

Physiological Research Pre-Press Article

Title: Chronic stress promotes the progression of pressure overload-induced cardiac dysfunction through inducing more apoptosis and fibrosis.

Short Title: Stress promotes hypertension-induced cardiac dysfunction

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Summary

Stress serves as a risk factor in the etiology of hypertension. The present study was designed to decipher the effect and mechanism of chronic stress on the progression of pressure overload-induced cardiac dysfunction. We used abdominal aortic constriction (AAC) to induce pressure overload with or without chronic restraint stress to establish the animal models. Echocardiographic analysis showed pressure overload-induced cardiac dysfunction was worsened by chronic stress. Compared with the AAC rats, there is a significant increase in cardiac hypertrophy, injury, apoptosis and fibrosis of the AAC + stress rats. Furthermore, we found the secretion of norepinephrine (NE) increased after the AAC operation, while the level of NE was higher in the AAC+ stress group. Cardiomyocytes and cardiac fibroblasts isolated from neonatal rats were cultured and separately treated with 1, 10, 100 μ M NE. The higher concentration NE induced more cardiomyocytes hypertrophy and apoptosis, cardiac fibroblasts proliferation and collagen expression. These results revealed that high level of NE-induced cardiomyocytes hypertrophy and apoptosis, cardiac fibroblasts proliferation and collagen expression further contributes to the effect of chronic stress on acceleration of pressure overload-induced cardiac dysfunction.

Key words: Chronic stress, pressure overload, cardiac dysfunction, norepinephrine

Introduction

Hypertension is one of the most common causes of cardiac remodeling and dysfunction, which eventually leads to heart failure (Cumbermack *et al.* 2011; Li *et al.* 2013; Manolis *et al.* 2013; Ribeiro *et al.* 2014). Heart failure is a final stage of most cardiovascular disease, which poses serious threats to human health and life (Levy *et al.* 2002). The progression of cardiac remodeling adversely promotes myocardial stiffness and left ventricular dilation, resulting in accelerating heart failure (Brilla *et al.* 1990). Cardiac remodeling is the convergent phenotype of various diseases that causes dysfunction of cardiac myocytes and cardiac fibroblasts (Brown *et al.* 2005; Kremneva and Abaturova 2003). Thus, preventing the progression of pressure overload is expected to suppress heart failure. A deeper understanding of the risk factors affecting the cardiac dysfunction in response to hemodynamic overload is essential to the development of effective preventative measures and therapies.

Stress is defined as the effects of environmental or psychosocial factors on physical or mental well-being. Stress overload can cause injury and even diseases, such as diabetes, gastric ulcer, obesity, cancer, and Parkinson's disease. Data suggest a relationship between stress and the risk of cardiovascular disease (Zhao *et al.* 2007). It has been found that the cardiovascular system is the major target of stress injury (Ippoliti *et al.* 2013; Wang *et al.* 2012). Stress has also been shown to be important to vascular hypertension (Spruill 2010; Yu *et al.* 2013), and it may serve as a risk factor, induce blood pressure spikes, or increase an already elevated blood pressure (Lutgendorf *et al.* 2000; Wenneberg *et al.* 1997). But whether chronic stress

participates in the progression of pressure overload-induced cardiac dysfunction remains poorly characterized. Therefore, the present study was undertaken to investigate the effect of chronic stress on the progression of the pressure overload-induced cardiac dysfunction, and explore the possible mechanism of the effects of chronic stress during the progression of pressure overload-induced cardiac remodeling.

Materials and Methods

Experimental animals

The male Wistar rats weighing 180-200g were randomly divided into the following groups: sham operation, abdominal aortal constriction (AAC) operation and AAC + stress. The operation group was subjected to abdominal aortal constriction (AAC) with a 7-gauge syringe needle under anaesthesia with sodium pentobarbital, the sham operation group was treated as previously described, and the AAC + stress group were subjected to chronic restraint stress for 6 hours per day after 1 week of the AAC operation. The rats were sacrificed 2, 3 and 4 weeks after the operations under anaesthesia. All animal experimental procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The protocol was approved by the committee on the Ethics of Animal Experiments of the Beijing Institute of Basic Medical Sciences (Permit Number: 2012-D-3096)

Echocardiography and blood pressure analysis

The rats were anesthetized by isoflurane and O₂ inhalation. Blood pressure

measurements were conducted using carotid artery cannulation with a MP150 polygraph (BIOPAC, USA). Echocardiographic measurements were conducted with a high-resolution echocardiography analysis system for small animals (Vevo770, Visual Sonics, Canada). A 2-dimensional short-axis view and M-mode tracings of the left ventricle (LV) were obtained with a 17.5-MHz RMV-716 transducer.

Histology

The left ventricle was separated, washed and fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin and sectioned at 5µm. The sections were stained with hematoxylin/eosin to detect myocardial hypertrophy and with Masson's trichrome staining to assess fibrosis.

Measurement the contents of Serum levels of CK-MB and TnI

Enzyme linked immunosorbent assay (ELISA) kits were used to determinate the contents of creatine kinase isoenzyme (CK-MB) and troponin I (TnI) in rats serum. All assays were performed according to the manufacture's instruction.

Caspase-3 activity assay

Caspase-3 activity in rat heart tissue was detected using the caspase-3 colorimetric activity assay kit (Genmed Scientific Inc., China). The assay was based on spectrophotometric detection of the pNA after cleavage from the labeled substrate Ac-DEVD-pNA and performed according to the manufacture's instruction (Jin *et al.* 2013)

Cell culture

Cardiac myocytes and cardiac fibroblasts were isolated from the left ventricles of 1-2-day-old Wistar rats as previously described (Tsuruda *et al.* 1999). Cardiomyocytes with more than 100 times of spontaneous beating were used for the experiment. The identities of the CFs were confirmed by immunostaining for DDR2. Passages 2-4 were used for the experiments.

Western blot analysis

The heart tissue and cells were lysed and subjected to SDS-PAGE and transferred to PVDF membranes. Western blot analyses were conducted with commercially available antibodies (anti-BNP: Santa Cruz; anti-collagen I and collagen III: abcam). They were visualized with the use of SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA) and images were captured with an ImageQuant LAS 4000 (GE,USA).

MTT assay

Cell viability was determined using the MTT assay (Sigma-Aldrich, St. Louis, MO). The cells were seeded onto a 96-well plate overnight. Then, cells were treated with the processing mode. After treatment, the medium was removed and 200 µl of the MTT medium (0.5 mg/ml MTT reagent in fresh medium) was added to each well, and the cells were incubated at 5% CO₂ and 37°C for 4 h. Finally, the MTT reagent was removed and 150 µl DMSO was added to each well following 10 min of gentle shaking. The absorbance was measured at 490 nm with a microplate reader. All experiments were performed in eight wells and repeated three times.

Flow cytometry

For the apoptosis assays, annexin V staining was performed using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, CA, USA) according to the manufacturer's recommendations. The samples were examined by flow cytometry (FACS Calibur, BD, USA), and the data were analyzed with CELLQuest software (FACS Calibur, BD, USA).

For the cell cycle assays, following the processing mode, cells were harvested, washed three times with PBS, centrifuged and fixed with 70% anhydrous ethanol overnight at 4°C. They were then incubated with RNAase for 30 min and stained with PI for 15 min at 37°C in the dark. The samples were examined by flow cytometry (FACS Calibur, BD, USA), and the data were analyzed with ModFit LT software (FACS Calibur, BD, USA).

Statistical analysis

The data are expressed as mean as the means \pm SEM. Comparisons were performed using Student's t test or ANOVA as appropriate. $p < 0.05$ was considered significant.

Results

1. Chronic stress worsens pressure overload-induced cardiac dysfunction.

To explore the impact of stress on the pressure overload-induced cardiac dysfunction, a microsurgical approach was used to induce hypertension by pressure overload and chronic restraint stress was used after 1 week of AAC operation. Compared with the sham group, the blood pressure of the AAC rats increased after 1 week and remained

high for 4 weeks (Fig.1A). Also, we found that chronic stress induced higher blood pressure than the AAC groups (Fig.1A). To recognize the cardiac function after the AAC or chronic stress, we used the echocardiographic assessment to detect the cardiac function. Echocardiographic assessment showed no significant decrease of the ejection fraction and fractional shortening induced by 3 weeks stress (Supplemental Fig.1). But, compared with the sham group, the left ventricle anterior wall and left ventricular mass increased after 4 weeks of the AAC rats or after 3 weeks of the AAC + stress rats, indicating that stress promoted pressure overload-induced cardiac hypertrophy (Fig.2A, B, C). Echocardiographic assessment showed no significant increase of the ejection fraction induced by AAC during our detected times (Fig.2D). But, the ejection fraction decreased after 3 weeks in the AAC + stress group (Fig.2D). Also, We found that the fractional shortening significantly decreased after 4 weeks in the AAC + stress group (Fig.2E). These results illustrated that chronic stress promotes pressure overload-induced cardiac dysfunction.

2. Pressure overload-induced cardiac hypertrophy and fibrosis can be increased by chronic stress

We detected the change of cardiac hypertrophy after AAC or stress. The heart weight to body weight ratios increased after 4 weeks of the AAC rats, but increased after 3 weeks of the AAC + stress rats (Fig.3A). Echocardiographic assessment demonstrated an evident increase in the left ventricle mass and the left ventricle anterior wall after 4 weeks of the AAC rats or after 3 weeks of the AAC + stress rats (Fig.2B, C). Hematoxylin and eosin staining demonstrated that the cardiomyocytes width of AAC

+ stress rat heart sections were significantly thicker than the AAC group (Fig.3B), indicating that chronic stress promoted pressure overload induced cardiac hypertrophy. Moreover, to determine the effect of stress on pressure overload-induced cardiac fibrosis, we detected the collagen accumulation in the heart sections. Masson staining showed pressure overload-induced collagen accumulation increased when the rats were subjected to chronic restraint stress (Fig.3C, D). Also, Western blot analysis revealed that the expression of collagen was markedly increased after 4 weeks of the AAC rats or after 3 weeks of the AAC + stress rats (Fig.3E). These results demonstrate that pressure overload-induced fibrosis was aggravated by chronic stress

3. Chronic stress aggravated pressure overload-induced cardiomyocytes injury

Cardiomyocytes apoptosis in the left ventricular tissue of rat was evaluated by detecting the caspase-3 activity. Compared to the sham group, there was an increase in LV tissue caspase-3 activity after 4 weeks of AAC operation. But, the increase of caspase-3 activity was found as early as 3 weeks in the AAC + stress group (Fig.3F). Caspase-3 activity in the AAC + stress group was significantly higher than that in the AAC group after 4 weeks of the operation. In order to determine the injury of cardiac myocytes, the amounts of CK-MB and TnI in the serum were detected by ELISA. The content of serum CK-MB and TnI significantly increased in the AAC group after 4 weeks of operation and in the AAC + stress group after 3 weeks of operation (Fig.3G, H). Moreover, it was significantly higher than in AAC group after 4 weeks of the operation in AAC + stress group (Fig.3G, H). These results demonstrated that chronic stress promoted the progression of pressure overload-induced cardiomyocyte injury.

4. NE induced cardiomyocytes hypertrophy and apoptosis

Under the condition of pressure overload or stress, the secretion of norepinephrine (NE) increased. Also, the level of NE was higher in the AAC + stress group than that in the AAC group (Fig.1B). To determine the effect of high concentrations of NE on cardiomyocytes hypertrophy and apoptosis, the neonatal rat cardiac myocytes were incubated with 10^{-6} - 10^{-4} mol/l NE for 48 h. Compared with the parental controls, protein content of cardiac myocytes increased in a dose-dependent manner (Fig.4A). BNP is an important biomarker of hypertrophy, western blotting analysis showed NE had a dose dependent stimulatory effect on BNP expression (Fig.4B). Furthermore, flow cytometry analysis showed there was a significant increase of cell apoptosis with a dose-dependent manner in the NE-induced cardiomyocytes (Fig.4C). These results indicated that cardiac myocytes hypertrophy and apoptosis were more obvious in the higher level of NE group, which may be the mechanisms of pressure overload-induced cardiac hypertrophy and injury worsened by chronic stress.

5. NE induced cardiac fibroblasts proliferation and collagen expression.

To determine the effects of NE on cardiac fibrosis, the neonatal rat cardiac fibroblasts were incubated with 10^{-6} - 10^{-4} mol/l NE for 24 h. Compared with the parental controls, the MTT assay showed a significant increase of the cell proliferation rate in a dose-dependent manner (Fig.5A). To identify a potential mechanism for NE-induced cell proliferation, the cell cycle distribution was assessed using flow cytometry, which showed that the percentage of cells in the DNA synthesis phase (S phase) significantly increased in a dose-dependent manner (Fig.5B). Furthermore, Collagen, the important

extracellular matrix (ECM) component, was also increased in a dose-dependent manner in the NE-induced cardiac fibroblasts (Fig.5C). These results indicated that higher level of NE induced higher proliferation rate and ECM protein expression, which may contribute to the process of that chronic stress promoted pressure overload-induced cardiac fibrosis.

Discussion

There have been numerous studies about stress, a concept describing the process in which environmental or psychosocial factors exceed the adaptive capacity of an organism, resulting in psychological and biological changes that may place persons at risk for disease (Sparrenberger *et al.* 2009; Taylor *et al.* 2010). For instance, stress plays a major role in immunological diseases and immune-related disease processes. In addition, inflammation, infection, autoimmune processes, and perhaps even the onset and development of tumors may occasionally be associated with the stress phenomenon (Esch *et al.* 2002; Kashani *et al.* 2012; Koh *et al.* 2008; Martocchia *et al.* 2013). Moreover, Stress has been considered as an important cause of hypertension among other potential risk factors such as low potassium consumption, low physical activity and sleep abnormalities (Heine and Weiss 1987). Hypertension is one of the most common worldwide diseases. Meanwhile, it is also the most important modifiable risk factor for cardiac remodeling and heart failure. Owing to the associated morbidity and mortality, and the cost to society, hypertension is a serious challenge to public health. Concerted effort on the part of the health care professionals

has led to decreased morbidity and mortality from the multiple organ damage arising from long-term and untreated hypertension (Ettner *et al.* 2012). Stress, as a risk factor for hypertension, should be highly concern. Our research showed that chronic stress increased the AAC-induced high blood pressure and made the pressure overload-induced cardiac dysfunction worsened.

Cardiac remodeling after long-term pressure overload results in ventricular dysfunction and heart failure and is considered to be a key determinant of clinical outcome in heart disease (Kehat 2012; Levy *et al.* 2002). Cardiac remodeling includes changes in heart size, shape and function in response to injury or stress stimulation. Pathological remodeling involves the reactivation of cardiomyocytes hypertrophy and death and cardiac fibrosis (Kudo *et al.* 2007; Saito *et al.* 2010). Our results demonstrated that chronic stress aggravated pressure overload-induced cardiac hypertrophy, cardiac fibrosis and cardiomyocytes apoptosis, which may contribute to the decline of cardiac function . Loss of cardiomyocytes through apoptosis is an important pathological change in myocardial injury (Fan *et al.* 2013). When the structure of cardiomyocytes damaged, amounts of CK-MB, LDH and TnI were released into blood, so the content of TnI and CK-MB level was usually used to identify the cell injury and membrane integrity in previous studies (Amani *et al.* 2013; Li *et al.* 2012). Our results showed that the contents of these enzymes in AAC + stress rat serum increased significantly, indicating that pressure overload-induced myocardial injury can be aggravated by chronic stress.

The impact of stress on the development of hypertension is believed to involve a

sympathetic nervous system response, in which the release of catecholamines leads to increased heart rate, cardiac output, and BP (Carroll *et al.* 2005; Spruill 2010). As shown in figure 1B, compared with the AAC group, the level of NE is higher in the AAC + stress group. The heart is comprised of a syncytium of cardiac myocytes and surrounding nonmyocytes, the majority of which are cardiac fibroblasts (Zhang *et al.* 2012). Cardiomyocyte hypertrophy and apoptosis, cardiac fibroblasts proliferation and ECM protein synthesis are considered to be the important cellular basis for pressure overload-induced cardiac remodeling (Fan *et al.* 2012; Wollert and Drexler 2002). As is well known, NE binds to specific adrenoceptor on the cell membrane to induce PKC activation and cause a variety of cellular effects. We are currently performing further investigations about the effect of high level of NE induced by chronic stress on the cardiac myocytes and fibroblasts. Compared with the parental controls, NE had a significantly stimulatory effect on Cardiomyocytes hypertrophy and apoptosis, cardiac fibroblasts proliferation and ECM protein synthesis in a dose-dependent manner, which may contribute to the high level of NE induced by chronic stress to promote pressure overload- induced cardiac dysfunction.

In summary, we demonstrated that chronic stress promotes the progression of pressure overload-induced cardiac dysfunction. We further verified that pressure overload-induced myocardial hypertrophy, cardiac fibrosis, cardiomyocyte injury and apoptosis could be aggravated by chronic stress. The high level of NE induced by chronic stress contributed to acceleration of pressure overload-induced cardiac dysfunction through inducing cardiac myocytes hypertrophy and apoptosis, cardiac

fibroblasts proliferation and collagen synthesis. These findings improved our understanding of the risk factors and mechanisms involved in the effect of stress on pressure overload-induced cardiac dysfunction and provided new insights into future therapeutic targets for hypertension-induced cardiac remodeling and heart failure.

Conflict of Interest: none declared.

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Figure legends

Fig. 1. The levels of blood pressure and NE during the progression of pressure overload. Rats were subjected to abdominal aortic constriction (AAC) surgery or chronic restraint stress. **A.** The mean arterial blood pressure. **B.** An ELISA kit (IBL, Germany) was used to detect the NE levels in the serum. Data represent means \pm SD. * $P \leq 0.05$ vs. same time sham, # $P < 0.05$ vs. same time AAC, n=8.

Fig. 2. Echocardiographic parameters of the rats during the progression of pressure overload. Rats were subjected to abdominal aortic constriction (AAC) surgery or chronic restraint stress. **A.** Representative left ventricular (LV) M-mode echocardiograms in short axis. **B.** Left ventricular anterior wall (LVAW). **C.** Left ventricular mass (LV mass) **D.** Left ventricular ejection fraction (%EF). **E.** Left ventricular fractional shortening (%FS). Data represent means \pm SD. * $P \leq 0.05$ vs. same time sham, # $P < 0.05$ vs. same time AAC, n=8.

Fig. 3. The biological characteristics of AAC or stress induced myocardial injury. **A.** Heart weight/body weight ratios. **B.** Representative histological images with hematoxylin and eosin staining of heart sections, The widths of 30 individual cardiomyocytes were measured across a line bisecting the nucleus, Bar graph shows the cardiomyocyte width. **C.** Representative histological images with Masson's

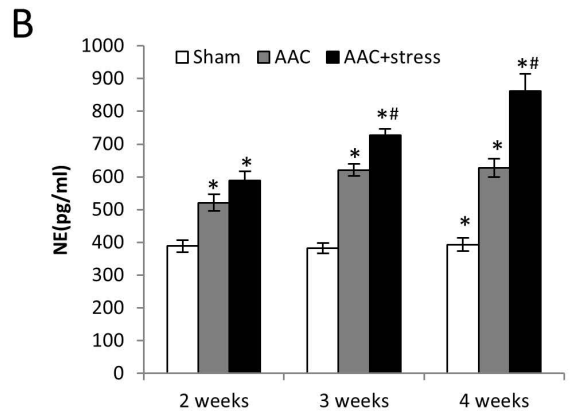
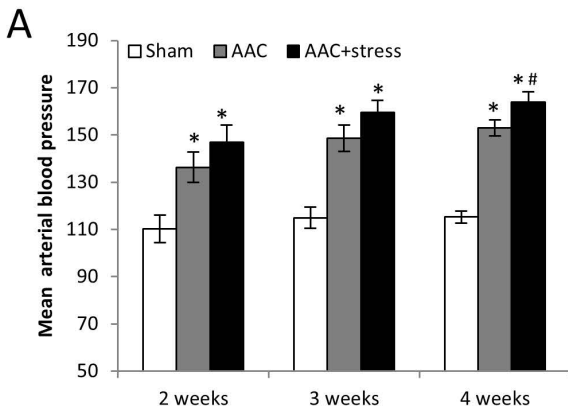
trichrome staining of heart sections. **D.** Quantification of collagen accumulation areas for Masson's trichrome staining . **E.** Western blot analysis of collagen expression. **F.** Caspase-3 activity in myocardial tissues. **G.** ELISA analysis of the level of CK-MB in the serum. **H.** ELISA analysis of the level of TnI in the serum. Data represent means \pm SD. * $P \leq 0.05$ vs. same time sham, # $P < 0.05$ vs. same time AAC, n=8.

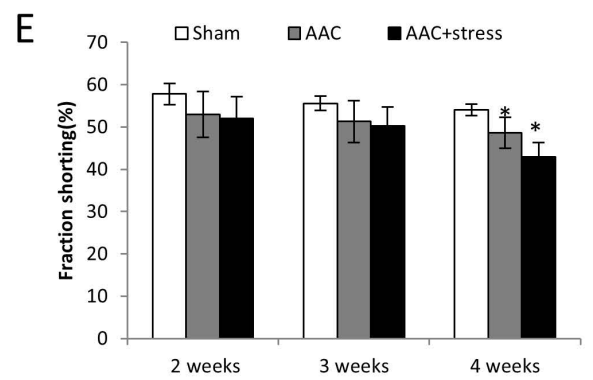
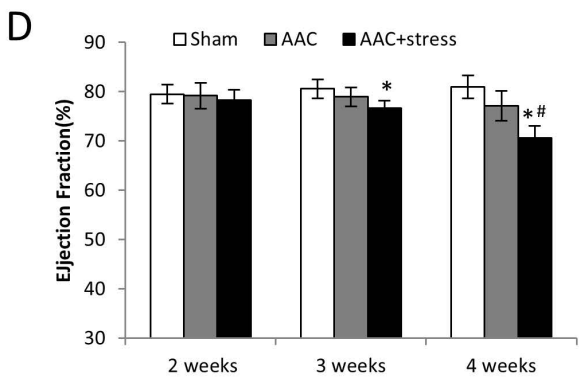
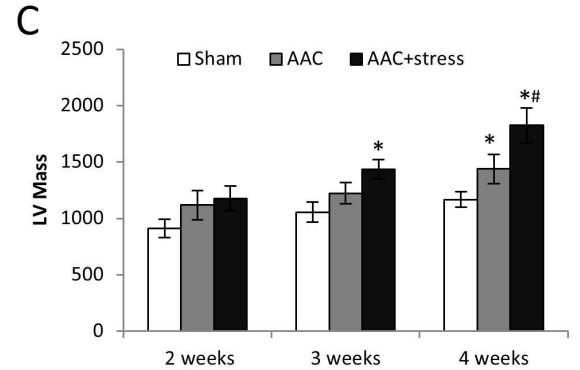
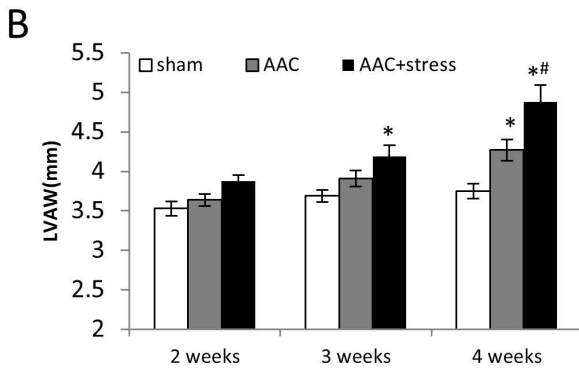
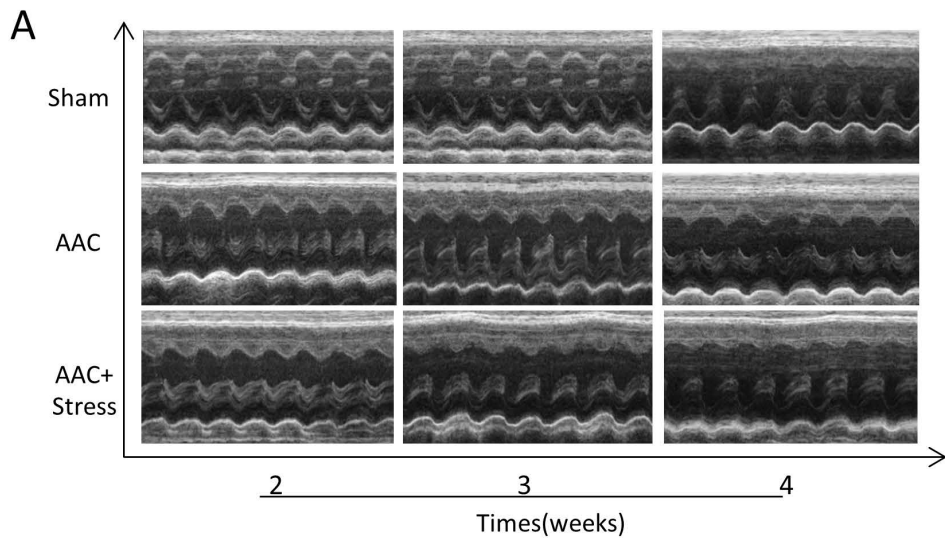
Fig. 4. The effects of NE on cell hypertrophy and apoptosis in cultured cardiac myocytes. Cardiomyocytes were treated with different concentrations of NE for the indicated times. **A.** The protein content of cell. **B.** Western blot analysis of NE-induced BNP expression. **C.** The cells were stained with propidium iodide and annexin V for flow cytometry and analyzed for apoptosis using CELLQuest software. Data represent means \pm SD. * $P \leq 0.05$ vs. Control. All experiments were performed 3 times.

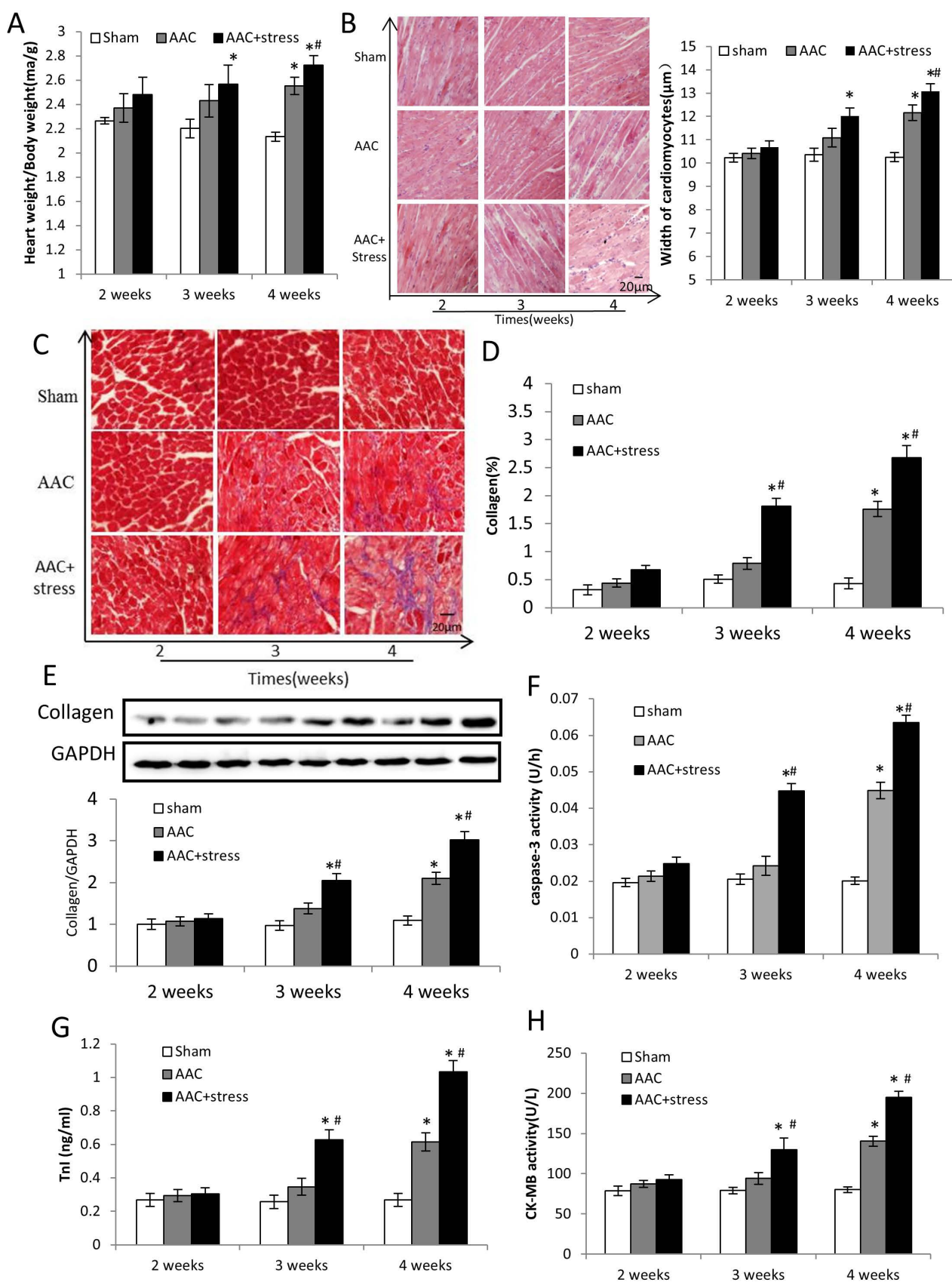
Fig. 5. The effects of NE on cell fibrosis in cultured cardiac fibroblasts (CFs). CFs were treated with different concentrations of NE for the indicated times. **A.** Proliferation was measured using the MTT assay. **B.** Cells were stained with PI and examined by FACS. The data were analyzed using the ModFit program. **C.** Western blot analysis of NE-induced collagen expression. Data represent means \pm SD. * $P \leq 0.05$ vs. Control. All experiments were performed 3 times.

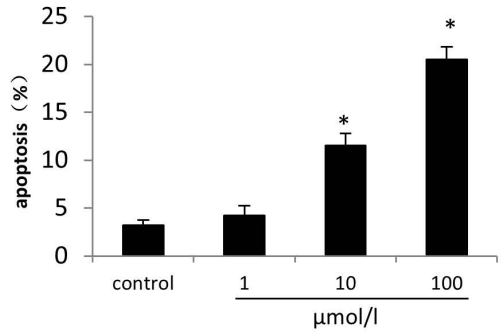
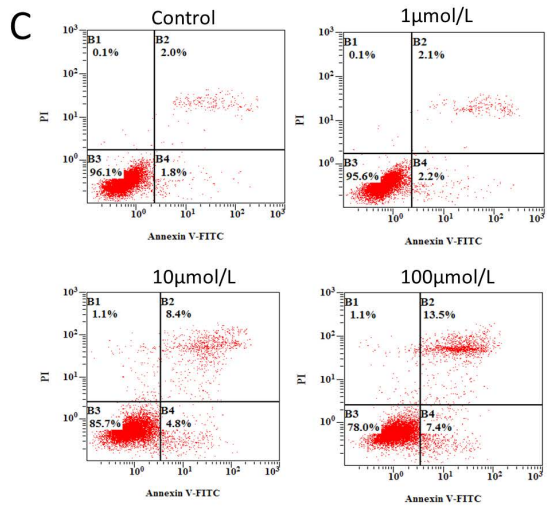
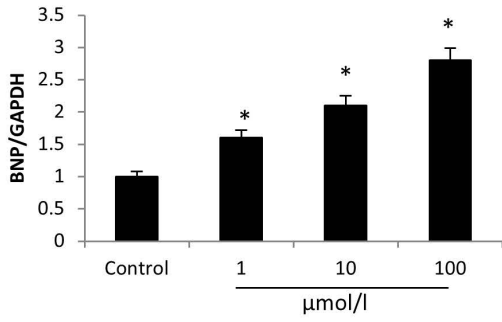
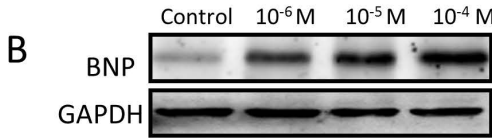
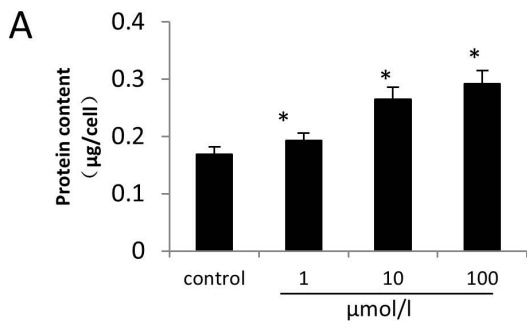
Supplemental Fig. 1 Echocardiographic parameters of the stress rats. The rats were subjected to chronic restraint stress for 6 hours per day. Echocardiographic assessment were used to detect the cardiac function after 3 weeks of stress. **A.** Representative left

ventricular (LV) M-mode echocardiograms in short axis. **B.** Left ventricular ejection fraction (%EF). **C.** Left ventricular fractional shortening (%FS). Data represent means \pm SD, n=8.

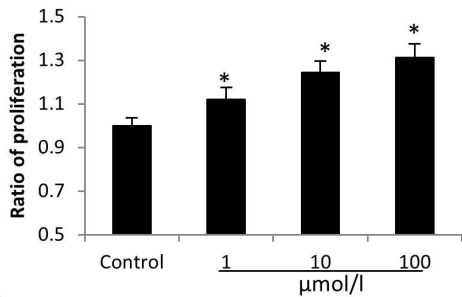




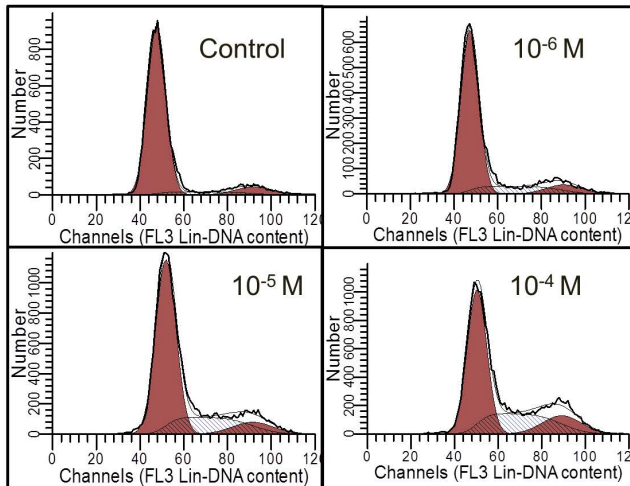




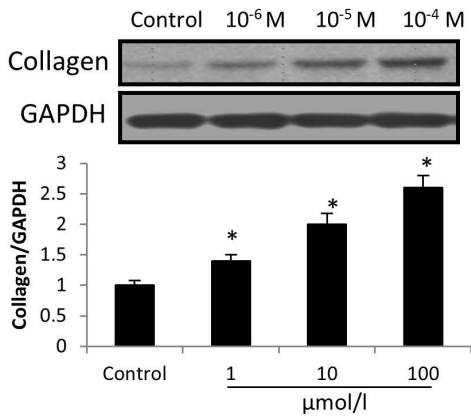
A



B



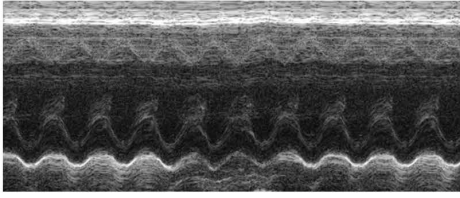
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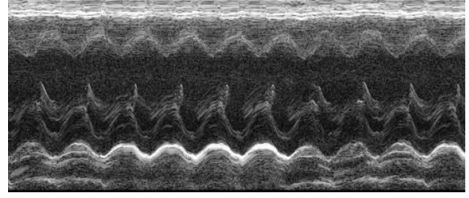
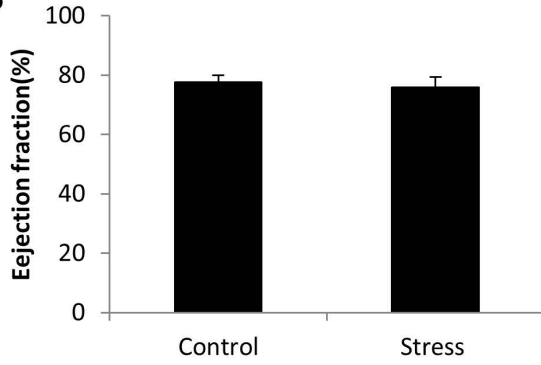
	Control	10 ⁻⁶ mol/l	10 ⁻⁵ mol/l	10 ⁻⁴ mol/l
G1	85.92%± 2.62%	77.02%± 1.52%	68.13%± 2.20%	57.52%± 2.43%
G2	8.04%± 0.95%	8.54%± 1.06%	9.32%± 0.87%	14.51%± 1.22%
S	6.02%± 0.73%	14.47%± 0.97%	22.56%± 2.74%	28.96%± 1.91%

A

Control



Stress

**B****C**