B-Type Natriuretic Peptide Enhances Mild Hypoxia-Induced Apoptotic Cell Death in Cardiomyocytes

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In the case of left ventricle remodeling after myocardial infarction, cardiomyocyte apoptosis is attributed to increased cardiac workload by the stimulus such as chronic hypoxia. B-Type natriuretic peptide, being known as a reliable prognostic of cardiovascular pathology, plays an important role in the myocardial infarction. However, the action of B-type natriuretic peptide on cardiomyocytes undergoing apoptosis is unclear. In the present study, B-type natriuretic peptide has exhibited the enhancing effects on the mild hypoxia-induced cardiomyocyte apoptosis with the manifestation of facilitating phosphatidylserine evagination and increasing typical fragmented nuclei. In addition, B-type natriuretic peptide aggravated the dissipation of ΔΨm, the depletion of intracellular ATP and the increase of caspase-3 activity. 8-Bromo-cGMP, which increased cGMP independent of B-type natriuretic peptide, could mimic B-type natriuretic peptide’s effects; whereas cGMP-dependent protein kinase inhibitor, Rp-8-br-cGMP inhibited that. Further study revealed the enhancing effect of BNP on down-regulation of Bcl-2 mRNA expression in the presence of mild hypoxia. In conclusion, the present study demonstrated that B-type natriuretic peptide aggravated the cardiomyocyte apoptosis by influencing hypoxia-induced mitochondrial death pathway, which is true at least in this oxygen deprivation model; and this effect was partially realized through intracellular cGMP.

Key words  B-type natriuretic peptide; apoptosis; hypoxia; cGMP

The natriuretic peptides are a family of small peptides that activates specific NP-bound guanylyl cyclase (GC) receptors and stimulates cGMP synthesis. Three main natriuretic peptides have been identified so far: atrial (ANP), B-type (BNP) and C-type natriuretic peptides (CNP). Among them, BNP having a common core structure of natriuretic peptides, which consists a 17-member disulfide ring with a highly conserved internal sequence of -FGXXXDRIGXXSGL-, plays an essential role in the maintenance of homeostasis such as natriuresis, diuresis, vasorelaxant effect and inhibits the activation of renin-angiotensin-aldosterone system under pathological conditions.5) Expression of the three natriuretic peptide receptors (NPR-A, NPR-B, NPR-C) has been confirmed in human and rat cardiac tissue.21) Commonly, BNP exhibits its effects by preferentially binding to the NPR-A (also known as GC-A) which is coupled to cytoplasmic C-terminal GC catalytic domain and signals via formation of cGMP.3,4) Although some studies have shown a compensatory role for BNP in heart disease, the precise contribution of BNP to the pathophysiology of heart is poorly understood.

Ischemia is one of the most common pathological conditions in many cardiovascular diseases. Apoptotic cell death has been shown to be one of the major pathological events in a variety of cardiovascular ischemic conditions such as myocardial infarction.5) As the stimuli of cardiomyocyte apoptosis, a range of stress including mild hypoxic,6) ischemic and neurohumoral stimulation7) has been illustrated in vitro. Particularly in several studies, hypoxia is the direct trigger and well-known stimulus of apoptosis independent of the neurohumoral factors in various physiologic and pathologic conditions.8) Some studies have indicated that ANP and CNP inhibit vascular smooth muscle growth9,10) and ANP induces apoptosis in cultured vascular smooth muscle cells,12) which infer the negative effect of natriuretic peptides on the cardiovascular system. In this regard, the role of BNP in mild hypoxia-induced cardiomyocytes apoptosis and the possible mechanism are of great interest to explore. The present study was therefore designed to examine whether BNP influenced apoptotic cardiomyocyte death under mild hypoxia which has been proved to trigger the intrinsic mitochondrial death signaling pathway, and whether cGMP was involved in the process.

MATERIALS AND METHODS

Primary Culture of Cardiomyocytes Primary culture of neonatal rat cardiomyocytes were prepared as described before.13) In brief, the ventricles of 3-d-old Sprague-Dawley rats were digested with 0.08% trypsin and 0.06% collagenase II (Sigma Chemical Co., U.S.A.). The cells in suspension were collected, washed and seeded in 24 well plates at a density of 2×10^5 cells. All cells were cultured in a medium containing 85% DMEM (Invitrogen, CA, U.S.A.) and 15% fetal bovine serum. The proportion of cardiomyocytes was enriched by the preplating method. BrdU (0.1 mm, Sigma, U.S.A.) was added within the first 48 h to inhibit fibroblast proliferation. Based on the approach, purity of cardiomyocytes were over 90% of total cells per well (24-well) for further experiments.

Preparation of Mild Hypoxic Cardiomyocytes A myocardial model of hypoxia was prepared as described previously with some modification.14,15) Briefly, experiments were performed in an air tight desiccator, maintained at 37°C and continuously supplied with a gas mixture of 3% O2/92% N2/5% CO2 to generate the hypoxic environment. Maintenance of O2 concentration of 3% was routinely monitored with an O2 gas sensor (JingMi Medical Instrument Inc., China).

Morphological Analysis of Apoptotic Cardiomyocytes After treatments with mild hypoxia and rat BNP-32 (>98% pure, Sigma-Aldrich, St. Louis, U.S.A.), Rp-8-Br-cGMP (BIOLOG Life Science Institute, Germany), 8-bromo-cGMP...
(Sigma-Aldrich, St. Louis, U.S.A.), morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by double-staining with Hoechst 33342 fluorochrome (2 mg/ml Sigma-Aldrich, St. Louis, U.S.A.) and Propidium iodide (PI, 2 mg/ml, Sigma-Aldrich, St. Louis, U.S.A.) for 10 min at room temperature, and then fixed with 4% paraformaldehyde. 1000—1500 cells of each group were imaged with a fluorescence microscope (Olympus IX70, Japan) under the UV light (365 nm/380 nm). The proportion of stained apoptotic or necrotic cells to total cells was calculated by an investigator without the knowledge of treatments. The total number of cells was calculated under light microscope. Intact homogeneous blue nuclei, condensed/fragmented blue nuclei, condensed/fragmented pink nuclei, and pink intact nuclei were considered viable, early apoptotic, late apoptotic, and necrotic cells, respectively.

Flow Cytometric Analysis of Apoptotic Cardiomyocytes After 12-h incubation with drugs under mild hypoxia, myocytes were harvested and digested by 0.25% trypsin in EDTA for 10 min, and centrifuged at 1000×g for collection. Briefly, the pelleted myocytes were fixed with 70% ice-cold ethanol in PBS (pH 7.2) at 4 °C, and then incubated for 1 h followed by re-suspending in 1× binding buffer at the 1×10⁶ cells/mL. The cells were transferred to a culture tube (100/μl), and then Annexin V-FITC apoptosis detection kit (BD Biosciences, U.S.A.) was added into cell suspensions in the dark at room temperature according to the manufacturer's instructions. After staining for 30 min, cells undergoing apoptosis or necrosis (totally 10000 cells) were determined by flow cytometry (FAC sort, Becton-Dickinson, U.S.A.) with an FL-1 fluorescence was collected at 525 nm. The fluorescence intensity analysis was performed by Laser Scanning Confocal Microscopy (LSCM, Carl Zeiss LSM 510, Germany) to observe the changes of unstained cells (basal fluorescence) was set to negative (UR). Pink intact nuclei were considered viable, early apoptotic, late apoptotic, and necrotic cells as Annexin V-positive and PI-negative (LL), apoptotic cells as Annexin V-positive and PI-negative (LR) and necrotic cells as Annexin V-positive and PI-positive (UR).

Mitochondrial Transmembrane Potential Analysis Cardiomyocytes were exposed to hypoxia for 12 h in the presence or absence of drugs, and then immediately incubated for 30 min with Rhodamine-123 (10 μM, Molecular Probe, U.S.A.) under hypoxic and normoxic conditions. Changes of Δψm have been considered an indicator of mitochondrial damage. Rhodamine-123 is potential-sensitive and widely used for determination of Δψm. The fluorescent intensity analysis was performed by Laser Scanning Confocal Microscopy (LSCM, Carl Zeiss LSM 510, Germany) to obtain the images at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. For FACs analysis, rhodamine-123 loaded cells were excited at 488 nm and Rhodamine fluorescence was collected at 525 nm. The fluorescence of unstained cells (basal fluorescence) was set to negative control.

Reverse-Phase HPLC Analysis of ATP Depletion The treated cells were collected, homogenized in PBS (pH 7.0), and then added HClO₄ with final concentration of 0.56 M. The mixture was added into the suspension, which was then sonicated three times with a Sonifier Cell Disruptor (Model 185, Branson) for 20 s and centrifuged at 3300×g for 10 min at 4 °C. The supernatant was harvested and adjusted to pH 6.7. Before the HPLC analysis, the reaction mixtures were centrifuged at 4 °C through a microultrafilter at 800×g to concentrate the protein. The reaction products were analyzed by a HPLC system (Shimadzu-10 AVP, Japan) with a column of Hypersil C18 (BDS; 250×4.6 mm, 5 μm). A standard curve was obtained by diluting the purified ATP (Amresco, Germany) into grads.

**Caspace-3 Activity Assay** Caspase-3 enzymatic activity was determined with a CPP32 assay kit (Beyotime Co., China). Cells were lysed in Caspase assay buffer containing 50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 10% sucrose and 5 mM DTT. Aliquots of 6 mg crude cell lysate were incubated with caspase-3 substrate Ac-DEVD-AMC at 37 °C for 30 min. The caspase-3 activity was monitored at 440 nm (excitation at 380 nm) by VERSA max™ microplate reader (Molecular Devices Co., U.S.A.).

**Preparation of Total RNA, Real-Time RT PCR** Total RNA was prepared from cultured cardiomyocytes by TRIzol reagent (Invitrogen Co., U.S.A.) and the phenol—chloroform single step isolation. Reverse transcription of RNA was performed with ExScript™ RT reagent kit (Takara Bio Co., Japan) according to the manufacturer's instructions. The cDNA was diluted in RNase free water and amplified using the random primers. Specific primers were designed by Primer Premier 5.0 version software (Premier Biosoft International, U.S.A.) containing minimal internal structure (i.e. hairpins and primer–dimer formation) for each selected gene and having compatible Tm’s (Table 1).

The DNA Engine Thermal Cycler with the Chromo 4™ real-time detector system ( Bio-Rad Laboratories, U.S.A.) and Opticon Monitor software (Bio-Rad Laboratories, U.S.A.) were used for real-time PCR analysis. This method is based on the phenomenon that SyBR Green I binds double-stranded DNA. During polymerization, the fluorescence signal increased and then fell down when the DNA was denatured. Plotting the fluorescence signal as a function of temperature generated a specific melting curve. Real-time PCR was carried out in 20 μl reaction volumes containing 2 μl SyBR Green I (20×, Invitrogen, U.S.A.,) 2 μl 10× Taq buffer with (NH₄)₂SO₄, 5 U/μl Taq DNA polymerase 0.2 μl, 25 mM MgCl₂ 2.8 μl, 2.5 mM dNTP 1.6 μl, 0.5 μM forward and 0.5 μM reverse primers, and 2 μl cDNA from the RT-PCR above, with the following cycle profile: one cycle of 95°C for 3 min, then 40 cycles of 95°C for 5 s, 60°C for 20 s, 72°C for 20 s and 82°C for 2 s. Melting curve was obtained from 60°C to 95°C and read every 0.2°C. All reactions were carried out in triplicate. Ct-value was defined as the cycle number in which the fluorescent signal was recorded above background. All data were normalized to the expression lev-

**Table 1.** Primers Used for Real-Time RT-PCR

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<thead>
<tr>
<th>Primers Used for Real-Time RT-PCR</th>
<th>Position</th>
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<th>Position</th>
<th>β-Actin</th>
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<tr>
<td>Forward</td>
<td>666—814</td>
<td>5'-TGTGCGCTTCTTTGAGTTCG-3'</td>
<td>696—968</td>
<td>5'-CATCCCAAGCTTCGTTATCC-3'</td>
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<tr>
<td>Reverse</td>
<td>148 bp</td>
<td>5'-GAGGGAAATCTGGCCTGAC-3'</td>
<td></td>
<td>5'-TTGGCATAGTAGTCTTCTACGG-3'</td>
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els of house-keeping $\beta$-actin gene.

**Statistical Analysis** Values were reported as means±S.E.M. Statistical comparisons by Student’s $t$-test were made to detect significant difference. Multi-comparison handled by ANOVA. $p<0.05$ was considered to be statistically significant.

**RESULTS**

**Morphological Assessment of Apoptosis** We first asked what the effects of BNP on hypoxia-induced apoptosis in cardiomyocytes were. As illustrated in Fig. 1A, the images of double staining by Hoechst 33342/PI revealed that after incubation of 12-h mild hypoxia, about (6.98±2.26%) percentage
of cardiomyocytes showed a typical apoptotic cell death as revealed by typical chromatin condensation observed under microscope. The percentage of stained cells was about 30% of the total cells in the present condition. Interestingly, BNP or 8-bromo-cGMP treatment resulted in the significant increase of hypoxia-induced apoptotic cells in a concentration dependent manner. In addition, the synergetic effect of BNP and hypoxia on apoptosis could be inhibited by Rp-8-br-cGMP (0.1 μM) (Fig. 1B, \( p<0.05 \) vs. hypoxia plus BNP; \( n=6 \)); whereas, for BNP alone, we couldn’t observe a significant difference in the apoptotic cell ratio in comparison with control (\( p>0.05; n=6 \)).

**Apoptotic Analysis by Flow Cytometry** Apoptosis was also confirmed by flow cytometry studies. As shown in Fig. 2A, mild hypoxia increased the apoptotic phase cells markedly (Annexin V−PI−). Moreover, BNP enhanced that

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**Fig. 2. Myocytes Death Analysis by Flow Cytometry**

The dot-plot typical graphs showed the normal, apoptotic and necrotic fractions in control myocytes (a), the myocytes under 12-h mild hypoxia (b), the myocytes treated with mild hypoxia plus 8-bromo-cGMP (0.1 μM) (c), the myocytes treated with mild hypoxia plus 1 μM BNP (d) and the myocytes treated with Rp-8-br-cGMP (0.1 μM) plus BNP (1 μM) under mild hypoxia (e). LL: represented the normal cells (AV−PI−) in the dot-plot graphs; LR: represented the apoptotic cells (AV+PI−); UR: represented the necrotic cells (AV+PI+). The X- and Y-axis represented the fluorescent density. **\( p<0.01 \) vs. control; *\( p<0.05 \) vs. hypoxia; **\( p<0.01 \) vs. hypoxia plus BNP (1 μM).
concentration-dependently (10.21±0.53%, 9.19±0.37% and 8.97±0.68%; n=6). In contrast, Rp-8-br-cGMP (0.1 μM) inhibited BNP’s effect to the value of 7.39±0.39%. Few necrotic cell ratios (Annexin V+/PI+) could be calculated to reach statistical significance in BNP group (7.59±0.46%, 7.66±0.38% and 7.86±0.59%), 8-bromo-cGMP group (0.1 μM, 7.58±0.38%) and hypoxia group (8.26±0.20%). The normal cells ratio (Annexin V−/PI−) remained identical in all groups (Fig. 2B).

Observation and Measurement of Δψm Dissipation Since hypoxia-induced apoptosis was known by mitochondria-dependent apoptotic pathway, we therefore evaluated whether this pathway was also influenced by BNP. Rhodamine-123, potentially a sensitive mitochondrial probe, is utilized to investigate Δψm. Rhodamine-123 is used because of its particular fluorescence property to self-quench. Δψm showed green mitochondrial staining in control myocytes, indicative of normal high membrane potentials. After 12-h mild hypoxia incubation, myocytes showed green dissipated fluorescence to the cytoplasm, indicating a loss of Δψm. In addition, Δψm dissipation of the cells subjected to mild hypoxia plus BNP was further enhanced significantly, whereas, Rp-8-br-cGMP inhibited this increase. (Fig. 3A). In order to analyze Δψm quantitatively, we measured Δψm by flow cytometry. As shown in the histogram (Fig. 3B), Δψm of the control group (9.55±0.09%) decreased significantly after exposing to the mild hypoxia for 12 h. BNP facilitated the loss of Δψm from the value of 7.29±0.91% to the values of 5.67±0.23%, 5.1±0.10% and 4.67±0.22%, depending on BNP concentrations, whereas, Rp-8-br-cGMP inhibited BNP’s effect to the value of 7.11±0.11%.

Reverse-Phase HPLC Analysis of ATP Depletion Intracellular ATP content is required for mitochondrial apoptotic pathways in hypoxic cardiomyocytes.18) In order to elucidate the potential influence of BNP on the energy of myocytes at exposure to hypoxia, ATP content was determined with HPLC analysis. As illustrated in Fig. 4, a significant difference of ATP content was observed between hypoxic cells and hypoxia plus BNP treated cells. Hypoxia plus BNP (1 μM) depleted ATP content to decreased ratio of 32.65±0.04% in comparison with hypoxia group (p<0.01), in contrast, Rp-8-br-cGMP (0.1 μM) blunted this effect from the value of 3.98±0.19 μM in BNP-treated cells to 5.88±0.08 μM (p<0.05).

Caspase-3 Activity Assay Caspase-3 activity in the cardiomyocytes exposed to 12-h hypoxia was significantly increased by 2.46±0.21 folds (Fig. 5), as compared with the control group. BNP enhanced this increase to 1.86±0.51, 1.93±0.52 and 2.07±0.84 folds of hypoxia group, respectively. Rp-8-br-cGMP blunted the activation of caspase-3 to 2.4±0.1 folds of hypoxia plus BNP group.

Bcl-2 mRNA Expression by Real-Time RT PCR We then determined the influence of BNP on Bcl-2 mRNA expression by real-time RT PCR analysis. Hypoxia significantly
increased the ΔCt value of Bcl-2 mRNA expression to 9.48±0.25 from 7.61±0.19 in the control group. BNP enhanced this increase from the ΔCt value of 9.48±0.25 in hypoxic group to 11.78±0.24, 11.59±0.37 and 10.95±0.15 in BNP concentrations, respectively (Table 2).

**DISCUSSION**

Our results clearly indicated that BNP enhanced mild hypoxia-induced apoptosis in the cardiomyocytes. We showed that mild hypoxia significantly induced phosphatidylserine evagination, triggered the loss of ΔΨₘ, depleted cytosolic ATP, activated caspase-3, and down-regulated the expression of Bcl-2 mRNA in mitochondria, which are all involved in the common pathway of apoptotic cell death in the myocytes. BNP significantly aggravated these death signaling pathways. In addition, our data suggest that the action of BNP worked in the mitochondrial pathway of apoptosis partially via activation of cGMP synthesis.

BNP, as an autocrine and/or paracrine hormone secreted from ventricles has been known to have natriuretic and diuretic actions in the cardiovascular system. Although it was initially assumed that cardioprotection by BNP is attributable to the opposition of renin-angiotensin-aldosterone system, the detailed mechanisms by which exist in the regulation of BNP on heart still remain elusive. Commonly, growth-promoting agents tend to promote survival. For example, angiotensin II inhibits smooth muscle cell apoptosis;39 whereas nitric oxide, the potent inhibitor of smooth muscle cell growth has been shown to induce apoptosis in cardiomyocytes via cGMP generation. Similar to nitric oxide, BNP has been shown to inhibit the growth of cardiac fibroblasts in culture.20 In addition, a recent report has indicated that exogenous BNP regulates pulmonary vascular response to hypoxia in rats via cGMP synthesis.21 In our study, hypoxic cardiomyocytes in the presence or absence of BNP that resulted in morphologically apoptotic changes were characterized with the induction of loss of ΔΨₘ and activation of caspase-3 and transposition of phosphatidylserine to the outer cell membrane. We also observed that cGMP analogue, 8-bromo-cGMP, facilitated hypoxia-induced apoptosis, which suggests that BNP acted on mitochondria-mediated intrinsic death signaling pathway via elevation of intracellular cGMP. This is coincided with the notion that sustained cellular cGMP induces apoptosis, delays cell cycle progression and usually produces negative functional and metabolic effects on cardiomyocytes.25

Perturbations in mitochondrial physiology appear to play an important role in apoptosis.23 Commonly, ΔΨₘ dissipation is triggered by permeability transition pore (PT pore) opening and then cells lose the ability to synthesis of mitochondrial ATP. Moreover, following PT pore opening, mitochondria releases cytochrome c into the cytosol where it forms a complex with Apaf-1. The complex then catalyzes the activation of caspase-3 to induce the apoptosis in an ATP-dependent manner.24,25 To the best of our knowledge, hypoxia accelerated mitochondria-mediated common cascade to myocyte apoptosis by down-regulating the expression of Bcl-2 mRNA,26,27 and provoking ATP depletion,8 enhancing the ΔΨₘ dissipation and PT pore opening significantly.28 Bcl-2 is constitutively localized at the outer mitochondrial membrane, which is known to regulate the opening of PT

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**Table 2. Ct values of Real-Time RT PCR Analysis of Bcl-2 mRNA Expressions**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Hypoxia</th>
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<tr>
<td>Ct β-Actin</td>
<td>17.10±0.11</td>
<td>17.12±0.15</td>
</tr>
<tr>
<td>Ct Bcl-2</td>
<td>24.81±0.11</td>
<td>26.6±0.23</td>
</tr>
<tr>
<td>ΔCt</td>
<td>7.61±0.19</td>
<td>9.48±0.25</td>
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* p<0.05 vs. control; ** p<0.01, *** p<0.005 vs. hypoxia.

**Fig. 4. Analysis of Cellular ATP Depletion**

The cellular ATP depletion was analyzed by HPLC, and then normalized by standard curves of pure ATP. Data are expressed as means±S.E.M. of six independent experiments. Standard curve equation was Y=13732X−39453, r²=0.951, * p<0.05 vs. control; ** p<0.01, *** p<0.005 vs. hypoxia; t p<0.01 vs. hypoxia plus BNP (1 μM); n=6.

**Fig. 5. Analysis of Caspase-3 Activity**

Myocytes were treated and caspase-3 activity was determined. Activation level of caspase-3 was shown as a percentage of change in mean value of at least six independent experiments. * p<0.01 vs. control; * p<0.001 vs. hypoxia; ** p<0.01, *** p<0.001 vs. hypoxia plus BNP (1 μM).
pore and inhibits the deterioration of \( \Delta \psi_m \) in vitro assay. \(^{29}\) The present data showed that mild hypoxia significantly decreased the expression of Bcl-2 mRNA in mitochondria. Furthermore, this decline was further enhanced by BNP and 8-bromo-cGMP. Thus, it seems likely that Bcl-2 could be regulated by cGMP and BNP under mild hypoxic conditions.

Acute hypoxia itself has been reported to trigger BNP mRNA expression. \(^{30}\) Nevertheless the BNP concentrations in our data were much higher than increased endogenous BNP by cardiomyocytes under hypoxia, therefore the endogenous BNP in the presence of a considerable amount of exogenous BNP contributed to few. On the other hand, since we have not tested the contribution of natriuretic peptide receptors (most likely NPR-A), which might be essential for the results, we therefore could not deny the contribution of NPR-A to the pro-apoptotic actions of BNP.

In conclusion, new insights have been added in this article to address the ongoing debate whether exogenous BNP on the cardiovascular system is or is not beneficial for chronic treatment. At least in this oxygen deprivation cell model, BNP potentiated the evagination of phosphatidylserine, the dissipation of \( \Delta \psi_m \), the depletion of ATP, the increase of caspase-3 activity and the down-regulation of Bcl-2 mRNA expression. These actions were probably via cGMP generation and the mitochondria dependent pro-apoptotic pathway. Since BNP is a reliable prognostic of cardiac pathology, the effects of this peptide on cardiomyocyte death progression might have significant implications in the development of heart pathology.

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