difference in COX-1 mRNA across groups. HF+VEH rats also had higher NF-κB p65 mRNA levels and lower IkB-α mRNA levels in SFO and PVN, compared with SHAM+VEH rats. These 2 components of NF-κB were normalized in the HF+PD and HF+SB rats. There were no differences across treatment groups in the gene expression of these inflammatory mediators in cerebral cortex.

Effects of MAPK Inhibitors on Plasma Norepinephrine

Plasma norepinephrine levels were higher in HF+VEH rats compared with those in SHAM+VEH rats (Figure 3). In HF+PD and HF+SB rats, the plasma concentrations of norepinephrine were significantly lower than those in the HF+VEH rats. The reduction in plasma norepinephrine levels in HF+SP rats did not reach statistical significance.

Effects of the p44/42 MAPK Inhibitor on ER Stress Proteins

To validate the significance of real-time polymerase chain reaction data, Western blot was used to measure the protein levels of several ER stress biomarkers, GRP78, XBP-1, and ATF4, in the SFO and PVN. These data confirmed the increased expression of ER stress in the SFO and PVN of the HF+VEH rats.
rats, compared with the SHAM+VEH rats, and a lower level of ER stress biomarkers in HF+PD rats.

Confocal immunofluorescent images revealed intense expression of GRP78 immunoreactivity in the SFO and PVN (Figure 5) in HF rats. The ER stress biomarkers XBP-1 and ATF4 were also expressed in the SFO and PVN but to a lesser extent. In the PVN, these ER stress biomarkers were expressed in dorsal parvocellular, medial parvocellular, ventrolateral parvocellular, and magnocellular regions, the 4 commonly recognized subdivisions. In the SFO, they were evenly expressed throughout the nucleus. In HF+PD rats, the expression of GRP78, XBP-1, and ATF4 in SFO and PVN was reduced.

**Effects of the p44/42 MAPK Inhibitor on Indicators of HF**

Echocardiography revealed no significant improvement in LVEF or LV volume/mass ratio in HF rats treated with PD, compared with HF rats treated with VEH (Figure 6).

Hemodynamic assessment at the completion of the experiments revealed that HF+VEH rats had a higher LV end-diastolic pressure, a lower LV peak systolic pressure, and maximal rate of rise of LV systolic pressure (LV dP/dt max) compared with SHAM+VEH rats. HF+PD rats had increased LV dP/dt max and decreased LV end-diastolic pressure compared with HF+VEH rats, but these values were still significantly different from those in SHAM+VEH rats (Figure 6). LV peak systolic pressure was not significantly different in HF+PD versus HF+VEH rats (Table S1).

The anatomic assessment exhibited that the heart weight/BW ratio, ventricular weight/BW ratio, and lung weight/BW ratio were substantially higher in HF+VEH rats compared with SHAM+VEH rats but were significantly reduced in the HF+PD rats (Figure 6).

**Discussion**

We previously reported that the expression of phosphorylated p44/42 MAPK, p38 MAPK, and c-Jun N-terminal kinase is upregulated in the SFO and PVN in HF and that increased activity of MAPK signaling in the brain plays an important role in the neurohumoral activation in HF rats. In part, this seems to be related to MAPK-mediated upregulation of brain RAS activity. This study sought to determine whether brain MAPK signaling in HF stimulates the activity of other central mechanisms that may contribute to sympathetic excitation. We observed increases in biomarkers of ER stress and inflammation in the SFO and PVN of HF rats that were significantly attenuated in HF rats treated with a continuous intracerebroventricular infusion of inhibitors selective for each of the major MAPK signaling pathways. Among these, inhibitors of the p44/42 and p38 MAPK signaling pathways were most effective. Peripheral indicators confirmed an associated reduction in plasma norepinephrine, a general indicator of sympathetic activity, and improvements in cardiac remodeling and cardiac function. These new findings, taken together with our previous results, suggest that brain MAPK signaling contributes significantly to the excitatory neurochemical milieu driving sympathetic activity in HF.

Emerging evidence suggests a role for brain ER stress in the pathophysiology of cardiovascular diseases. Young et al reported that ER stress in the SFO plays an important role in...
mediating the progression of Ang II–induced hypertension in mice. Chao et al.\textsuperscript{23} reported that ER stress is upregulated in the rostral ventrolateral medulla of spontaneously hypertensive rats and contributes to the development of hypertension. However, the presence of ER stress is not uniformly associated with sympathetic activation. One study in deoxycorticosterone acetate (DOCA)–salt mice reported that ER stress does not contribute to the blood pressure response although it does mediate the DOCA-induced saline intake.\textsuperscript{24} Another reported an increase in mRNA for biomarkers of ER stress but no increase in ER stress proteins in SFO and PVN of DOCA-salt mice and no effect of intracerebroventricular treatment with the ER stress inhibitor tauroursodeoxycholic acid on the development of hypertension.\textsuperscript{25} Thus, the contribution of ER stress to sympathetic excitation may vary depending on the experimental model studied.

The excitatory milieu of the HF brain might be considered highly conducive to the induction of ER stress. Increased RAS activity, inflammation, and reactive oxygen species—all present in cardiovascular regulatory regions of the brain in that setting—\textsuperscript{17–20} are all reported to induce ER stress.\textsuperscript{26} This study confirms that ER stress is present in the SFO and PVN of rats with ischemia-induced HF, but its contribution to the augmented sympathetic activity in this model remains to be determined.

This study also highlights an underappreciated relationship between MAPK signaling and ER stress. Although MAPK signaling is known to occur downstream from ER stress,\textsuperscript{4,5} many of the factors that drive ER stress are products of MAPK signaling. Phosphorylated MAPKs act on the transcription factors activator protein-1 and NF-κB,\textsuperscript{33–35} which can upregulate the expression of angiotensinogen, the precursor of Ang II and the Ang II type 1 receptor, as well as the PICs TNF-α, IL-1β, and COX-2.\textsuperscript{36–38} Thus, inhibition of MAPK signaling in HF may interrupt a feed-forward mechanism driving ER stress and its downstream consequences. The observation that the MAPK inhibitors reduce the expression of the inflammatory mediators TNF-α, IL-1β, and COX-2 and markers of ER stress in the PVN and SFO in HF rats is consistent with that hypothesis. Inhibition of MAPK signaling also normalized the NF-κB activity in the SFO and PVN, suggesting that MAPK-driven NF-κB activity contributes to the production of cytokines in the HF brain.

Chronic intracerebroventricular treatment of HF rats with either the p44/42 MAPK or the p38 MAPK inhibitor significantly reduced circulating norepinephrine levels, a general indicator of the level of sympathetic nerve activity. Because central inhibition of p44/42 MAPK signaling is known to have beneficial effects in HF\textsuperscript{21} and Ang II–induced hypertension,\textsuperscript{22} we examined the effects of central inhibition of MAPK activity on cardiac function. Consistent with previous studies of the effects of central interventions that reduce sympathetic drive,\textsuperscript{39–41} there was no improvement in echocardiographically defined LVEF or LV volume/mass ratio. However, LV end-diastolic pressure, wet lung weight/BW, and right ventricle/BW ratios were reduced and LV dP/dt max was improved, likely
reflecting reduced sympathetic drive to the kidneys and the vasculature with accompanying reductions in preload and afterload. Similar improvements of LV function were observed in previous studies in which targeted interventions in brain RAS activity reduced sympathetic activity in HF rats.40–43

Perspectives

This study extends understanding of the role of brain MAPK signaling in HF. To our knowledge, this is the first evidence that MAPK signaling drives ER stress and the unfolded protein response in cardiovascular regulatory regions of the brain in HF, likely by affecting transcriptional upregulation of RAS activity and the production of PICS. Interrupting this apparent feed-forward central circuit by targeting brain MAPK signaling—and particularly p44/42 or p38 MAPK signaling—may simultaneously treat multiple central mechanisms that contribute to the augmented sympathetic nerve activity in HF.

How to achieve inhibition of brain MAPK signaling remains problematic. Microparticle approaches providing sustained circulating drug levels and nanoparticle approaches to facilitating passage across the blood–brain barrier offer promise. In addition, advantage might be taken of the vascular vulnerability of circumventricular organs such as the SFO, whose activity influences intraparenchymal nuclei regulating sympathetic drive. Because of the many similarities in the central neurochemical abnormalities and neural mechanisms in HF and hypertension, the findings of this study may also provide important new insights into potential therapeutic interventions in hypertension.

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Disclosures

None.

References

25. Chao YM, Lai MD, Chan YJ. Redox-sensitive endoplasmic reticulum stress and autophagy at rostral ventrolateral medulla contribute to hypertension.


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**Novelty and Significance**

**What Is New?**

- Expression of endoplasmic reticulum stress biomarkers, including glucose-regulated protein 78, activating transcription factor 6, RNA-activated protein kinase P58IPK, C/EBP homologous protein, X-box binding protein 1, and activating transcription factor 4, is upregulated in subhilar cellular and paraventricular nucleus, 2 key cardiovascular autonomic regions of the brain, in rats with heart failure.
- Chronic central inhibition of mitogen-activated protein kinase (MAPK) signaling reduces markers of endoplasmic reticulum stress and inflammation in paraventricular nucleus and subhilar organ of rats with heart failure.
- Chronic central inhibition of MAPK signaling decreases sympathetic nerve activity and reduces the peripheral manifestations of heart failure.

**What Is Relevant?**

- In addition to its known effects to upregulate the brain renin-angiotensin system, MAPK signaling augments the expression of endoplasmic reticulum stress and inflammatory mediators in cardiovascular regions of the brain in heart failure.
- Similar mechanisms may contribute to neurogenic hypertension.

**Summary**

MAPK signaling contributes to endoplasmic reticulum stress and inflammation in cardiovascular regions of the brain in heart failure. Inhibition of brain MAPK signaling reduces sympathetic activation and the progression of cardiac dysfunction in heart failure. Brain MAPK signaling is a potential novel therapeutic target in heart failure.
Inhibition of Brain Mitogen-Activated Protein Kinase Signaling Reduces Central Endoplasmic Reticulum Stress and Inflammation and Sympathetic Nerve Activity in Heart Failure Rats
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SUPPLEMENT

to

Inhibition of brain mitogen-activated protein kinase signaling reduces central endoplasmic reticulum stress and inflammation and sympathetic nerve activity in heart failure rats

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Running Title: Brain MAPK and ER Stress in Heart Failure
MATERIALS AND METHODS

Induction of heart failure

Heart failure (HF) was produced 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.1, 2 Sham-operated (SHAM) rats underwent the same surgical procedure but did not undergo coronary ligation.

Assessment of heart failure

Within 24 hours and 4 weeks after coronary artery ligation or SHAM surgery, rats were tranquilized with ketamine (60 mg/kg, IP) and left ventricular (LV) function was evaluated by two-dimensional echocardiography as previously described.2 The ischemic zone (IZ) as a percent of LV circumference (% IZ), LV ejection fraction, and LV end-diastolic volume were measured.

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

ICV infusions

Drug or vehicle was infused via a 31-gauge stainless steel cannula with the tip positioned in the left lateral cerebral ventricle (coordinates AP, −1.0 mm; DV, −4.5 mm; and ML, -1.5 mm, with bregma as a reference).

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

Drugs administered

The p44/42 MAPK inhibitor PD98059, the p38 MAPK inhibitor SB203580 and the JNK inhibitor SP600125 were obtained from Tocris (Ellisville, MO). These inhibitors were dissolved in dimethyl sulfoxide (DMSO) first, and then diluted in aCSF to make a 5% final DMSO concentration. The ICV vehicle (VEH) was aCSF containing 5% DMSO.

Brain tissue preparation

At the completion of each protocol, the animals were decapitated while under anesthesia to obtain brain tissue for the designated studies. To obtain tissues for Western blot and real time PCR, the brains were immediately removed, frozen in liquid nitrogen and stored at −80 °C for subsequent use. The frozen brain was cut into 300-μm coronal sections and target tissues including SFO and PVN were obtained using a punch device (inner diameter 1.5 mm, Stoelting, Wood Dale, IL). The punched tissues were homogenized in cell lysis buffer (Cell Signaling Technology, Beverly, MA) to extract protein for Western assay or in TRI reagent (Molecular Research Center, Cincinnati, OH) to extract RNA for real-time PCR. To obtain tissues for immunostaining, rats were anesthetized with
urethane (1.5 g/kg, IP) and transcardially perfused with 4% paraformaldehyde. Brains were then embedded with OCT and rapidly frozen in acetone chilled dry ice. Coronal sections (16 μm) of target brain tissues were made with a cryostat and stored at −80 °C.

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

The mRNA levels of ER stress biomarkers ATF6, GRP78, CHOP and p58IPK and PVN were measured with real-time PCR following reverse transcription of total RNA. The sequences for primers and probe used are shown in Table S2. TaqMan primer and probe for rat GAPDH were purchased from Applied Biosystems (Foster City, Calif.). Real-time PCR was performed using the ABI prism 7700 Sequence Detection System (Applied Biosystems). GAPDH mRNA was quantified as an internal control for each sample. The value for each sample was normalized to GAPDH and expressed as a fold difference relative to the control.

**Western blot**

The protein levels of ER stress biomarkers GRP78, XBP-1 and ATF4 were detected using polyclonal rabbit antibodies against GRP78 (AB21685, 1: 500, Abcam, MA), XBP-1(SC-7160, 1: 200, Santa Cruz, CA) and ATF4 (SC-200, 1: 200, Santa Cruz, CA) respectively. The second antibodies were goat anti-rabbit IgG-HRP (SC-2054, 1:5000, Santa Cruz). Immunoblots were visualized with an enhanced chemiluminescence reagent. The immunoblotting was quantified with NIH ImageJ software. The content of ER stress biomarkers GRP78, XBP-1 and ATF4 was normalized by the total β-actin detected by rabbit monoclonal antibody (#4970, 1:1000; Cell Signaling Technology, Inc., Danvers, MA).

**Immunofluorescence**

Immunofluorescent staining was used to examine the ER stress expression in PVN and SFO in HF rats treated with vehicle or p44/42 MAPK inhibitor PD98059. The sections were incubated with the primary antibodies, the rabbit polyclonal antibodies to GRP78 (AB21685, 1: 200, Abcam, MA), XBP-1(SC-7160, 1: 100, Santa Cruz, CA) and ATF4 (SC-200, 1: 100, Santa Cruz, CA) respectively, followed by secondary antibodies Alex Fluor 488 goat anti-rabbit IgG (A-11070, 1:200, Invitrogen). Immunofluorescent staining was visualized with a confocal laser-scanning microscope (Zeiss LSM 710, Carl Zeiss, Inc).

**ELISA measurement of plasma norepinephrine concentration**

Blood samples were collected from trunk blood in EDTA tubes and were centrifuged for 20 min at 4°C. The plasma was harvested and stored at -80°C for future use. Plasma norepinephrine concentrations were measured using Noradrenaline Research ELISA kit (CAT# BA 10-5200, LDN, Germany). The experimental procedures for ELISA were performed according to the manufacturer’s manuals.
REFERENCES


Table S1. Echocardiographic, Hemodynamic and Anatomical Measurements

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<td>0.79 ± 0.12*</td>
<td>0.81 ± 0.11*</td>
</tr>
<tr>
<td>LV Vol/Mass (µl/mg)</td>
<td>0.62 ± 0.04</td>
<td>1.09 ± 0.11*</td>
<td>1.05 ± 0.09*</td>
<td>1.02 ± 0.10*</td>
<td>1.01 ± 0.11*</td>
</tr>
<tr>
<td>Echocardiographic Variables at 4 weeks (n=6)</td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>%IZ</td>
<td>-----</td>
<td>45.2 ± 3.8</td>
<td>44.6 ± 3.7</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>LVEF</td>
<td>0.78 ± 0.05</td>
<td>0.33 ± 0.04*</td>
<td>0.45 ± 0.06*</td>
<td>-----</td>
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</tr>
<tr>
<td>LVEDV (ml)</td>
<td>0.45 ± 0.05</td>
<td>1.54 ± 0.17*</td>
<td>1.27 ± 0.11*</td>
<td>-----</td>
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</tr>
<tr>
<td>LV Vol/Mass (µl/mg)</td>
<td>0.65 ± 0.06</td>
<td>1.67 ± 0.11*</td>
<td>1.39 ± 0.12*</td>
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<tr>
<td>Hemodynamic Variables at 4 weeks (n=6)</td>
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</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>4.6 ± 2.2</td>
<td>19.9 ± 2.5*</td>
<td>14.9 ± 1.9*†</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>LV dP/dt max (mmHg/s)</td>
<td>7904 ± 262</td>
<td>4148 ± 168*</td>
<td>6206 ± 185*†</td>
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<td>-----</td>
</tr>
<tr>
<td>LV PVS (mmHg)</td>
<td>121 ± 5</td>
<td>96 ± 4 *</td>
<td>103 ± 5 *</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Anatomical Variables at 4 weeks (n=6)</td>
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<tr>
<td>RV/BW (mg/g)</td>
<td>0.75 ± 0.12</td>
<td>1.39 ± 0.16*</td>
<td>0.98 ± 0.11*†</td>
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<tr>
<td>HW/BW (mg/g)</td>
<td>3.25 ± 0.22</td>
<td>4.48 ± 0.21*</td>
<td>3.79 ± 0.19*†</td>
<td>-----</td>
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<tr>
<td>LW/BW (mg/g)</td>
<td>5.59 ± 0.24</td>
<td>8.72 ± 0.25*</td>
<td>6.47 ± 0.32*†</td>
<td>-----</td>
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</tr>
</tbody>
</table>

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; HW: heart weight; LW: lung weight; LVPSP: LV peak systolic pressure; LVEDP: LV end-diastolic pressure. LV dP/dt max: maximum rate of rise of LV pressure. Values are expressed as mean ± SEM. * p<0.05 versus SHAM, † p<0.05 versus HF+VEH.
Table S2. Sequences for primers and probes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers and Probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP78 (SYBR)</td>
<td>Forward primer: 5’-AAG GTG AAC GAC CCC TAA CAA A-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’-GTC ACT CGG AGA ATA CCA TTA ACA TCT-3’</td>
</tr>
<tr>
<td>CHOP (SYBR)</td>
<td>Forward primer: 5’-GCC TTT CGC CTT TGA GAC AGT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’-TGA GAT ATA GGT GCC CCC AAT-T-3’</td>
</tr>
<tr>
<td>ATF6 (SYBR)</td>
<td>Forward primer: 5’-GAT TTG ATG CCT TGG GAG TC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’-GGA CCG AGG AGA AGA GAC AG-3’</td>
</tr>
<tr>
<td>p58IPK (SYBR)</td>
<td>Forward primer: 5’-ATT AAA GCA TAC CGA AAG TTA GCA C-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’-AGA GGG TCT TCT CCG TCA AA-3’</td>
</tr>
<tr>
<td>GAPDH (SYBR)</td>
<td>Forward primer: 5’-AAG GTC ATC CCA GAG CTG AA-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’-ATG TAG GCC ATG AGG TCC AC-3’</td>
</tr>
</tbody>
</table>