Increased B-type-natriuretic peptide promotes myocardial cell apoptosis via the B-type-natriuretic peptide/long non-coding RNA LSINCT5/caspase-1/interleukin 1β signaling pathway

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Abstract. Chronic heart failure (CHF) is the final stage of various heart diseases, and is increasingly recognized as a major health problem in the elderly. Previous studies demonstrated that B-type-natriuretic peptide (BNP) is an established biomarker of CHF. Furthermore, BNP also regulates cell proliferation, differentiation and apoptosis. Recent evidence has revealed that BNP affects myocardial cell apoptosis during myocardial ischemia-reperfusion injury. Long non-coding RNAs (lncRNAs) are emerging as novel molecular compounds involved in gene regulation, and have important roles in numerous human diseases. However, the mechanism underlying the BNP and lncRNA-induced regulation of myocardial cell apoptosis remains to be elucidated. The present study reported that lncRNA LSINCT5, upregulated by BNP, is able to regulate myocardial cell apoptosis via the activation of the caspase-1/interleukin (IL)-1β signaling pathway. BNP-induced apoptosis of HCM cells was observed using flow cytometry, and involved caspase-1. In addition, expression profiling using a human lncRNA polymerase chain reaction array revealed that LSINCT5 was highly expressed in BNP-treated myocardial cells, as compared with untreated cells. The role of lncRNA LSINCT5 in HCM cell apoptosis was also investigated. The results of the present study indicated that LSINCT5 silencing by small interfering RNA inhibits caspase-1/IL-1β signaling, and suppresses apoptosis in BNP-treated HCM cells. Therefore, high expression levels of BNP promote the apoptosis of myocardial cells through the lncRNA LSINCT5 mediator, which activates the caspase-1/IL-1β signaling pathway. These findings uncovered a novel pathogenic mechanism, and provided a potential therapeutic target for CHF.

Introduction

Chronic heart failure (CHF) is the final stage of various heart diseases, and is the primary cause of mortality in elderly individuals worldwide (1). CHF affects approximately four million individuals in China, and an increasing number of new cases are diagnosed annually (2). CHF is recognized as a major and increasing health problem in China. However, there is a shortage of studies regarding the underlying pathogenesis, diagnosis and treatment of CHF. Numerous factors, including chronic myocardial lesions, hypertension and atherosclerotic disease, are important in CHF (3).

B-type-natriuretic peptide (BNP) is a cardiac hormone secreted from the atrial and ventricular myocardium, following ventricular overloading or volume expansion. At present, the association between BNP and the risk of CHF is well-established, and BNP is the most established biomarker of CHF (4,5). The measurement of BNP expression levels is used clinically as a diagnosis for CHF, as BNP levels are elevated in the plasma of patients with CHF (6,7). BNP has also been investigated as a potential therapeutic target of CHF (8). Although extensive studies on BNP have been conducted, the detailed molecular mechanism underlying the involvement of BNP in CHF is only partially understood.

Apoptosis is the process of programmed cell death often characterized by cell shrinkage, nuclear fragmentation, and chromosomal DNA fragmentation (9). Cell apoptosis has been identified to be important in numerous human diseases, including cancer (10), Alzheimer’s disease (11) and tuberculosis (12). Previous studies have demonstrated that apoptosis of human myocardial cells has an important role in the pathogenesis of CHF (13,14). It is evident that myocardial apoptosis results from numerous factors, including hyperlipidemia, oxidized lipoproteins, myocardial ischemia-reperfusion injury, hypoxia, and other heart injury-inducing factors (15-18). However, the detailed molecular mechanisms underlying the effect of myocardial apoptosis on CHF remain to be elucidated. Deng et al (19) reported that BNP affects myocardial cell apoptosis during myocardial ischemia-reperfusion injury. However, whether BNP is also associated with myocardial cell apoptosis in CHF remains unclear.

Protein-coding genes only constitute a small portion of the human genome, and the majority of transcripts are non-coding RNA (ncRNAs) (20). ncRNAs include small ncRNAs and...
long ncRNAs (lncRNAs). Although small ncRNAs, such as microRNAs, small interfering (si)RNAs and piwi-interacting RNAs have been well-studied, lncRNAs are less well-characterized. However, an increasing number of studies have reported that lncRNAs have important roles in cancer progression and metastasis, as well as cellular processes, such as cell proliferation and apoptosis (21,22). Therefore, identifying the association between lncRNAs regulated by BNP and myocardial apoptosis may aid in understanding the function of BNP in the pathogenesis of CHF.

The present study aimed to demonstrate how increased BNP may induce myocardial cell apoptosis. Human lncRNA polymerase chain reaction (PCR) arrays were used to compare the lncRNA expression profiles between BNP-treated cells and control cells. Finally, the mechanism underlying the regulation of myocardial cell apoptosis in vitro by lncRNA LSINCT5 was investigated.

Materials and methods

Reagents. The following mouse monoclonal antibodies were purchased from Abcam (Cambridge, MA, USA): Anti-caspase-1 (cat. no. ab17815), anti-caspase-3 (cat. no. ab158030), anti-caspase-7 (cat. no. ab1580933), anti-caspase-8 (cat. no. ab39731) and anti-interleukin (IL)-1β (cat. no. ab2105). Rabbit anti-mouse immunoglobulin G (IgG) polyclonal horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. no. ZB-2305) and mouse anti-human GAPDH monoclonal antibodies (cat. no. TA-08) were purchased from Beijing Zhongshan Jingqiao Biotechnology Co., Ltd. (Beijing, China). BNP was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Cell culture. HCM human myocardial cells were purchased from ScinCell Research Laboratories (Carlsbad, CA, USA). The HCM cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin (both HyClone, GE Healthcare Life Sciences, Logan, UT, USA), and incubated at 37°C in an atmosphere containing 5% CO₂.

Determination of apoptosis levels. The levels of apoptosis were determined by flow cytometry using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer’s instructions. The cells were added to various concentrations of BNP (50, 100, 150 and 200 pg/ml) in the treated groups, and untreated groups served as controls. After 48 h, the cells were harvested and washed twice with phosphate-buffered saline (PBS). The cells were then stained with 1 ml Annexin V binding buffer containing 10 µl propidium iodide (PI) solution and 5 µl Annexin V-fluorescein isothiocyanate, for 10 min at room temperature, and analyzed by flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA).

Western blot analysis. The cells were harvested and homogenized using cell lysis buffer (Beyotime Institute of Biotechnology). The homogenates were then centrifuged for 30 min at 4°C, 10,500 x g, and the supernatants were collected as protein samples. Protein levels were measured using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Equal quantities of protein samples were separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred onto polyvinylidene difluoride membranes (Beyotime Institute of Biotechnology). The membranes were subsequently incubated in 5% skimmed milk in Tris-buffered saline with Tween-20 (Sangon Biotech Co., Ltd., Shanghai, China) blocking solution at room temperature for 1 h. The membranes were then washed three times with TBST (20 mM Tris-HCl, pH 7.6; 137 mM NaCl; 0.1% Tween-20). The membranes were incubated with agitation at 4°C overnight with the following specific mouse anti-human monoclonal primary antibodies: Anti-caspase-1 (1:1,000), anti-caspase-3 (1:1,000), anti-caspase-7 (1:2,000), anti-caspase-8 (1:1,500), anti-IL-1β (1:1,000) and anti-GAPDH (1:1,000). The membranes were washed again, and then incubated with rabbit anti-mouse IgG polyclonal HRP-conjugated secondary antibodies (1:1,000), at room temperature for 50 min. Finally, the protein bands were visualized using an Enhanced Chemiluminescence Western Blotting Detection system (GE Healthcare Life Sciences, Chalfont, UK).

Human lncRNA PCR array and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and purified with an RNasey MinElute Cleanup kit (Qiagen GmbH, Hilden, Germany). RT-qPCR was subsequently performed using one Step SYBR® PrimeScript™ RT-PCR kit II (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer’s instructions. The Human lncRNA Profiler, which included 94 lncRNAs associated with numerous human diseases, was designed and

| Primer name | Primer sequence |
|-------------|----------------|
| Caspase-1 | F: 5'-AGGAGGGAATATGTTGGG-3' | R: 5'-AACCTTGGGCTTGTCTT-3' |
| Caspase-3 | F: 5'-ATCCACGAGCAGTCAAAGG-3' | R: 5'-CCTTGTAACACTGTCACACACAC-3' |
| Caspase-7 | F: 5'-CAACCAGGAGGACGAGAGT-3' | R: 5'-TCAGGATGGAGGAGGACGAGG-3' |
| Caspase-8 | F: 5'-TACATTCCAGAAGTTGGGACG-3' | R: 5'-TGGAAATGAGTTGTTGGC-3' |
| IL-1β | F: 5'-CCTTGTCGAAATGGGCAGT-3' | R: 5'-TTCTGTCGACATGTGCCT-3' |
| GAPDH | F: 5'-GGAATACGACAACTCAGCGG-3' | R: 5'-AGCCACATCGCCTACAGACAC-3' |
| LSINCT5 | F: 5'-TTCCGGCAAGCTCCTTTCTTA-3' | R: 5'-GCCCAAGTCTCCAAAAAAGTCTT-3' |
produced by the Shanghai Funeng Biological Technology Co., Ltd. (Shanghai, China). The sample preparation and IncRNA PCR array were performed according to the manufacturer’s instructions. Purified RNA was reversed transcribed into cDNA using a Human lncRNA Profiler cDNA synthesis buffer (Shanghai Funeng Biological Technology Co., Ltd). RT-qPCR and PCR array amplification were conducted using an IQ5 machine (Bio-Rad Laboratories, Inc., Hercules, CA, USA), with the following thermocycling conditions: 42˚C for 5 min, 95˚C for 10 sec, 40 cycles at 95˚C for 15 sec, 60˚C for 15 sec, and 72˚C for 20 sec. PCR was followed by melt curve analysis to determine the reaction specificity. Relative gene expression levels were calculated using the  \( \Delta\Delta^Ct \) method (23).

Primers used in RT-qPCR are shown in Table I.

Small interfering (si)RNA. In order to investigate the mechanism underlying the regulation of myocardial cell apoptosis by IncRNA LSINCT5, the HCM cells were transfected with siRNA targeting IncRNA LSINCT5 (si-LSINCT5) or negative control (si-Scramble) using Lipofectamine® 2000 transfection reagent (Invitrogen Life Technologies). Briefly, HCM cells at 50% confluence were transfected with 100 nM of either siRNAs targeting LSINCT5 or scrambled negative controls using Lipofectamine® 2000, according to the manufacturer’s instructions. siRNAs were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The sequences of si-LSINCT5 were as follows: si-LSINCT5-1, 5’-TACAGCCTTCTAGGCGAGATT-3’; si-LSINCT5-2, 5’-GACACTTACTTTACCTCAGATCA-3’; and si-LANCT5-3, 5’-CAATACAGTCAGCCTGAATCTTCT-3’.

Immunofluorescence. The cells were washed three times with cold PBS, and fixed for 10 min with 4% paraformaldehyde dissolved in PBS at room temperature. The fixed cells were blocked for 1 h in 5% skimmed milk dissolved in PBS. The cells were then incubated with anti-GAPDH antibody overnight at 4˚C in blocking solution, and washed three times with PBS with Tween-20. Following washing, the cells were incubated with goat anti-mouse IgG monoclonal antibody conjugated to FITC dye (cat no. KC-MM-095; 1:50; Kangchen, Shanghai, China) for 1 h at room temperature. Finally, the cells were stained with 10 µg/ml DAPI (Beyotime Institute of Biotechnology) for 10 min. Images were observed and captured using a Nikon fluorescence microscope (TE2000-U; Nikon Corporation, Tokyo, Japan).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical significance was determined using Student’s t-test (two-tailed) and one-way analysis of variance using SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

*Increased BNP induces human myocardial cell apoptosis.*

HCM cell apoptosis was measured by an Annexin-V FITC/PI double staining assay. As shown in Fig. 1A, the BNP-induced increase in HCM cell apoptosis levels occurred in a dose-dependent manner. In the BNP-untreated group, the percentage of apoptotic cells was ~2.4%. However, following cell treatment with various concentrations of BNP for 24 h, the percentage of apoptotic cells increased to 3.0, 4.4, 8.1 and 13.1%, respectively. These results suggest that BNP significantly promotes the apoptosis of HCM cells.

*Caspase-1 is involved in BNP-induced apoptosis of HCM cells.* To determine the mechanism underlying BNP-induced
apoptosis, the expression levels of caspase-1, -3, -7 and -8 were analyzed in 200 pg/ml BNP-treated HCM cells. As shown in Fig. 1B, only the expression levels of caspase-1 were upregulated by BNP in a dose-dependent manner. No marked changes were identified in the expression levels of the other caspases in BNP-stimulated HCM cells. These data provide evidence that BNP-induced apoptosis is associated with the caspase-1 signaling pathway.
IncRNA expression profiles of BNP-treated HCM cells. To further investigate the molecular mechanism underlying BNP-induced apoptosis, a human IncRNA PCR array was used to compare the IncRNA expression profiles between BNP-treated and untreated cells. A heat map of differential IncRNA expression was generated using a method of hierarchical clustering by GeneSpring GX software 7.3 (Agilent Technologies, Inc., Santa Clara, CA, USA). As shown in Fig. 2A, a total of 33 IncRNAs were found to be differentially expressed between the two groups. Among them, eight and 25 IncRNA probes were upregulated and downregulated, respectively, in BNP-treated cells. IncRNA LSINCT5 exhibited the greatest change in expression in BNP-treated cells.

RT-qPCR was used to further investigate the upregulation of LSINCT5 in BNP-treated cells. As shown in Fig. 2B, compared with untreated cells, the expression levels of LSINCT5 were significantly increased (~10-fold) in a dose-dependent manner in BNP-treated cells.

LSINCT5 silencing inhibits caspase-1/IL-1β signaling in BNP-treated HCM cells. To investigate the role of IncRNA LSINCT5 in the apoptosis of HCM cells, LSINCT5 was silenced in BNP-treated cells using siRNA. Cells transfected with si-scramble were used as negative controls. As shown in Fig. 3A, the expression levels of LSINCT5 in siRNA-transfected cells decreased by 0.8, 0.3 and 0.4-fold, respectively, as compared with the scramble group. These results indicate that LSINCT5 was efficiently silenced in HCM cells by siRNA-LSINCT5-2. Therefore, siRNA-LSINCT5-2 was used for all subsequent LSINCT5 silencing experiments.

The expression levels of caspase-1, -3, -7 and -8 in siRNA-LSINCT5-2-treated groups were analyzed using RT-qPCR and western blotting. Compared with cells treated with si-scramble, the expression levels of caspase-1 were significantly downregulated in siRNA-LSINCT5-2-treated groups; however, the expression levels of the other caspases were not significantly altered (Fig. 3B and C).

In order to investigate the role of LSINCT5 in the caspase-1/IL-1β signaling pathway, the expression levels of caspase-1 and IL-1β were quantified in HCM cells containing silenced LSINCT5. As shown in Fig. 3D and E, caspase-1 and IL-1β were downregulated in LSINCT5 knockdown cells. The data suggest that LSINCT5 silencing inhibits caspase-1/IL-1β signaling in BNP-treated HCM cells.
Knockdown of LSINCT5 prevents apoptosis in BNP-treated HCM cells. The expression levels of LSINCT5 were increased in BNP-treated HCM cells. Therefore, LSINCT5 may be important in HCM apoptosis. To investigate this hypothesis, siRNA knockdown was performed in HCM cells.

To facilitate the observation of cell proliferation, immunochemistry was used for visual observation. As shown in Fig. 4A, cell proliferation was significantly suppressed in the BNP and BNP + siRNA-scramble-treated groups following three days treatment. However, in BNP-treated cells transfected with siRNA-LSINCT5, a marked increase in cell proliferation was observed, as compared with the BNP-treated control group. HCM cell apoptosis was measured by an Annexin-V FITC/PI double staining assay. The expression levels of caspase-1 and IL-1β were quantified in all groups. The expression of caspase-1 and IL-1β was overexpressed in BNP-treated and BNP + siRNA-scramble-treated cells. However, these values were markedly downregulated in cells transfected with siRNA-LSINCT5 (Fig. 4B). As shown in Fig. 4C, the percentage of apoptotic cells in the BNP and BNP + siRNA-scramble-treated groups was ~10.6 and 11.0%, respectively. However, this value in the BNP + siRNA-LSINCT5-treated group decreased to 4.3%. These results suggest that knockdown of LSINCT5 prevents apoptosis in BNP-treated HCM cells via the inhibition of caspase-1/IL-1β signaling pathway.

Discussion

To the best of our knowledge, the present study is the first to report that BNP promotes apoptosis of myocardial cells through IncRNA LSINCT5 in vitro. Previous studies have suggested that BNP, a peptide neurohormone secreted predominantly in the heart, has an important role in numerous cardiovascular diseases (24,25). Patients with type 2 diabetes mellitus exhibit elevated levels of BNP in plasma (26). In acute coronary syndromes, elevated levels of BNP are a prognostic factor for adverse outcome (27). It is well-established that BNP is useful as a biomarker for the diagnosis of CHF, and even as a therapeutic target in CHF (6,8). This implies that BNP may have an important role in the development and progression of CHF.

An increasing number of studies have demonstrated that myocardial cell apoptosis is involved in the pathogenesis of CHF (28,29). Hirota et al (30) reported the presence of considerable myocardial cell apoptosis in rats, which was induced by the absence of membrane protein gp130, resulting in the rapid development of CHF. Song et al (31) showed that inhibition of myocardial cell apoptosis may prevent CHF progression in the rat model. Although numerous studies on myocardial cell apoptosis have been conducted, the molecular mechanisms underlying myocardial cell apoptosis remain to be elucidated.

Caspases, a family of cysteine proteases, have a regulatory function in cell apoptosis by cleaving their specific substrates. Previous studies have indicated that caspases are implicated in the development and progression of heart failure (32,33). Narula et al (34) proposed that caspase-3 activated by the release of mitochondrial cytochrome c is a predictive factor of adverse outcomes in patients with CHF. Liu et al (35) reported that manipulation of the caspase-12 and c-Jun N-terminal kinase signaling pathways may alter the outcome of heart failure. Recent studies have reported concordant results, demonstrating that caspase-1 has an important role in cardiovascular disease (36,37). Merkle et al (38) demonstrated that overexpression of cardiomyocyte-specific caspase-1 in mice may lead to heart failure. All these findings support a critical role for caspase-1-mediated myocardial apoptosis in the progression of CHF. However, little is known regarding the mechanism underlying the regulation of caspase-1 in myocardial apoptosis in patients with CHF.

Accumulating evidence suggests that IncRNAs have important roles in the regulation of cell apoptosis in numerous diseases. DeOcesano-Pereira et al (39) revealed that IncRNA INXS, transcribed from the opposite genomic strand of B-cell lymphoma-extra-large, induces apoptosis in kidney tumor cell lines. Xu et al (40) reported that high expression levels of IncRNA URNA may inhibit apoptosis by suppressing ZAK expression, a regulator of the extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway, in hepatocellular carcinoma. Wang et al (22) demonstrated that IncRNA CARL inhibits anoxia-induced apoptosis in cardiomyocytes by impairing microRNA-539-dependent downregulation of prohibitin 2, which is able to inhibit mitochondrial fission and apoptosis. Although IncRNAs have been recognized as regulators of gene expression in apoptosis, the possible regulation of caspase-1 by IncRNAs has yet to be determined.

In the present study, a human IncRNA PCR array kit, the Human IncRNA Profiler, was used to compare the IncRNA expression profile between BNP-treated and control cells. The results demonstrated that the expression levels of IncRNA LSINCT5 were upregulated by BNP in cardiomyocytes. In addition, suppression of LSINCT5 by siRNA was able to reduce cell apoptosis. Therefore, LSINCT5 may have an important role in cardiomyocyte apoptosis. In order to further investigate the molecular mechanisms underlying myocardial cell apoptosis, caspase expression levels were quantified in myocardial cells with LSINCT5 knockdown. The results indicated that the expression of caspase-1 was regulated by LSINCT5 during myocardial apoptosis. To further investigate these results, the present study also quantified the expression levels of IL-1β, cleaved by caspase-1 from pro-IL-1β. The results demonstrated that the expression levels of IL-1β were concordant with those of caspase-1.

In conclusion, the results of the present study demonstrated that IncRNA LSINCT5 was upregulated in apoptotic myocardial cells induced by BNP. In addition, LSINCT5 has an important role in the regulation of cardiomyocyte apoptosis through the regulation of caspase-1/IL-1β expression. These findings may aid in improving the understanding of the molecular mechanism underlying CHF. Future research may focus on whether IncRNA LSINCT5 can be used as a potential diagnostic tool, or even a therapeutic target for CHF.

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