Nesfatin-1 protects against diabetic cardiomyopathy in the streptozotocin-induced diabetic mouse model via the p38-MAPK pathway

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ABSTRACT
Nesfatin-1 is a novel anorexigenic peptide that possesses antihyperglycemic and cardiovascular effects. We hypothesized that nesfatin-1 has a beneficial protective effect against diabetic cardiomyopathy (DC). We investigated the therapeutic effect of nesfatin-1 on diabetes-associated cardiac dysfunction in the high-fat diet (HFD)/streptozotocin (STZ)-induced diabetic mouse model. We found that the cardiac nesfatin-1 level was lower in diabetic mice than in normal mice. Nesfatin-1 treatment (180 mg/kg/day for two weeks) improved insulin sensitivity and mitigated diabetic dyslipidemia. Nesfatin-1 ameliorated the diabetes-related myocardial hypertrophy and heart dysfunction, as revealed by the reduced hypertrophy index, heart rate, mean arterial pressure (MAP), creatine kinase (CK)-MB, and aspartate aminotransferase (AST) levels. Nesfatin-1 exerted antioxidant and anti-inflammatory activity in diabetic mice, as shown by decreased reactive oxygen species (ROS), oxidative lipid product malondialdehyde (MDA) levels, increased superoxide dismutase (SOD) and glutathione (GSH), decreased cardiac and plasma interleukin-1 β (IL-1β) and tumor necrosis factor-α (TNF-α) levels. Mechanistically, we found that nesfatin-1 inhibited the cardiac p38-MAPK pathway activation and subsequent glucagon-like peptide-1 (GLP-1) level. Collectively, our data shows nesfatin-1 exerted protective effects against diabetic cardiomyopathy. Our study suggests that nesfatin-1 therapy has therapeutic implications against diabetic cardiomyopathy.

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1. Introduction

Diabetes mellitus is a chronic metabolic disorder that poses tremendous health, social, and economic burden globally [1]. Epidemiological data indicate that the prevalence of diabetes mellitus is rapidly growing to an estimated 330–380 million by 2025 [2]. In diabetes mellitus patients, prolonged hyperglycemia damages multiple organs such as the heart, kidneys, eyes, and nerves, which are closely associated with high mortality [3].

Diabetes mellitus is recognized as one of the major risk factors for cardiomyopathy, termed diabetic cardiomyopathy (DC) [4]. Multiple diabetes-associated risk factors including mitochondrial uncoupling, lipotoxicity, glucotoxicity, and fibrosis, contribute to the development of DC and result in cardiac dysfunction or heart failure (HF) [2]. The pathogenesis of DC is undoubtedly multifactorial and complex, and multiple mechanisms are involved in this pathophysiological condition [4]. Exposure of the heart to hyperglycemia results in increased advanced glycation end-product (AGE) formation, reactive oxygen species (ROS) production, lipid level, and dysregulated insulin signaling, all occurring with detrimental effects on cardiac function [4]. Protecting the heart from these abnormal events could be a viable approach to DC therapeutic intervention.

Nesfatin-1 is an amino acid peptide derived from the secreted precursor nucleobindin-2 [5]. It was first reported as a novel anorexigenic peptide due to its fat-reducing orphan ligand. In addition to its involvement in malnutrition, weight loss, and appetite regulation, nesfatin-1 regulates food intake and glucose homeostasis [6]. In recent years, nesfatin-1 has been found to be abundantly expressed in pancreatic islets, exerting a direct glucose-dependent insulinotropic action [7]. Increasing evidence has revealed that nesfatin-1 possesses an antihyperglycemic effect. Injection of nesfatin-1 stimulates glucose uptake and prevents hepatic glucose formation, implicating it in the treatment of diabetes [8]. In addition, it is also involved in managing diabetic complications. Nesfatin-1 exhibits a recuperative effect on type-2 diabetes-associated reproductive dysfunction in high-fat diet (HFD)/streptozotocin (STZ)-induced diabetic mice [9]. Moreover, it has been shown to play roles in the cardiovascular system, including regulation of cardiomyocytes metabolism and viability, endothelial function, blood pressure, and heart physiology [10,11]. These evidences suggest the level of nesfatin-1 in vivo might have an impact on diabetic complications, particularly cardiac complications.

We hypothesized that the increased nesfatin-1 level might have a beneficial effect on diabetes-related cardiac complications. The therapeutic effect of nesfatin-1 on diabetic cardiomyopathy has not been fully investigated. This study aims to assess the potential therapeutic applications of nesfatin-1 on diabetic cardiomyopathy in experimental mice models.

2. Materials and methods

2.1 The diabetes mice model and treatment

The diabetes mice model was generated based on a previous study [12]. The six-week-old male C57BL/6 J mice were randomly divided into three groups (10 mice per group): the control group, mice were given a regular diet; diabetic model group, and mice were fed with a high-fat diet (HFD) for 8-weeks, on the first day of the last week, mice were intraperitoneally injected with STZ (180 mg/kg) once. On the second day of last week the mice were intraperitoneally injected with 0.9% saline for 14 consecutive days; and the nesfatin-1 treatment group: mice were fed with HFD for 8-weeks, on the first day of the last week, mice were intraperitoneally injected with STZ (180 mg/kg) once, and then intraperitoneally injected with nesfatin-1 (20 μg/kg/day) [9] for 14 consecutive days. The body weight of the mice was measured and recorded weekly during the experimental period. The survival rate in the control, T2DB, T2DB+ Nesfatin-1 groups is 100%, 90%, and 90%, respectively.
2.2 Analysis of fasting blood glucose and insulin level

On the last week of treatment, blood samples were immediately taken from ophthalmic vein after fasting overnight (12 h). The plasma was collected after centrifugation at 1000 × g for 10 min at 4°C. Fasting blood glucose (FBG) and fasting insulin levels (FINS) were determined using commercial kits (Nanjing SenBeJia BioTech., Nanjing, China). The formula for calculating insulin sensitivity (INS) was: INS = 1/FBG * FINS.

2.3 Blood biochemical analysis

At the end of the experiments, the mice were fully anesthetized and blood samples were collected by the heart punctures. Serum samples were prepared for the determination of total cholesterol (TC), triglycerides (TAG), low-density lipoprotein (LDL), creatine kinase (CK)-MB, and aspartate aminotransferase (AST) using commercial kits (Nanjing Jiancheng BioTech., Nanjing, China).

2.4 Analysis of cardiac function

Heart rate (HR) and mean arterial pressure (MAP) were measured after full anesthetization with the Blood Pressure Analysis System (BP-2000; Visitech Systems, Apex, NC) using a noninvasive tail-cuff blood pressure analyzer. Finally, the mice were sacrificed by decapitation and the hearts were excised and weighted. Cardiac hypertrophy (hypertrophy index) was evaluated by heart mass:bodyweight ratios.

2.5 Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from the heart tissues and purified, and the gene transcripts of nesfatin-1 were determined using qRT-PCR as described previously [13]. Briefly, 1 µg of total RNA was used for First-strand cDNA synthesis with a reverse transcription kit (Promega, Madison, WI). The primer pairs are: Nesfatin: 5’-AGACCGATGGTGACTCTGGAG-3’, 5’-CCTCGGTAATACTGGTGCTGG-3’; β-actin: 5’-CATTTGCTGACAGGATGCAAGG-3’, 5’-TGCTGGAAGGTGGACAGTGAGG-3’. The real-time PCR was performed using obtained cDNA, primers, and SYBR green PCR master mix (Bio-Rad Laboratories, Hercules, CA) on a Roche Light Cycler system (Roche, Germany) with the primers listed above. The reaction conditions consisted of denaturation of 95 C for 60 sec, followed by 40 cycles of 94 C for 5 sec, 60 C for 10 sec, and 72 C for 15 sec, followed by melting curve analysis. The mRNA expression level of nesfatin-1 was normalized to GAPDH.

2.6 Western blot analysis

Whole proteins from heart tissues were extracted and the protein concentration was measured using a BCA protein assay kit (Thermo Scientific Inc. USA). A total of 20 µg protein for each sample was resolved by a 4–12% precast SDS-PAGE gel (BioRad, USA), then transferred to a nitrocellulose membrane. After 1 h incubation in 5% BSA to block the nonspecific bindings, membranes were incubated overnight at 4°C with antibodies against nesfatin-1 (1:1000), glucagon-like peptide-1 (GLP-1) (1:1000), p38 (1:1000), p-p38 (1:1000) or β-tubulin (1:10,000). All the primary antibodies were obtained from a commercial source (Cell Signaling Technology, Danvers, MA). Then the membranes were probed with anti-mouse secondary antibody goat anti-rabbit IgG antibody (Cell Signaling Technology) for 1 h. After being rinsed, Chemiluminescence Reagent Plus (Thermo Fisher Scientific, Waltham, MA) was used to develop the bands, which were further analyzed with Image J [14].

2.7 Wheat germ agglutinin (WGA) staining

The hearts were sliced in half by forming a transverse slice between the atrioventricular sulcus and the apex, then fixed in 4% paraformaldehyde for 24 h. Then the tissues were embedded in paraffin and cut into sections.
(5 μm). WGA (5 μM; Thermo-fisher Scientific, USA) staining was performed to assess the cross-sectional area of cardiomyocytes (CSA). Images captured by fluorescence microscope were analyzed by Image J software (NIH, USA) to check for hypertrophy.

2.8 ELISA
Heart tissues were chopped and mixed with PBS buffer, and then homogenized at 4°C with a homogenizer to obtain cardiac homogenates. After centrifugation at 4000 rpm for 10 min, the supernatants were collected. The TNF-α and IL-1β levels in cardiac homogenates and plasma were determined using ELISA assay and commercial kits (Nanjing Jiancheng BioTech).

2.9 Analysis of the oxidative status
To examine the oxidative status of heart tissues, oxidative stress markers including malondialdehyde (MDA), reactive oxygen species (ROS), superoxide dismutase (SOD), and glutathione (GSH) levels in cardiac homogenates were measured using commercial kits (Nanjing Jiancheng BioTech).

2.10 Data analysis
The data and statistical analysis were performed using GraphPad Prism 5 software. Quantitative data from at least three replicates were presented as mean ± standard error of mean (S.E.M.). Statistically significant differences were assessed using analysis of variance (ANOVA) followed by post-hoc Bonferroni correction. A p-value less than 0.05 was determined as statistically significant.

3. Results
In the current study, we found diabetic mice had a reduced cardiac expression of nestifin-1 and then pursued its therapeutic effect in STZ-induced diabetic mice. We show that nesfatin-1 treatment mitigated the increase in insulin and glucose levels and altered lipid profiles in diabetic mice. Nesfatin-1 ameliorated diabetes-associated myocardial hypertrophy and heart dysfunction. In heart tissue, Nesfatin-1 exerted antioxidant and anti-inflammatory effects. At the molecular level, nesfatin-1 therapy suppressed the activation of the cardiac p38-MAPK pathway and increased the GLP-1 level in diabetic animals.

3.1 The changes of nesfatin-1 expression in STZ-induced diabetic mice
As compared to control mice (1 ± 0.108), diabetic mice showed a significant decrease (0.34 ± 0.038) in the mRNA level of nesfatin-1 (Figure 1(a)). Meanwhile, it is apparent that a decreased protein level of nesfatin-1 (0.41 ± 0.045) was observed in diabetic mice.

Figure 1. The expression of cardiac Nesfatin-1 in control and type 2 diabetes (T2DB) mice. (a) mRNA level and (b) Protein level of Nesfatin-1 in the heart in control and diabetes mice. (****, P < 0.0001 vs. Vehicle group).
when compared with control mice (1 ± 0.11) (Figure 1(b)).

3.2 Effects of nesfatin-1 on insulin sensitivity and hyperlipidemia in STZ-induced diabetic mice

The result in Figure 2(a) indicated a significant increase in body mass in T2DM mice fed HFD compared to control mice. The T2DM mice treated with nesfatin-1 displayed obvious decline in body mass compared to untreated T2DM mice (Figure 2(b)). A considerable increase in FBG (354.7 ± 42.5 mg/dL) was observed in diabetic mice, compared to control mice (95.3 ± 11.3 mg/dL). Treatment of diabetic mice with nesfatin-1 showed a significant reduction (254.6 ± 25.7 mg/dL) in FBG (Figure 2(c)). Fasting insulin sensitivity in diabetic mice (33.6 ± 3.9 mU/L) was significantly higher than that of control mice (4.3 ± 0.48 mU/L). Treatment of diabetic mice with nesfatin-1 significantly reduced the change in fasting insulin level (11.5 ± 1.5 mU/L) (Figure 2(d)). Accordingly, insulin sensitivity was decreased in diabetic mice, and nesfatin-1 treatment evoked insulin sensitivity (Figure 2(e)). Next, the changes in blood lipids were examined. The data in Figures 2(f-h) show that the serum levels of TC, TAG, and LDL were significantly increased in diabetic mice, whilst they were reduced by nesfatin-1 treatment.

3.3 Effects of nesfatin-1 on heart function in STZ-induced diabetic mice

In comparison with control group mice (457.3 ± 12.5 beat/min), diabetic model mice exhibited an increased heart rate (564.8 ± 23.8 beat/min). Treatment of diabetic mice with nesfatin-1 decreased the heart rate to 512.5 ± 43.5 beats/min (Figure 3(a)). Diabetic mice showed an elevated CK-MB level up to 134.6% when compared with control mice, whilst nesfatin-1 treatment caused a significant reduction
in the CK-MB level (Figure 3(b)). The AST level in diabetic mice was significantly increased to 161.5% in comparison with the control mice. On the other hand, treatment with nesfatin-1 reduced the AST level by 19.2% in diabetic mice (Figure 3(c)). The MAP level in the diabetic model group (146.8 ± 12.5 mmHg) was found to be significantly higher than that in the control group (108.5 ± 11.4 mmHg), whereas that in the nesfatin-1 treatment group (122.6 ± 14.2 mmHg) was significantly lower than that in the diabetic model group (Figure 3(d)).

3.4 The protective effect of nesfatin-1 on myocardial hypertrophy in STZ-induced diabetic mice

In addition, slight but significant differences were observed in the hypertrophy index among the three groups (Figure 4(a)). Representative images of WGA staining in the CSA are shown in Figure 4(b). According to the quantification analysis, CSA was increased 2.8-fold in diabetic mice as compared to control mice. However, the increase in CSA was inhibited by nesfatin-1 treatment with a 0.68-fold change (Figure 4(c)).

3.5 Nesfatin-1 reduces the inflammation in STZ-induced diabetic mice

As shown in Figure 5(a), diabetic mice had significantly increased cardiac IL-1β (2.1-fold) and TNF-α levels (2.6-fold) compared to control mice. Treatment with nesfatin-1 decreased the cardiac IL-1β and TNF-α levels by 29.6% and 29.5%, respectively. Furthermore, the plasma IL-1β and TNF-α levels in the diabetic mice were also elevated compared to those in
Figure 4. Protective effects of Nesfatin-1 on myocardial hypertrophy in type 2 diabetes (T2DB) mice. (a) Hypertrophy index; (b) Images of wheat germ agglutinin (WGA) staining in the cardiomyocyte cross-sectional area; (c) Quantification analysis of Cardiomyocyte area (****, $P < 0.0001$ vs. vehicle group; ## $P < 0.01$ vs. streptozotocin (STZ) mice).
the control mice, which were also attenuated by nesfatin-1 (Figure 5(b)).

3.6 Nefatin-1 reduces the oxidative stress in STZ-induced diabetes mice

Regarding oxidative stress, diabetic mice had significant increases in cardiac MDA and ROS levels by 2.3- and 3.4-fold, respectively, as compared to control mice. Treatment with nesfatin-1 showed a significant reduction in cardiac MDA and ROS levels by 0.82- and 0.62-fold, respectively, as compared to diabetic mice (Figures 6(a-b)). On the other hand, cardiac SOD and GSH levels were significantly decreased by 44.4% and 29.1%, respectively, in the diabetic mice compared with those in the control mice. However, mice in the nesfatin-1 treatment group showed increases in cardiac SOD and GSH levels by 1.3- and 1.5-fold (Figures 6(c-d)), respectively.

3.7 Nefatin-1 increases the expression of GLP-1 in STZ-induced diabetic mice

Compared with the control group, the cardiac GLP-1 expression level in the diabetic model group was found to be significantly decreased by 55.0%. However, the decreased expression level of cardiac GLP-1 in the diabetic mice was elevated by nesfatin-1 with a 1.6-fold change (Figure 7).

3.8 Nefatin-1 protects against cardiac damages in STZ-induced diabetic mice through the P38-MAPK pathway

As indicated by Western blot analysis, the protein expression of p-p38-MAPK was markedly upregulated 2.3-fold in the diabetic mice, compared with that in the control mice. By contrast, the p-p38-MAPK protein expression in diabetic mice was decreased by 30.4% after nesfatin-1 treatment (Figure 8). There were no apparent differences in total p38-MAPK expression among the three groups.

4. Discussion

Diabetic cardiomyopathy (DC) is a specific type of CVD that is promoted by the events commonly occurring in diabetes, including hyperglycemia, hypertension, dyslipidemia, and insulin resistance [15]. This understanding provides directions for evaluating the potential therapeutic approaches to DC. It is known that β-cell dysfunction and reduced insulin sensitivity play essential roles in the pathogenesis of diabetes [16]. A decrease in insulin sensitivity causes insulin resistance, which triggers the pathophysiological mechanisms of DC [17]. Specifically, hyperglycemia and insulin resistance in heart tissues cause increased AGEs production, oxidative stress, mitochondrial dysfunction, inflammation, cardiac lipotoxicity, altered insulin metabolic signaling, dysregulation of exosomes, and impaired cardiomyocyte...
autophagy, impairment of calcium homeostasis, and increased cardiomyocyte death [18–22]. These alterations result in cardiac dysfunction characterized by a dysregulated heart rate and blood pressure, as well as alterations in the cardiac structure such as hypertrophy and interstitial fibrosis [22].

We first evaluated the role of nesfatin-1 in regulating insulin sensitivity and found that nesfatin-1 treatment elevated the Insulin sensitivity in diabetic mice. Atherogenic dyslipidemia, a major cause of CVD, is characterized by elevated plasma concentrations of TC, TAG, LDL, triglyceride-rich lipoproteins (TRLs), and low high-density lipoprotein (HDL) cholesterol [15]. Excessive accumulation of lipids and lipid metabolites contributes to diastolic dysfunction, inflammation, cardiac insulin resistance, and fibrosis [23]. Our results show that nesfatin-1 treatment attenuated diabetic dyslipidemia with reduced TC, TAG, and LDL levels in diabetic mice. Next, the protective effects of nesfatin-1 on cardiac function and structure in diabetic mice were demonstrated, as proven by the reduced heart rate and MAP level, decreased CK-MB and AST levels, and mitigated myocardial hypertrophy.

Hyperglycemia induces a protein glycation reaction and leads to an increase in AGEs, which are involved in promoting the production of ROS [24]. Oxidative stress is caused by the imbalance between ROS production and the scavenging ability [25]. In patients with diabetes, ROS-mediated oxidative stress is involved in the pathogenesis of diabetes-induced heart dysfunction since it can cause mitochondrial dysfunction and decrease fatty acid oxidation capacity, resulting in diastolic dysfunction, lipids accumulation, fibrosis, and heart failure [26–28]. Here, we found that nesfatin-1 exerted antioxidant activity in STZ-induced diabetic mice with decreased ROS and MDA.
levels, as well as increased SOD and GSH levels in heart tissues. In addition, AGEs also trigger NF-κB activation and therefore induce the secretion of proinflammatory cytokines, which play key roles in cardiac remodeling and fibrosis in the progression of DC [22,29]. We found that nesfatin-1 reduced inflammation in STZ-induced diabetic mice, as shown by the decreased cardiac and plasma IL-1β and TNF-α levels.

MAPK signaling pathways include p38-MAPK MAPK, JNK, and ERK1/2. MPAK kinases are upregulated by insulin resistance, cardiac hypertrophy, and heart failure [30,31]. In particular, the connection between p38-MAPK and DC has been extensively investigated. Growing evidence reveals that p38-MAPK is aberrantly activated under both experimental and clinical diabetic conditions, and its inhibition prevents the development of DC [31]. Previous studies imply that p38-MAPK may serve as a novel therapeutic target for DC. A recent study has proven that nesfatin-1 protects cardiomyocytes from cobalt chloride (CoCl₂)-induced hypoxic injury through inhibiting the MAPK signaling pathways [32]. In this study, we also found that p38-MAPK was markedly activated in diabetic mice. At the same time, nesfatin-1 significantly inhibited its activation, implying that nesfatin-1 might exert its protective effect on diabetes-mediated cardiac damages via regulating p38-MAPK.

In light of the above discussion, the limitation of the study has to be addressed. First, the current work focused on the effect of nesfatin-1 on cardiac function. As a neuropeptide, nesfatin-1...
regulates food intake and nutritional metabolism [6,10–12], which has effects on whole-body homeostasis and other organs other than the heart. Secondly, the molecular mechanism of nesfatin-1 in cardiac tissue remains to be fully understood. Although we show nesfatin-1 acted to suppress the cardiac p38-MAPK pathway and GLP-1 level, the precise mechanism remains unknown. Nesfatin-1 regulates multiple hormones and their receptors. The specific receptors in heart tissue remain largely unknown [33]. Future identification of the tissue-specific receptors will be critical to understanding the physiology of nesfatin-1.

5. Conclusion
In conclusion, this study provides evidence that nesfatin-1 exerted protective effects on diabetic cardiomyopathy in the STZ-induced mouse model. Nesfatin-1 exhibits pleiotropic effects on diabetic-associated dyslipidemia, insulin resistance, myocardial hypertrophy, and heart dysfunction. In cardiac tissue, nesfatin-1 possesses antioxidant and anti-inflammatory effects. Mechanistically, nesfatin-1 suppresses the activation of the cardiac p38-MAPK pathway and increases the GLP-1 level. The clinical implication of nesfatin-1 on diabetic cardiomyopathy remains to be fully investigated.

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Ethical statements
This study conformed to the ethical guidelines of the Youth Science Foundation of National Natural Science of China.

Consent to publication
All the authors have read and approved the final submission of this study.

data-availability
Data of this study are available upon reasonable request to the corresponding authors.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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Author Contribution
Zhanwei Fan, Jianjiang Dong, and Xian Liu contributed to the study design. Zhanwei Fan, Jianjiang Dong, and Yindong Mu contributed to the experimental conduction and data collection. All authors critically reviewed the manuscript and approved the final draft.

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