HNP-1 Reverses Hypertensive Left Ventricular Hypertrophy by Inhibiting the NF-κB Signaling Pathway

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Author Contributions

Xiaorong Duan carried out the main experiments and drafted this manuscript. YU Zhenqiu directed the experimental research and manuscript revision.

Conflict-of-interest statement

We declare that none of the work contained in this manuscript is published in any language or currently under consideration at any other journal. And there are no conflicts of interest to declare. All authors have read and approved this submitted manuscript in its current form. Our manuscript has also been edited by a native English-speaking expert to ensure its English is good enough for publication.

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Abstract

Background: Human neutrophil peptide-1(HNP-1) is a commonly investigated therapeutic agent. However, its role in hypertensive left ventricular hypertrophy(HLVH) remains unclear.

Methods: We measured HNP-1 levels in patients with hypertension and treated HLVH rat and H9c2 cell hypertrophy models with HNP-1. Cardiomyocyte hypertrophy indexes (i.e., single-cell surface area, left ventricle fibro area, BNP levels, and β-MHC levels) were measured using hematoxylin-eosin and Masson's trichrome staining and WB. NF-κB signaling factors (i.e., IKKβ, p-IKKβ, IκBα, p-IκBα, p65, and p-p 65) were measured by WB and qPCR. Lastly, inflammatory factors (i.e., IL-6, IL-1α, and TNF-α) were measured by ELISA.

Results: HNP-1 levels were lower in the exposure vs. control groups (M (95% CI), 48.83 (45.64–52.26) vs. 59.03 (55.62–62.54), p = 0.000). A decrease in HNP-1 was associated with HLVH occurrence in patients. HLVH rat and H9c2 cell hypertrophy models revealed elevated cardiomyocyte hypertrophy indexes and NF-κB signaling and inflammatory factors. However, each HNP-1 treatment group experienced a decline in the aforementioned indices as compared with the model groups.

Conclusion: HNP-1 decrease is a risk factor for HLVH. HNP-1 treatment may reverse HLVH by inhibiting NF-κB signal pathways.

Introduction

The prevalence of hypertension has risen annually in China [1–5], resulting in an increase in cardiovascular disease-related morbidity and mortality [6–10] and imposing a significant financial and health burden [11–18]. Left ventricular hypertrophy is a marker of hypertensive target-organ damage and a principal pathological process in heart failure [19–22]. Reversing hypertensive left

ventricular hypertrophy (HLVH) is a primary therapeutic goal to prevent heart failure and an effective method to reduce the risk of cardiovascular disease [23–26]. However, its reversal rate is generally low [27, 28]. Human neutrophil peptide-1 (HNP-1, sequence: ACYCRIPACIAGERRYGYCIYQGRLWAFCC) is also called alpha-defensin 1. HNP-1 is usually produced by immune cells such as neutrophils, lymphocytes, monocytes, and eosinophils [29, 30]. By regulating nuclear factor-κB (NF-κB) signaling pathways, HNP-1 plays a critical role in autoimmune disorders, anti-infection, and peripheral nerve injury repair [31–42]. However, the role of HNP-1 in cardiovascular disease is contentious. On the one hand, HNP-1 levels have been linked to the severity of coronary atherosclerosis [43] and may increase the risk of thrombosis by altering fibrin shape, structure, and stability [44]. Conversely, not only may HNP-1 prevent atherosclerosis progression, it may also reduce plasma low-density lipoprotein cholesterol levels [45] and reduce atherosclerosis [46]. Moreover, HNP-1 may reduce arterial thrombosis by inhibiting the aggregation of von Willebrand factor [47]. Whether HNP-1 plays a role in the process of HLVH is still unclear. Thus, to potentially identify a target spot for improving HLVH, this study aimed to explore the association between HNP-1 and HLVH.

Materials and Methods

1. Study participants

A total of 861 patients with hypertension were recruited from the Affiliated Hospital of Guizhou Medical University between May and December 2021. All patients were at least 18 years old and had been diagnosed with hypertension using the 2018 Chinese Guidelines for Prevention and Treatment of Hypertension [48]. After excluding those with secondary hypertension, infectious diseases, rheumatic immune diseases, tumors, diabetes, stroke, coronary atherosclerotic heart disease, heart failure, and chronic kidney disease, 216 patients with hypertension were included in the study. Ethics

approval for the study was obtained from the Ethics Committee of the Affiliated Hospital of Guizhou Medical University (No. 2021-167, March 2, 2021).

2. Exposure and control groups

The 216 study participants were divided into exposure and control groups according to their left ventricular mass index (LVMI). The exposure group comprised 100 patients with HLVH whose LVMI was > 115 g/m^2 in men and > 95 g/m^2 in women [28]. The control group comprised 116 patients with hypertension and without left ventricular hypertrophy whose LVMI was ≤ 115 g/m^2 in men and ≤ 95 g/m^2 in women.

3. Rat subjects

Forty male Wistar rats aged five-to-six weeks were obtained from the Guizhou Medical University Laboratory Animal Center. All rats were fed SPF-rated foods and kept in 12-hour light-dark cycles at 25 ± 1 °C. After one week of adaptive feeding, 39 healthy Wistar rats were selected for this study. The animal study protocol was approved by the Animal Care and Welfare Committee of Guizhou Medical University (No. 2201437, March 31, 2022).

4. Establishing the HLVH rat model

To establish the norepinephrine (NE)-induced HLVH model in rats, 39 healthy male rats were randomly divided into control, NE, and NE plus HNP-1 groups. Rats in the control group received intraperitoneal injections with 0.9% saline for eighteen weeks. Rats in the NE group received intraperitoneal injections with NE (R030760, Rhawn, Shanghai, China) at 1.5 mg/kg/day [49] for ten weeks, then received intraperitoneal injections with 0.9% saline for eight weeks. Rats in the NE plus HNP-1 groups received intraperitoneal injections with NE at 1.5 mg/kg/day for ten weeks, then received intraperitoneal injections with HNP-1 (HSA10038, Hsynthbio, Hefei, China) at 10 μg, 20 μg, 30 μg, and 40 μg, respectively, once every two days for eight weeks.

5. Blood pressure monitoring in rats

Blood pressure (BP) was measured by a non-invasive tail-cuff method (Non-Invasive Blood Pressure System, CODA, Kent Scientific Corp., Austin, TX, USA). BP was monitored weekly during NE injections and once every two weeks during HNP-1 injections. Prior to blood pressure testing, rats were restrained on a warming table set to 35°C and allowed to stabilize for 10 minutes. Next, BP measurements were taken 15 cycles in the conscious rats, not under anesthesia, with the average value used to calculate the BP.

6. Myocardial tissue staining in rats

Before being embedded in paraffin and sectioned into 4 - 5 μm thick slices, cardiac ventricles were fixed with 10% paraformaldehyde for 24–48 hours. Then hematoxylin-eosin (HE) and Masson's trichrome (MASSON) staining were performed according to the manufacturers' instructions. Left ventricle HNP-1 expression was identified through immunohistochemical (IHC) staining, which was performed with rabbit anti-rat alpha-defensin 1 mAb (NBP3-05562, Novus, 1:80, Colorado, USA). Finally, the sections were digitized with a fully automatic digital slice scanner.

7. Establishing the H9c2 cell hypertrophy model

H9c2 cells (BNCC353655, BNCC, Beijing, China) were cultured with Dulbecco's Modified Eagle's Medium (DMEM, HyClone, USA) at 37 °C in a 5% CO2 incubator (BPN-80CW, Shanghai, China). After cell density reached 50%, the H9c2 cells were randomly divided into control, Ang II, and Ang II plus HNP-1 groups. Cells in the control group received treatment with 10% fetal bovine serum (FBS, 10099-141, Gibco, California, USA) for five days. Cells in the Ang II group received treatment with 1 μM Ang II (HY-13948, MCE, New Jersey, USA) for 48 hours [26] and then received treatment with 10% FBS for 72 hours. Cells in the Ang II plus HNP-1 groups received treatment with 1 μM Ang II for 48 hours and then received treatment with a different dose of HNP-1 for 72 hours.

8. H9c2 cell viability assay

A Cell Counting Kit-8 (CCK-8, KGA317, KeyGEN, Nanjing, China) was used to determine cell viability. After stimulation with HNP-1 at 0, 5, 10, 15, and 20 μg/mL for 24 hours, 48 hours, and 72 hours, a 10 μL CCK-8 reagent was added to each well at 37 °C for two hours. The absorbance was then measured at 450 nm.

9. H9c2 cell immunofluorescence (IF) staining

After fixing with 4% paraformaldehyde for 15 minutes, the H9c2 cells were blocked with 5% bovine serum albumin (BSA) for 30 minutes. Next, mouse anti-rat F-actin mAb (OM252288, OmnimAbs, 1:100, Shanghai, China) was added to the H9c2 cells. Finally, the H9c2 cells were observed with a fluorescence microscope.

10. Western blotting(WB)

HNP-1, BNP, and β-MHC levels were measured with rabbit anti-rat alpha-Defensin 1 mAb (NBP3-05562, Novus, 1:2000, Colorado, USA), rabbit anti-rat BNP mAb (WL02126, Wanlei, 1:1000, Shenyang, China), and rabbit anti-rat β-MHC mAb (NBP2-74079, Novus, 1:2000, Colorado, USA), respectively. IKKβ, p-IKKβ, IκBα, p-IκBα, p 65, p-p 65, and GAPDH levels were measured with rabbit anti-rat IKKβ, p-IKKβ, IκBα, p-IκBα, p65, p-p65 mAb (all 1:500, Affinity, USA), and GAPDH mAb (1:10000, Affinity, USA), respectively.

11. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from H9c2 cells using Trizon Reagent (CW0580S, CWBIO, Beijing, China). Next, total RNA was reverse-transcribed into first-strand cDNAs with HiScript II Q RT SuperMix (R223-01, Vazyme, Nanjing, China). Finally, qPCR was performed using ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme, Nanjing, China) with fluorescence captured in an iCycler IQ system. The relative expressions of mRNA were calculated based on Ct values. GAPDH was used as the reference gene. All primers are listed in Tab. 1.

12. Enzyme-linked immunosorbent assay (ELISA)

An ELISA kit (JL20063, JIANGLAI, Shanghai, China) was used to measure the concentration of serum HNP-1 in patients, following manufacturer instructions. ELISA kits(MM-0193R1, MM-0193R1, MM-0180R1, MEIMIAN, Shanghai, China) were also used to measure interleukin 1α (IL-1α), interleukin 6 (IL-6), and tumor necrosis factor α(TNF-α) levels, in accordance with manufacturer instructions.

Statistical Analysis

Statistical analyses were conducted using SPSS (21.0, IIBM, USA) and GraphPad Prism 8.4 (CA, USA). The integrated density of the image was calculated with Image J software (Media Cybernetics, USA). The Student's t-test, Wilcoxon signed rank test, and one-way ANOVA were used to test for between-group differences. Pearson or Spearman correlation analyses and regression analyses were used to compare the associations between HNP-1, BP, and left ventricular hypertrophy indexes. $p < 0.05$ was taken to indicate statistical significance.

Results

1. Participants' descriptive characteristics and HNP-1 levels

There were no statistically significant differences between the exposure and control groups in terms of age, gender, body mass index (BMI), and cardiovascular risk factors, including smoking,TG, TC, L-DLC, H-DLC, glucose, Hcy, and history of hypertension (Tab. 2). There were also no statistically significant differences in the proportion of patients using anti-hypertensive drugs, including beta-blockers, CCBs, ACEI/ARB, and diuretics (Tab. 2). However, BP and HLVH indexes were higher in the exposure vs. control group (FIG. 1, Tab. 3), while HNP-1 levels were significantly lower in the exposure vs. control group (M (95% CI), 48.83 (45.64–52.26) vs. 59.03 (55.62–62.54), $p = 0.000$)(FIG. 1). In addition, HNP-1 level was significantly negatively correlated with BP and left ventricular hypertrophy indexes (Tab. 4).

2. HNP-1 decrease was a risk factor for HLVH in patients

Multiple linear regression analyses revealed that HNP-1 decrease was significantly correlated with LVMI increase after adjusting for age, gender, SBP, DBP, 24 h SBP, 24 h DBP, D SBP, D DBP, N SBP, N DBP, and PP(Tab. 5). Binary logistic regression analyses showed that HNP-1 decrease was significantly correlated with HLVH after adjusting for the aforementioned variables (Tab. 6).

3. HNP-1 treatment reduced BP in NE-induced HLVH rats

After injection with NE in the fifth week, SBP and DBP began to be significantly higher in the NE group than in the control group (FIG. 2 A and B). In the NE group, SBP increased by 22.43 mm Hg and DBP increased by 25.96 mm Hg (from baseline BP) after injection with NE for ten weeks (FIG. 2 E). However, after HNP-1 injection, SBP and DBP decreased over time (FIG. 2 C, and D). Finally, compared to the NE group, SBP and DBP decreased considerably in each NE plus HNP-1 treatment group (FIG. 2 F).

4. HNP-1 treatment increased left ventricle HNP-1 expression in NE-induced HLVH rats

IHC staining showed that HNP-1 was mainly expressed on the cardiomyocyte membrane in the left ventricle of rats. Furthermore, left ventricle HNP-1 expression decreased in the NE group. Compared to the NE group, left ventricle HNP-1 expression increased in the NE plus HNP-1 groups in a dose-dependent manner (FIG. 3).

5. HNP-1 treatment improved ventricular hypertrophy and fibrosis in NE-induced HLVH rats

HW/BW and HW/TL ratios were significantly higher in the NE group than the control group and significantly lower in each NE plus HNP-1 treatment group than the NE group (FIG. 4 A). HE staining revealed that left ventricle single cardiomyocyte surface area was larger in the NE group than the control group. Further, as compared with the NE group, left ventricle single

cardiomyocyte surface area was smaller in each NE plus HNP-1 treatment group (FIG. 4 B). In addition, MASSON staining indicated that left ventricle fibrosis area increased in the NE group, as compared with the control group. However, compared with the NE group, left ventricle fibrosis area was reduced to a varying degree in each NE plus HNP-1 treatment group (FIG. 4 B).

6. HNP-1 treatment decreased the expression of BNP, β-MHC, and NF-κB in NE-induced HLVH rats

WB revealed that left ventricle expression of BNP, β-MHC, and phosphorylative NF-κB signaling factors (including p-IKKβ/IKKβ, p-IκBα/IκBα, and p-p 65/p65) was higher in the NE group than in the control group. However, compared with the NE group, expression of the aforementioned proteins decreased to a varying degree in the NE plus HNP-1 treatment groups (FIG. 5).

7. HNP-1 treatment promoted H9c2 cell growth activity and reversed Ang II-induced H9c2 cell hypertrophy

CCK8 indicated that HNP-1 promoted H9c2 cell growth viability. Furthermore, H9c2 cell growth activity was higher after stimulation with HNP-1 at 10 μg/mL and 15 μg/mL for 72 hours than after stimulation with HNP-1 at 5 μg/mL and 20 μg/mL for 24 hours and 48 hours, respectively (FIG. 6 A). IF revealed that single-cell surface area was larger in the Ang II group than in the control group. However, compared with the Ang II group, single cell surface area was smaller to a varying degree in each Ang II plus HNP-1 group (FIG. 6 B).

8. HNP-1 treatment reduced the expression of BNP, β-MHC, NF-κB, and inflammatory factors in Ang II-induced H9c2 cell hypertrophy

WB showed that HNP-1 levels decreased in the Ang II group and increased in the Ang II plus HNP-1 treatment groups (FIG. 7 A). WB and qPCR also revealed that BNP, β-MHC, p65, and p-p 65/p65 expression increased in the Ang II group and decreased in each Ang II plus HNP-1 group to a varying

degree (FIG. 7 A and B). Additionally, compared with the Ang II group, IL-1α, IL-6, and TNF-α levels were lower in the Ang II plus HNP-1 groups, especially when treated with HNP-1 at 15 μg/mL and 20 μg/mL (FIG. 7 C).

Discussion

This study found that HNP-1 levels decreased in patients with HLVH and that HNP-1 decrease was a risk factor for HLVH. In the experimental HLVH model, HNP-1 treatment decreased NF-κB and other heart hypertrophy markers. This indicates that HNP-1 could reverse HLVH and is a potential therapeutic target for cardiovascular disease. HNP-1 is the first cell-synthesized peptide that was demonstrated to directly enter a target cell to affect translation and regulate protein expression [50]. Furthermore, HNP-1 is widely found in various tissues and organs but mainly expressed in human neutrophil lineage cells [31, 51–56]. It has been reported that the HNP-1 gene is located on chromosome 8p23.1 [56–59]. In neutrophil lineage cells, HNP-1 is translated into a 94-amino-acid precursor form in promyelocytes and sequentially cleaved into 30-amino-acid mature HNP-1 in the Golgi apparatus [60, 61]. The mature HNP-1 is stored in the blue granules and can be released by cell degranulation or secretion [56].

Previous studies have reported decreased HNP-1 levels in the elderly, smokers, those who are toothless or have tooth decay, and those with lipid metabolism disorders or vasodilation responses [62-64]. HNP-1 decrease is associated with aging, decreased immune resistance, lipid metabolism disorders, and a reduced vasodilation response [65,66]. Similar to previous studies, this current study found that HNP-1 levels decreased in patients with HLVH and that HNP-1 decrease was a risk factor for HLVH.

This current study also found that HNP-1 treatment may reduce BP in HLVH rats, although BP did not change in a dose-dependent manner. This finding has three possible explanations. First, it can be speculated that HNP-1's BP-lowering effect may be related to baseline BP levels. Second,

HNP-1 may have a “bipolar effect.” For example, inflammatory factor expression may be enhanced by a high dose of HNP-1 (100 μg/d) and inhibited by a healthy dose of HNP-1 (5 μg/d or 10 μg/mL) [67]. Third, studies have reported that regulating the inflammatory response effectively controls BP and improves HLVH [67–73]. However, NF-κB is a key regulatory signaling pathway for inflammatory immune responses [74–77], and NF-κB inhibition may improve hypertension and HLVH [78–88]. This current study determined that HNP-1 may inhibit NF-κB activation, but this inhibitive effect may not be dose-dependent.

According to this current study, HNP-1 treatment increased HNP-1 expression in a HLVH rat model. In contrast, Kou et al. [36] reported HNP-1 treatment did not increase HNP-1 expression in RSC 96 cells. There are two possible explanations for these differences in findings. The first is related to differences in research subjects. Specifically, Kou et al. [36] studied RSC 96 cells in the nervous system, whereas this current study focused on rat left ventricular tissue and H9c2 cells. Second, HNP-1 treatment dose and duration varied between the studies. Kou et al. [36] treated RSC 96 cells with HNP-1 at 4 μg/mL and 8 μg/mL for 36 hours. This current study treated HLVH rats with HNP-1 at 10 μg, 20 μg, 30 μg, and 40 μg for eight weeks and H9c2 cells with HNP-1 at 10 μg/mL, 15 μg/mL, and 20 μg/mL for 72 hours. Furthermore, it is worth noting that HNP-1 expression was related to the treatment dose of HNP-1. HNP-1 expression was weak when HNP-1 treatment dose was 10 μg and 10 μg/mL and enhanced as the treatment dose of HNP-1 increased. Future studies are warranted to explore whether this effect was due to a rise in endogenous HNP-1 or exogenous HNP-1.

This current study found that HNP-1 treatment inhibited the activation of NF-κB in the HLVH model. However, other studies have reported differing effects of HNP-1 on NF-κB signaling pathways. For example, Kou et al. and Wang et al. [36, 31] found that p65 expression was higher in Schwann cells and CAL-1 cells after treatment with HNP-1. However, mRNA expression of AKT in

Schwann cells showed lower activity when treated with 8 μg/mL vs. 4 μg/mL of HNP-1. Other studies have reported that A549 cells and CD4 lymphocytes showed no differences in p65 expression after treatment with HNP-1 [35, 45]. Lastly, studies have also found that HNP-1 may enhance [89] or inhibit [90] the activation of complement classical pathways. Therefore, we speculate that HNP-1 may have different regulatory effects on NF-κB signaling pathways in various tissues and cells under various experimental conditions. In other words, this study identified HNP-1's protective role in HLVH, but the specific mechanisms through which this occurs warrant further study.

Conclusion

HNP-1 decline was a risk factor for HLVH in patients. HNP-1 treatment may reverse HLVH and H9c2 cell hypertrophy by inhibiting NF-κB signaling pathways.