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Neuroendocrine mechanisms of left ventricular dysfunction stimulated by anger stress in rats with atherosclerosis-a putative role of natriuretic peptide

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ABSTRACT

Objective: To investigate the role of natriuretic peptide in the process of left ventricular dysfunction caused by emotional stress. **Methods:** Adult male SD rats (n=30) and Wistar rats (n=60) were selected in this study. Atherosclerosis models were induced with high-fat diet and excess VD3 injection (eight consecutive weeks), and anger stress models were prepared by resident-intruder stress experiment (two consecutive weeks). Furthermore, left ventricular functions were examined by high-resolution echocardiograph, after which left ventricular myocardium and coronary arteries were prepared for pathological section and observed with electron microscope. At the same time, the hypothalamus, medulla oblongata and left ventricular myocardium were also prepared for pathological sections to detect the localization and expression of ANP, BNP and NPR-A with immunofluorescence and western blot. **Results:** We found that left ventricular functions of atherosclerosis or emotional stress modeled rats were both inferior to the healthy ones and superior to the combined (atherosclerosis and emotional stress) modeled ones (P<0.05). We also found that atherosclerosis and emotional stress could both cause morphological changes of left ventricular cells and capillary which contribute to apoptosis and hyperblastosis. Further more, there was NPR-A distributed in hypothalamus, medulla oblongata, as well as left ventricular tissues with the same express trend between groups, with atherosclerosis modeled rats the highest and the healthy rats the lowest. **Conclusions:** The results of our study suggest that anger stress could cause an excess consumption of ANP, BNP and NPR-A in nervous and cardiovascular system which inhibit the compensatory self-repair function of atherosclerosis rats, leading to a promotion of fibrosis and lipid peroxidation, offering insight into the neuroendocrine mechanisms of left heart function obstacle.

1. Introduction

Emotional response is one of the products of nervous system development which activity is dependent of both the autonomic nervous and endocrine system[1]. In addition to the debilitating neurobehavioral consequences, there is a strong association between negative emotional stress and serious medical disorders including irritable bowel syndrome, diabetes and cardiovascular disease[2-4]. It is believed that neuroendocrine system activation induced by negative emotional stress, especially the anger, has tendency to aggravate cardiac load, which plays a catalytic

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role in the occurrence and development of cardiovascular disease[5]. Over secretion of endocrine hormones such as angiotensin, adrenocortical hormone and endotheliolysin derived from sympathetic-adrenal medulla system (SAMS) and hypothalamus-pituitary-adrenal cortex system (HPACS), which caused dysarteriotony and endothelial damage is confirmed as the main theory of its pathological mechanism, but little is known of the mediators underlying this pathophysiology.

Natriuretic peptide (NPs) is one of the peptide hormones widely expressed in cardiovascular system with three main subtypes (ANP, BNP, CNP) in mammals. There is little expression of ANP, BNP and CNP in healthy circulatory system, while patients with cardiovascular disease could get obvious increase multiplied the normal to maintain the structure and function of cardiovascular system[6-8]. ANP and BNP are both key endocrine factors to maintain arterial blood pressure and blood volume, and ANP is stimulated mainly by atrial stretch with a typical performance against myocardial hypertrophy[9], while BNP gets positive effect to alleviate fibrosis and hypertension[10]. It is demonstrated that natriuretic peptide receptors (NPR-A, NPR-B, NPR-C) are essential for biological activity of NPs, and the most important one against myocardial hypertrophy and fibrosis among is NPR-A[11,12]. In addition, NPs is also widely distributed in nervous system with abilities of adjusting neurotransmitter's release and uptake, as well as signal transmission between synapsis[13]. Whether there are interrelations between neural NPs, cardiovascular NPs and emotional stress is still a medical issue needing in-depth investigation. In this experiment, we used both emotional stress and atherosclerosis rat models to do comparative studies. ANP, BNP and NPR-A expression in brain and cardiac tissues were both detected. Combined with left ventricular morphology observation, we aim to reveal the mediating role of emotional stress in left ventricular dysfunction, which provides theoretical foundation for the neuroendocrine mechanism of cardiovascular damage caused by emotional stress.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (180-220 g, n=30) were used as intruders, and male Wistar rats (300-350 g) served as controls (n=15) or residents (n=30), and there were also male Wistar rats (n=15) served as atherosclerosis models. All rats were singly housed with a 12 h light:dark cycle (lights on at 20:00 every night) in a climate-controlled room. Food and water were available ad libitum. And all experimentations were conducted between 08:30 and 12:00 in the day.

2.2. Model preparation

Male Wistar rats (n=15) were selected as atherosclerosis models group (AMG) and given high fat diet composed with cholesterol (3%), sodium cholic acid (0.5%), propylthiouracil (0.2%), sugar (5%), lard (10%) and basal feed (81.3%); at the same time, ectogenic vitamin D3 (Shanghai fudan zhaohui pharmaceutical co., LTD) were injected intraperitoneally according to weight ratio (600 000 IU/Kg) before feeding at 8:00 and 20:00 every day. After eight weeks, aortic morphological changes were observed for model evaluation. At the same time, Male Wistar rats (n=15) were selected as emotional stress model group (ESMG) and exposed to resident-intruder stress (two consecutive weeks) in the singly housed of 7th weeks. The fighting situation was record with infrared cameras, and model evaluation was synchronous with AMG according to angry behavior, open field and saccharine preference score. Further more, Male Wistar rats (n=15) were selected as combined models group (CMG) and exposed to resident-intruder stress on the basis of high fat diet and vitamin D3 injection.

2.3. Determination of left ventricular function

High-resolution ultrasound system (Vevo770, VisualSonics) was used in this detection. Rats were fixed on heating plate in order to maintain the body temperature; at the same time, isoflurane was channeled into respiratory tract through gas inhalation anesthesia system with an initial concentration of 2%-5%. When the rats get stable heart rate in spontaneous breathing, a stable concentration of isoflurane (2%) was given to record cardiogram. left ventricular end-diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), left ventricular end-diastolic volume (LVEDV), left ventricular end systolic volume (LVESV), left ventricular ejection fraction (LVEF) and left ventricular shortening fraction (LVFS) were calculated according to the average of three cardiac cycle.

2.4. Histomorphology observation

Rats were anesthetized with pentobarbital sodium (1%) and

did myocardial perfusion with normal saline (200 mL) and paraformaldehyde solution (4%, 40-60 min). Left ventricular myocardial tissue and coronary artery were separated and soaked in the paraformaldehyde solution overnight to prepare frozen pathological section. Morphological changes of the pathological sections were observed under optical microscope (CX21BIM-SET5, Olympus) after hematoxylin staining, and all the images were acquired by the system AnalysisImage pro plus IPP 7.0.

2.5. Immunofluorescence assay for NPR-A

Hypothalamus, medulla oblongata and left ventricular myocardium tissues of rats were prepared into frozen pathological sections. After rinsed with PBS (0.01 M, pH 7.2-7.4) and soaking with fetal bovine serum (4%), the frozen pathological sections were incubated with anti-natriuretic peptide receptor A antibody (ab70848, Abcam, 1:300) at 4 ℃ for 72 h. Then, they were rinsed with PBS again and incubated with Goat Anti-Rabbit IgG (ab150077, Abcam, 1:600) at 37 ℃ for 2 h. The distribution of NPR-A was observed and photographed with fluorescence microscope (CX31-32RFL, Olympus), and all the images were acquired and analyzed by the system AnalysisImage pro plus IPP 7.0.

2.6. Western blot assay for ANP, BNP and NPA-A

For immunoblots, Hypothalamus, medulla oblongata and left ventricular myocardium tissues of rats were collected in SDS sample buffer and heated to 100 ℃ for 4 min. After cooling on ice and centrifuging at 12 000 r/min for 4 min, total proteins were separated by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis (4 ℃) with a 4% stacking gel for 30min at 80V and then a 10% separating gel for 1 h at 120 V. After SDS-PAGE gel electrophoresis, the proteins were electrophoretically transferred onto a nitrocellulose membrane (37 ℃) for 1 h at 100 V. Then, the nitrocellulose membrane was blocked for 1h in TBST buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.4) containing 1% low-fat milk, the membrane was incubated overnight at 4 ℃ in TBST containing NPs antibodies [(Anti-ANP antibody (ab91250, Abcam, 1:200), anti-BNP antibody (ab19645, Abcam, 1:500), or Anti-Natriuretic Peptide Receptor A antibody (ab70848, Abcam, 1:300)], After three washes of 10min each in TBST, the membranes were incubated for 1 h at 37 ℃ with horseradish-conjugated peroxidase-labeled Goat Anti-Rabbit IgG Fc diluted 1:2 000 (ab6702, Abcam, 1:600) in TBST. The membrane was washed three times in TBST and then processed using the enhanced chemiluminescence (ECL) detection system. We used SDS sample buffer as the negative control. All the images were acquired and analyzed by the system AnalysisImage pro plus IPP 7.0.

2.7. Statistical analysis

All experiments were performed at least three times, and the results of a representative experiment are presented. The significance of the difference between experimental and control groups was analyzed with Student's *t*-test, and the significance of the difference between experimental groups was analyzed with one-way ANOVAs. Differences of $P<0.05$ were considered significant.

Table 1

Angry aggression, open-field, and saccharine preference score (mean±SD, n=15).

Index	CG	ESMG	AMG	CMG
Fight number	-	48.10±11.70	26.30±7.52	47.70±12.60
Threat number	-	15.30±3.74	26.20±11.30	13.50±6.74
Climbing number	-	19.70±4.94	23.30±14.70	23.10±11.40
Victory number	-	14.10±4.47	6.35±1.73	12.50±4.19
Defeat number	-	--	1.76±0.94	2.42±1.37
Angry aggression score	-	96.40±17.60	79.40±17.30	91.30±21.70△
Open-field	79.20±21.60	121.00±37.80*	84.60±29.80	127.00±39.70△
Saccharine preference	0.37±0.14	0.26±0.11*	0.21±0.07	0.13±0.06△

Angry aggression score = Fight number + Threat number + Climbing number + Victory number-Defeat number; Saccharine preference = [Sugar water consumption (g) / Sugar water consumption (g) + Pure water consumption (g)] ×100%; ESMG *vs.* CG, *$P<0.05$; CMG *vs.* AMG, △$P<0.05$.

3. Results

3.1. Model preparation

To evaluate the atherosclerosis model, aortas of the controls and atherosclerosis models were prepared into frozen pathological sections and stained with hematoxylin, and then observed under optical microscope. We found that aortic endothelial tissues of atherosclerosis modeled rats showed a fracture, and parts of the vascular inner surface wall were adhered by lipid plaques leading to hyperplasia liked construction (Figure 1). To evaluate the anger stress model, the angry behavior, open field and saccharine preference scores were assessed. We found that anger stress modeled rats got higher behavior and open field scores while lower saccharine preference scores than the controls (Table 1).

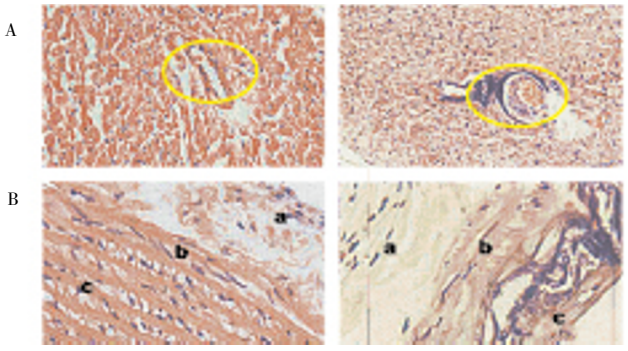

Figure 1. Morphological characteristics of the coronary artery under electron microscope.
(A) Transverse section of coronary artery; (B) Longitudinal section of coronary artery, (a) adventitia, (b) medial arterial, (c) endarterium. Magnification, ×400.

3.2. Left ventricular morphology and function

Rats modeled by atherosclerosis or emotional stress were increased in left ventricular transection diameter and intercellular space; at the same time, capillary density decreased because of rupture, damage or losses (Figure 2). To compare left ventricular functions between groups, we detected LVEDD, LVESD, LVEDV, LVESV, LVEF and LVFS with echocardiography. Statistical results showed (Figure 3) that left ventricular functions of AMG and EMSG were significantly lower than that of CG; at the same time, the left ventricular functions of CMG rats was significantly lower than of AMG or EMSG (P<0.05).

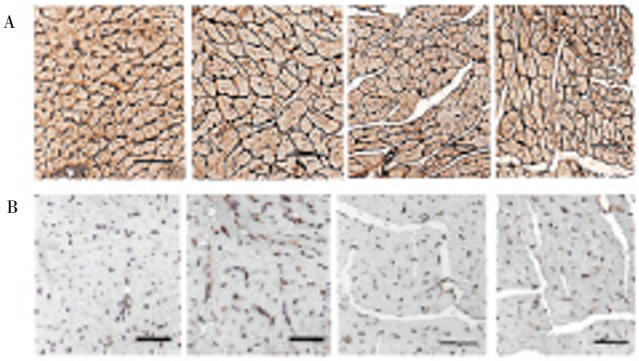

Figure 2. Morphological characteristics of left ventricular myocardial tissue under electron microscope.
(A) Cellular morphology; (B) Capillaries morphology. Magnification, × 400.

3.3. Distribution and expression of NPs

There was NPR-A in hypothalamus, medulla oblongata and left ventricular myocardium in immunofluorescence images (Figure 4); and fluorescence intensities were different between groups (P<0.05) with AMG the highest while CG the lowest. ANP, BNP and NPR-A protein expression got the same trend in hypothalamus, medulla oblongata and left ventricular myocardial tissues in west-blot images (Figure 5-7), and there was difference (P<0.05) between groups in ANP, BNP and NPR-A protein expression with AMG the

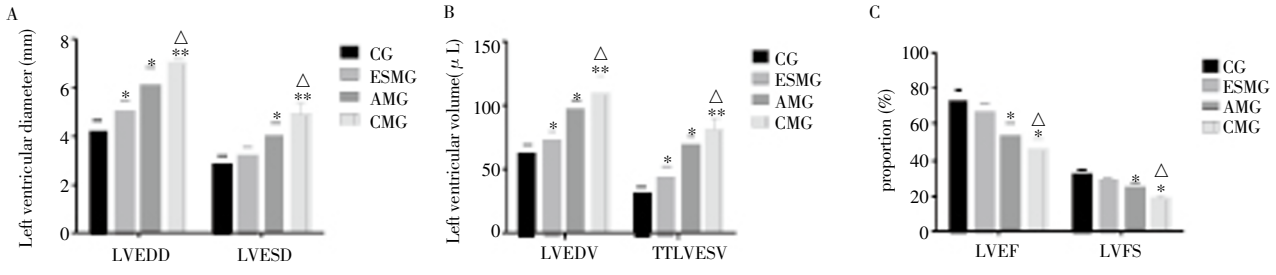

Figure 3. Left ventricular function indexes between groups.
(A) Left ventricular diameter in diastolic and systolic phase; (B) Left ventricular volume in diastolic and systolic phase; (C) Left ventricular ejection and shortening fraction. ESMG *vs.* CG, *P<0.05; AMG *vs.* CG, **P<0.01; CMG *vs* AMG, $^{\triangle}P$<0.05.

highest while CG the lowest.

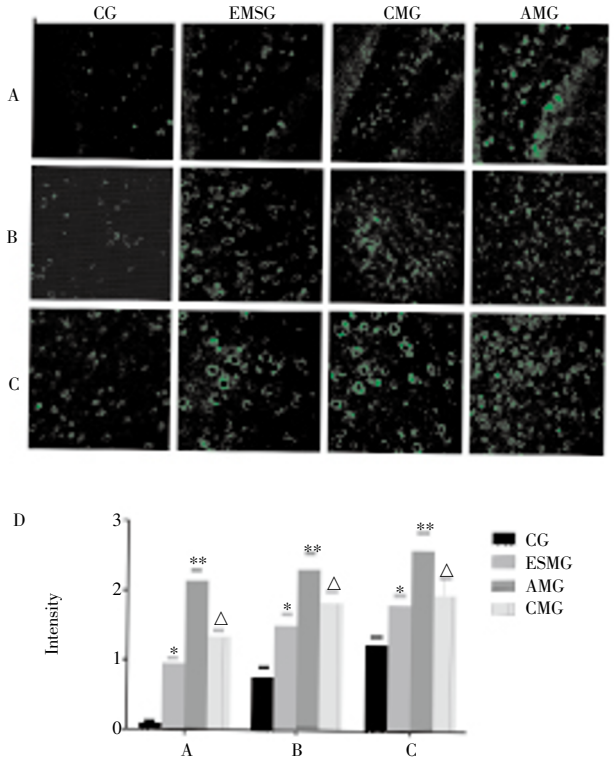

Figure 4. IF images (A, B, C) and intensity statistics (D) of freezing sections between groups.
(A) Sections of left ventricular tissue (B) Sections of medulla tissue (C) Sections of hypothalamus tissue. Magnification, ×400. ESMG *vs.* CG, **P*<0.05; AMG *vs.* CG, ***P*<0.01; CMG *vs.* AMG, △*P*<0.05.

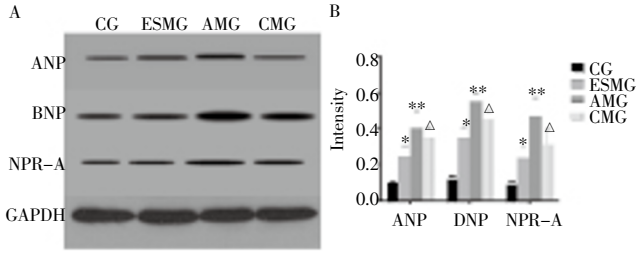

Figure 5. Protein expression of ANP, BNP, and NPR- A in left ventricular myocardial tissues between groups.
(A) Western blot images (B) Western blot statistics. ESMG *vs.* CG, **P*<0.05; AMG *vs.* CG, ***P*<0.01; CMG *vs.* AMG, △*P*<0.05.

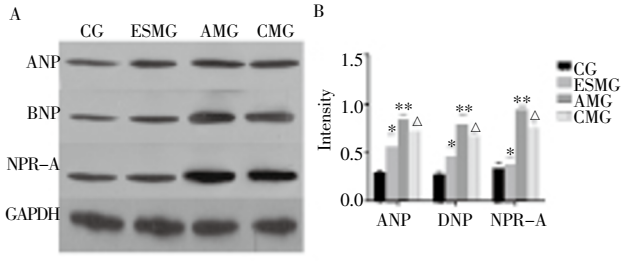

Figure 6. Protein expression of ANP, BNP, and NPR- A in hippocampus tissues between groups.
(A) Western blot images (B) Western blot statistics. ESMG *vs.* CG, **P*<0.05; AMG *vs.* CG, ***P*<0.01; CMG *vs.* AMG, △*P*<0.05.

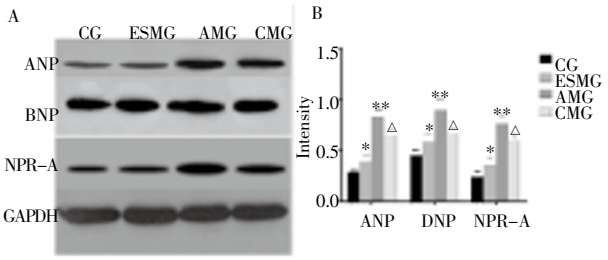

Figure 7. Protein expression of ANP, BNP, and NPR- A in medulla oblongata tissues between groups.
(A) Western blot images (B) Western blot statistics. ESMG *vs.* CG, **P*<0.05; AMG *vs.* CG, ***P*<0.01; CMG *vs.* AMG, △*P*<0.05.

4. Discussion

The correlation between emotional stress and cardiovascular disease has been reported a lot[14]; at the same time, NPs were found to be biomarkers of cardiovascular damage[15]. Further studies revealed that both emotional stress and NPs could affect the occurrence, development and prognosis of cardiovascular disease through the central nervous system[16]. This study set focus on the doubt that whether emotional stress could lead to left ventricular dysfunction in cardiovascular disease by NPs mediation. Used anger stress and atherosclerosis rats models, we detected ANP, BNP and NPR-A expression in brain and cardiac tissues; More over, left ventricular and aorta morphology were observed to reveal the mediating role of emotional stress in cardiovascular system damage. Our study showed that both therosclerosis and anger stress had effect to reduce left ventricular functions of rats, and the concurrence of them could produce synergy resulting in severer left ventricular dysfunction, and the morphological characteristics had typical consistency with its functions. In addition, the ANP, BNP, and NPR-A expression got similar trends in hypothalamus, medulla oblongata and left ventricular myocardium: the highest in AMG and the lowest in CG.

This experiment results explained the correlation between anger stress and atherosclerosis from myocardial morphology as well as left ventricular myocardial functions. It has been demonstrated that emotional stress has electrophysiological effect on several encephalic regions such as brainstem, hypothalamus and limbic system through SAMS and HPCS. Activated SAMS releases excess adrenal cortical hormone, angiotensin and prostaglandin that induce cardiac arrhythmias, conduction block and high pressure[17,18]; and

activated HPCS could promote the secretion of cholesterol and triglyceride that weaken endothelial function and result in atherosclerosis[19,20]. This study showed from the perspective of atherosclerosis that anger stress and cardiovascular disease had interaction in the process of left ventricular myocardial injury.

This experiment results also showed that myocardial morphology changes and left ventricular dysfunction induced by anger stress or atherosclerosis both had influence on the expression of NPs. Despite left ventricular myocardial tissue, we chose hypothalamus and medulla oblongata, the encephalic regions most closely related to SAMS and HPCS. With the aid of immunofluorescence assay, we found that there was NPR-A distributed in the hypothalamus, medulla oblongata, as well as left ventricle. With the aid of western blot assay, we also found that ANP, BNP and NPR-A had the same expression trend in the brain and cardiovascular tissues. NPs is a kind of polypeptide hormone widely spreading in the central and the circulatory system with positive functions such as excreting sodium, drainage, and relaxing blood vessels; thus maintain arterial blood pressure and blood volume[21]. It has been demonstrated that NPs barely express in healthy cardiovascular organizations. ANP against myocardial hypertrophy and BNP against fibrosis were released in the state of myocardial pathological morphological change to alleviate hyperpiesia and promote myocardial tissue's compensatory self-repair[22,23]. NPs in circulatory system need highly specific cell surface receptors (NPR) to play biological activities. There are three subtypes (NPR-A, NPR-B, NPR-C) already known in mammals, and NPR-A is the one most closely related with cardiovascular system. ANP-NPR-A system plays an inhibition role in regulating cardiac muscle cell growth, and ANP knockout mice show typical defect with holistic proliferative hypertrophy in ventricular volume, which is independent of blood pressure regulation[24]. BNP-NPR-A system has certain effect on regional pressure regulation, and BNP knockout mice release excess angiotensin converting enzyme and show a fibrosis tendency in partial region while the ventricular mass index is normal, which is different from ANP[25]. In consideration of the different characteristics of ANP and BNP deficiency, we detected both ANP and BNP expression, and found that the expression of ANP and BNP were changed with the same trend. This result indicated that left ventricle damage induced from emotional stress or atherosclerosis is not limited to local myocardial organization.

In addition, from the perspective of NPs expression, ANP, BNP and NPR-A expressions of rats exposed to single anger stress or atherosclerosis were all significantly higher than those of the controls. This is because in the constitution of vascular endothelial and left ventricular function damage, NPs were released to alleviate and blood vessel pressure compensatorily[26,27]. However, ANP, BNP and NPR-A expressions of rats exposed to both anger stress and atherosclerosis were significantly lower than those exposed to single anger stress or atherosclerosis while the pathological severity was increased. We indicate that under the condition of atherosclerosis pathology, anger stress could promote NPs consumption in the central and cardiovascular system, which is disadvantaged for compensatorily self-repair of rats.

In a word, anger stress could promote NPs (ANP, BNP and NPR-A) consumption, thus inhibit the compensatorily vicarious self-repair of atherosclerosis rats and promote fibrosis and peroxide damage, which may be one of its neuroendocrine mechanisms to increase left heart function obstacle for cardiovascular disease.

Conflict of interest statement

We declare that we have no conflict of interest.

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