Original Paper

Long-Term Administration of Neuropeptide Y in the Subcutaneous Infusion Results in Cardiac Dysfunction and Hypertrophy in Rats

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Key Words

Neuropeptide Y • Cardiac hypertrophy • Calcineurin • p38 MAPK

Abstract

Background/Aims: The purpose of the present study was to clarify whether chronically elevated plasma neuropeptide Y (NPY) might affect heart function and cardiac remodeling in rats. **Methods:** Male Wistar rats were administered NPY (85 µg for 30 days) by mini-osmotic pump subcutaneously implanted between the scapulae. Associated indices for heart function, cardiac remodeling and hypertrophy were evaluated. **Results:** Compared to the sham group, the baseline systolic blood pressure (SBP) in rats administered NPY was significantly increased; cardiac function was significantly decreased, as indicated by reduced ejection fraction (EF), left ventricular end-systolic pressure (LVESP), maximum change velocity of left ventricular pressure in the isovolumic contraction or relaxation period ($\pm dp/dt_{max}$) and increased left ventricular end-diastolic pressure (LVEDP); hematoxylin-eosin (H&E) staining detection displayed enlarged cell areas and a consistent increase in heart-to-body weight ratios (HW/BW) was observed; quantitative real time PCR (qRT-PCR) and Western blot analysis showed markedly increased expressions of β -myosin heavy chain (β -MHC), calcineurin (CaN) and phosphorylated p38 proteins, while no changes were found in the expressions of p38 total protein and the phosphorylations of JNK and ERK. **Conclusion:** This study reported for the first time that long-term elevated plasma concentration of NPY could induce cardiac dysfunction and cardiac hypertrophy and this phenomenon could, in part, be mediated by the Ca^{2+} /CaM-dependent CaN pathway and p38 mitogen-activated protein kinase (MAPK) signal pathway in rats.

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Introduction

Neuropeptide Y (NPY), a 36-amino-acid sympathetic neurotransmitter, is known to be widely distributed in the mammalian central and peripheral nervous system [1, 2] and is an important modulator in a variety of physiological actions, including food intake, stress, immune function and cardiovascular diseases [3-5]. It is reported that circulating NPY is increased in patients with various diseases including diabetes, hypertension, cardiac hypertrophy, heart failure and end-stage renal disease [6-11], suggesting a close relationship between persistently high levels of NPY and these chronic diseases. Notably, plasma NPY has been considered a potential biomarker for the diagnosis of cardiovascular diseases [10, 12, 13]. However, the functional significance of elevated plasma NPY is not entirely clear and needs to be investigated.

NPY mediates its actions via a group of 7 transmembrane domain receptors, including Y1, Y2, Y3, Y4, Y5 and Y6 receptors [14-20]. Y1, Y2 and Y5 receptors are distributed widely in the cardiovascular system and play an important role in mediating cardiac functions [5]. Previous studies have demonstrated that NPY stimulated vasoconstriction by activating Y1 receptors [21], promoted angiogenesis by Y2 receptors [22], and induced cardiomyocyte hypertrophy by Y1, Y2 and Y5 receptors [9, 23, 24]. Further studies revealed that NPY induced cardiac hypertrophy by activating the Ca^{2+} /CaM-dependent calcineurin (CaN) signal pathway and mitogen-activated protein kinase (MAPK) pathway in cultured primary cardiomyocytes [23, 25, 26]. It is worth mentioning that NPY is a peptide with slow release and persistent actions under physiological conditions, whereas most previous reports focus mainly on the acute and transient effects on isolated cardiomyocytes. Therefore, more research needs to be undertaken on the gradual and long-lasting effects of plasma NPY *in vivo*. Unfortunately, very few studies have been conducted to evaluate the potential effects of long-term elevations of plasma NPY on the structure and function of the heart. *In vivo* studies are limited to the finding that plasma levels of NPY are increased and correlated with left ventricular hypertrophy in patients with hypertension or end-stage renal disease [10, 27]. There is no report on the direct effects and the associated mechanisms of sustained elevated plasma NPY on the heart in a normal animal model.

In the present study, we clarified that the long-term subcutaneous administration of NPY could induce cardiac dysfunction and cardiac hypertrophy in rats which was associated with the Ca^{2+} /CaM-dependent CaN and phosphorylated p38 MAPK pathway.

Materials and Methods

Animal models

Twelve male Wistar rats weighing 250 to 300 g were obtained from the Animal Center of the Second Affiliated Hospital of Harbin Medical University and housed in a standard animal room (temperature: $23 \pm 1^\circ\text{C}$; humidity: 55~60%) with food and water freely administered under a 12 h-12 h light-dark cycle. The rats were randomly divided into two groups of 6 rats each. Each rat in the control or NPY group was surgically implanted with an osmotic pump filled with vehicle (PBS) or NPY (SciLight Biotechnology, LLC, Beijing, China), respectively. For surgery and the detection of heart function, the rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneal) and anesthesia maintained by administering 0.5-1.0% isoflurane. The depth of anaesthesia was monitored by detecting reflexes, heart rate and respiratory rate. Samples for qRT-PCR, Western blot assay and histological analysis were obtained from the left ventricle of the rats after anesthesia with sodium pentobarbital (100 mg/kg, intraperitoneal) and confirmation of death by exsanguination. All animal procedures were approved by the Institutional Animal Care and Use Committee at Harbin Medical University (No. HMUIRB-2008-06) and the Institute of Laboratory Animal Science of China (A5655-01). All procedures conformed to the Directive 2010/63/EU of the European Parliament.

Implantation of the ALZET mini-osmotic pump

The implantation of ALZET mini-osmotic pumps were performed as previously described [28-31]. Briefly, mini-osmotic pumps (Model 2004, Durect Corporation, Cupertino, CA, USA) filled with either sterile PBS or 85 µg NPY [28, 29] (SciLight Biotechnology, LLC, Beijing, China) were primed in sterile 0.9% saline solution at 37°C for at least 40 h. The pumps were surgically implanted subcutaneously in rats as follows: firstly, a small incision was made in the skin between the scapulae on the animals and a small pocket was formed by separating the subcutaneous connective tissues with a hemostat; secondly, the filled pump was inserted into the pocket with the flow moderator pointing away from the incision; finally, the skin incision was closed with absorbable sutures. The fillers in the pumps could persistently and steadily release for 30 days.

Detection of plasma NPY levels

Plasma NPY level was detected using an ELISA kit according to the manufacturer's instructions (Cat.# EK-049-03, Phoenix Pharmaceutical Company Belmont, CA, USA).

Measurement of haemodynamic parameters

Haemodynamic measurements were performed with the BL-420 Data Acquisition & Analysis System as described by our previous study [32]. After the rat was anesthetized, a catheter was inserted into the left ventricle of the rat to record cardiac haemodynamic parameters via the right common carotid artery. Ejection fraction (EF), left ventricular end-systolic pressure (LVESP), left ventricular end-diastolic pressure (LVEDP), and maximum change velocity of left ventricular pressure in the isovolumic contraction or relaxation period ($\pm dp/dt_{\max}$) were analyzed. Blood pressure was recorded through the right femoral artery using a blood pressure transducer (model MIT0699, AD Instruments Pty Ltd Castle Hill, NSW Australia) and the baseline systolic blood pressure (SBP) was acquired.

Histological analysis

Heart tissue was fixed in 10% formalin/PBS and embedded in paraffin. Five-micron-thick sections were stained with hematoxylin-eosin (H&E) as indicated to evaluate morphology and cellular dimensions. Photomicrographs were obtained by light microscopy (Nikon, Tokyo, Japan). The cardiomyocyte area was measured using an image analysis program (Image-Pro Plus 6.0). The areas of at least 100 cardiomyocytes were determined in randomly selected visual fields at 400-fold magnification. The final results were expressed as relative level by normalizing the data to control values.

Quantitative real time PCR (qRT-PCR) analysis

The total RNA samples were extracted using Trizol from rat left ventricular tissues. cDNA synthesis was performed according to the manufacturer's instructions (Reverse Transcription System, Cat#A3500, Promega) and as described previously [33]. The SYBR Green PCR Master Mix Kit (Applied Biosystems, Cat#4309155) was used in qRT-PCR for relative quantification of mRNAs in our study with GAPDH as an internal control. qRT-PCR was performed on 7500 FAST Real-Time PCR System (Applied Biosystems). The sequences of PCR primers were listed as follows: GAPDH forward primer 5'-TCT ACA TGT TCC AGT ATG ACTC-3' and reverse primer 5'-ACT CCA CGA CAT ACT CAG CACC-3'; β -MHC forward primer 5'-AAC CTG TCC AAG TTC CGC AAG GTG-3' and reverse primer 5'-GAG CTG GGT AGC ACA AGA GCT ACT-3'. The expression levels were indicated with $2^{-\Delta\Delta Ct}$.

Western blot analysis

The protein samples were extracted from the left ventricle. The protein content was determined by BCA Protein Assay Kit (Bio-Rad, Mississauga, ON, Canada). The proteins under study were: CaN (59 kDa), phosphorylated p38 (p-p38) and total p38 (t-p38) (38 kDa), p-JNK and t-JNK (46 kDa), p-ERK and t-ERK (42/44 kDa), and GAPDH (36 kDa). Protein samples were fractionated by SDS-PAGE (10%~20% polyacrylamide gels) and transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA). Samples were incubated with primary antibodies specific for each protein (Santa Cruz, CA, USA) diluted at 1:200 in PBS buffer for 1 h at 22°C~23°C. After washing, samples were incubated with fluorescence-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen, USA) at a dilution of 1:4,000. Western blot bands were quantified using Odyssey infrared imaging system (LI-COR, Lincon, NE, USA) by measuring the band intensity

(area $\times$ OD) in each group and normalizing to GAPDH. The final results were expressed as relative level by normalizing the data to control values.

Data analysis

All the data are presented as means $\pm$ SEM. Significance was determined by using the t-test with SPSS 13.0 software. A value of $P < 0.05$ was considered statistically significant. Figures were constructed by GraphPad Prism 5.0 software.

Results

Chronic elevation of NPY increased baseline SBP

After one-month of exogenous administration of NPY in the subcutaneous infusion, the plasma level of NPY was significantly increased compared to the sham group (Sham: 5.52 ± 0.21 ng/ml; NPY: 9.54 ± 0.38 ng/ml, $n=6$, $P < 0.05$) (Fig. 1A). The baseline SBP in NPY rats was increased to 119.58 ± 2.03 mmHg from 102.91 ± 1.61 mmHg in sham rats ($n=6$, $P < 0.05$) (Fig. 1B).

Chronic elevation of NPY impaired cardiac function in rats

In the present study, we observed that EF, LVESP and $+dp/dt_{\max}$, indicators of cardiac contractile function, were significantly decreased in rats administered NPY compared to the sham group (EF: sham: 68.17 ± 1.04 %; NPY: 52.67 ± 1.16 %; LVESP: sham: 14.28 ± 1.06 kPa; NPY: 12.90 ± 1.17 kPa; $+dp/dt_{\max}$: sham: 480.9 ± 59.73 kPa/s, NPY: 334.8 ± 91.91 kPa/s, $n=6$, $P < 0.05$) (Fig. 2A, 2B and 2C). LVEDP and $-dp/dt_{\max}$ reflect cardiac diastolic function. We observed that $-dp/dt_{\max}$ reduced to -304.3 ± 102.9 kPa/s in the NPY group from -460.1 ± 45.64 kPa/s in the sham group, and the LVEDP was increased to 0.27 ± 0.39 kPa in the NPY group from -0.36 ± 0.072 kPa in the sham group ($n=6$, $P < 0.05$) (Fig. 2D and 2E).

Chronic elevation of NPY induced cardiac morphological remodeling in rats

Based on the above data, we further examined the effect of sustained elevation of NPY levels on cardiac histology. As in Fig. 3A, H&E staining displayed disarrayed cardiomyocytes and enlarged nuclei in NPY-treated rat hearts. Furthermore, since previous studies have reported that NPY could induce cardiac hypertrophy in isolated cardiomyocytes [34], we evaluated the cell areas in rats following long-term NPY administration. As indicated in Fig. 3B, cell areas were significantly increased in hearts from the NPY group compared to those from the sham group ($n=6$, $P < 0.05$). And strikingly, heart-to-body weight ratios (HW/BW)

Fig. 1. Elevated plasma NPY level increased baseline systolic blood pressure (SBP). (A) Plasma NPY level was increased after long-term NPY administration in rats. (B) The SBP was increased in rats treated with NPY compared to rats in the sham group. Values are means $\pm$ SEM. * $P < 0.05$ vs. sham group, $n=6$.

Fig. 2. Effect of NPY on haemodynamics parameters after 30 days of exogenous administration of NPY in rats. (A) EF was decreased after long-term elevation of NPY. (B) LVESP of NPY-treated rats was significantly lower than in the sham group. (C) The +dp/dt_{max} in rats from the NPY group was reduced compared to rats in the sham group. (D) The -dp/dt_{max} in rats from the NPY group was decreased compared to rats in the sham group. (E) Long-term elevation of NPY induced increase in LVEDP level. EF: ejection fraction; LVESP: left ventricular end-systolic pressure; LVEDP: left ventricular end-diastolic pressure; +dp/dt_{max}: maximal rise of left ventricular pressure; -dp/dt_{max}: maximal decline of left ventricular pressure. Values are expressed as means ± SEM. **P*<0.05 vs. sham group, n=6.

Fig. 3. Long-term elevation of plasma NPY level induced cardiac hypertrophy in rats. (A) Hematoxylin and eosin (H&E) staining showed disarrayed cardiomyocytes and enlarged nuclei and cardiomyocytes in NPY-treated rat hearts. The sections were visualized at 200×magnifications. (B) Statistical analysis displayed increased cardiomyocyte areas in NPY-treated rat hearts compared to the sham group. (C) Heart-to-body weight ratios (HW/BW) were increased in the NPY group. (D) The mRNA level of β-MHC was significantly upregulated in NPY-treated rats by real time PCR and normalized to a reference mRNA (GAPDH). Values are expressed as means ± SEM. **P*<0.05 vs. sham group, n=6. (E) The β-MHC protein were found to be increased in the NPY-treated group by western blot and normalized by the house-keeping protein GAPDH. Values are expressed as means ± SEM. **P*<0.05 vs. sham group, n=6.

Fig. 4. Effects of NPY on the expressions of calcineurin (CaN) and mitogen-activated protein kinase (MAPK) activation in rats. (A) CaN protein level was increased in rats treated with NPY compared to the sham group. (B) There were no differences in the phosphorylation of ERK protein between the two groups. (C) There was no significant difference in phosphorylated JNK protein levels between the sham rats and NPY rats. (D) NPY upregulated phosphorylated p38 (p-p38) protein levels in cardiac tissue but not total p38 (t-p38) protein. Values are expressed as means $\pm$ SEM. * $P < 0.05$ vs. sham group, $n = 6$.

were increased in the NPY group compared to the sham group (Fig. 3C, $n = 6$, $P < 0.05$). Because up-regulation of β -myosin heavy chain (β -MHC) is considered as an early, sensitive and persistent marker for cardiac hypertrophy [35], we evaluated the expression of β -MHC, and found that, both the mRNA and protein expressions of β -MHC were significantly increased (Fig. 3D and 3E, $n = 6$, $P < 0.05$). All these data suggested that one-month of exogenous administration of NPY in the subcutaneous infusion could induce cardiac hypertrophy.

Effects of NPY on the expressions of CaN and MAPKs in rats

Based on the findings above, we examined whether the long-term increased plasma NPY had an effect on the expression of proteins associated with hypertrophy pathways. In the present study, we demonstrated that long-term NPY elevation *in vivo* increased the expression of CaN (Fig. 4A). Since MAPK pathway is considered as a peptide activated pathway [36], we evaluated the expressions of phosphorylation of JNK, ERK and p38 which are the key downstream sites of the MAPK pathway. We found that the phosphorylated ERK and JNK were not changed compared to the sham group (Fig. 4B and 4C, $n = 6$, $P > 0.05$). Further, though the level of p38 total protein (t-p38) did not change, the phosphorylation level of p38 (p-p38) was markedly increased in hearts from rats treated with NPY (Fig. 4D, $n = 6$, $P < 0.05$).

Discussion

In the present study, we report for the first time that long-term administration of NPY in the subcutaneous infusion could result in cardiac dysfunction and hypertrophy in rats. The potential molecular mechanism is elevated expressions of CaN and phosphorylated p38.

Previous studies have reported that NPY is involved in the progression of various chronic diseases such as hypertension, diabetes, and cardiovascular diseases [37-41]. Considering that NPY is a peptide with graduated release and persistent effects, the consequences of a sustained increase in plasma NPY levels are equally if not more physiologically relevant than its acute and transient effects. Little information is available about the effects of long-term elevated plasma NPY on heart function and cardiac hypertrophy *in vivo*. In the present study, we focused on the chronic effect of NPY on a normal rat model and demonstrated that exogenous administration of NPY could impair cardiac function, suggesting that the elevated plasma NPY might participate in the regulation of heart function. Interestingly there is a conflicting report that NPY improved myocardial perfusion and function in a swine model of hypercholesterolemia and chronic myocardial ischemia [42]. In the light of plasma NPY being increased in patients with multiple diseases [6-11], we hypothesize that NPY has bidirectional impacts on cardiac function. In the normal heart, NPY, especially if chronically administered, has adverse effects while in diseased conditions and under chronic stimuli, NPY plays a protective role on cardiac function. This is an important homeostatic mechanism in the whole body for self-protective functions. Xie et al found that long-term NPY administration induced abnormal baroreflex sensitivity but reversed chronic stress-induced baroreflex hypersensitivity in rats [28, 43], providing the evidence of the double controlled role of NPY depending on specific conditions. It's very meaningful to illustrate the effect of NPY on heart function at different time points. In our study, for the purpose of long-term study, we delivered NPY for 30 days and didn't observe the shorter time point. We don't exclude the possibility of a compensatory phase of cardiac hypertrophy with increased heart function. Therefore, greater mechanistic information is required in order to truly assess the potential for treatment of cardiac diseases using NPY-based drugs.

The present study also found that long-term administration of NPY induced myocardial hypertrophy in rats. *In vitro* research demonstrates that NPY induces hypertrophic responsiveness of cardiomyocytes via NPY receptors or the CaN signaling pathway [24, 25, 34, 44]. *In vivo* studies are limited to the finding that plasma levels of NPY are increased and correlate with left ventricular hypertrophy in patients with hypertension or end-stage renal disease [10, 27]. However the mechanisms remain unclear. For the first time, our data reveal the effect of long-term NPY on normal rat hearts, and we propose what we think is the main mechanism below. Strikingly, NPY administration also resulted in an increased level of SBP in the rat. As we know, hypertension can lead to cardiac hypertrophy in a hemodynamic afterload-dependent mechanism [5], which activates the phosphorylation process of MAPK pathway including p-p38, p-JNK, p-ERK [45-48] and upregulates CaN expression [49]. It has been reported that NPY is closely related to hypertension [7, 37] and may secondarily induce hypertrophy via upregulating CaN expression and activating the MAPK pathway. In this study, although we cannot completely exclude hypertension-induced hypertrophy involving the activation of p-p38 and upregulation of CaN, unchanged p-JNK and p-ERK in this model indicate minor effects of NPY-induced hypertension on hypertrophy. Therefore, in our study, the occurrence of myocardial hypertrophy could be mainly induced by the actions of NPY on the heart or partially affected by the upregulated SBP level, which remains to be illuminated in future study. Furthermore, we found a reduction in LVESP indicating decompensated cardiac contractile function, which seems inconsistent with increased SBP. The factors that determine SBP include peripheral resistance and vascular elasticity besides cardiac ejection. Our previous study demonstrated that the increased NPY contributed to dyslipidemia [28] and could be a potential risk factor for arteriosclerosis. We speculate that the inconsistency between the increased SBP and decreased LVESP might be explained by the decreased vascular compliance induced by arteriosclerosis in NPY rats.

Fig. 5. Schematic diagram of the signaling pathways that mediate cardiac hypertrophy induced by elevated plasma NPY. Elevated NPY binding with its receptors upregulates the expression of calcineurin (CaN) resulting in NFAT dephosphorylation and the promotion of phosphorylation of p38 through activation of the small G protein Ras by activating MAPKs. NPY had no effect on other members of the MAPK family such as ERKs and JNKs in the present study. The activities of nuclear transcription factors are stimulated by dephosphorylated NFAT and phosphorylated p38, which then induce the expressions of hypertrophic response genes, and thereafter trigger cardiac hypertrophy. The occurrence of myocardial hypertrophy could also be induced by the upregulated SBP level due to the vasoconstriction (Need further verification). NFAT: nuclear factor of activated T cells; MAPK: Mitogen-activated protein kinase; ERKs: extracellular signal-regulated kinases; JNKs: c-jun N-terminal protein kinases.

The potential molecular mechanisms of cardiac hypertrophy have been extensively investigated in recent years, and different stimuli were found to produce cardiac hypertrophy by mediating different signaling pathways, including insulin growth hormone, β -agonists, peptide growth factors, and so on [36]. Upregulation of CaN protein was found in a variety of pathological states [50-52]. In the present study, we found that the expression of CaN protein was significantly increased in rats following long-term NPY administration and cardiac hypertrophy was induced through the Ca^{2+} /CaM-dependent CaN signal pathway, which was consistent with a previous study in cultured neonatal rat cardiomyocytes [25]. Since MAPK activation was found to be a key pathway mediating peptide growth factors-induced hypertrophy [36], we evaluated the phosphorylation levels of ERK, JNK and p38. Interestingly, our data showed that long-term administration of NPY *in vivo* could result in the elevation of phosphorylated p38 but not the phosphorylation levels of ERK and JNK. These results were different from the findings reported previously, in which all three kinases were activated significantly by NPY in primary cardiomyocytes from mouse neonatal heart [23]. This discrepancy may be explained by the notion that long-term NPY *in vivo* exerts its function by modulating the regulatory function of the whole body rather than only via a direct action on cardiomyocytes. This suggests that the long-term administration of NPY *in vivo* may have significantly different effects from those observed in isolated cardiomyocytes and could very well uncover hitherto unknown biological roles that might have therapeutic applications. Taken together, our results indicated that chronic elevation of plasma NPY might induce hypertrophy involving CaN and p38 MAPK signaling pathways. We can't rule out the role of blood pressure, which remains to be illuminated in future study. (Fig. 5).

As we know, NPY exerts its function through six identified receptors subtypes [14-20]. Y1, Y2 and Y5 receptors appear to mediate the main functional responses in the heart [5]. Some *in vitro* research has been conducted to study certain receptor subtypes involved in the regulation of cardiac hypertrophy. NPY stimulates vasoconstriction, vascular and cardiomyocyte hypertrophy via Y1R and angiogenesis via Y2R [9]. NPY elicits positive and negative contractile effects in cardiomyocytes through Y1 and Y2 receptors, respectively [27]. While Nicholl SM et al report that initiation of cardiomyocyte hypertrophy by NPY requires activation of both Y1 and Y2 receptors [24]. There are other reports that NPY, via Y5 receptors, induces hypertrophic responsiveness in the cardiomyocytes of spontaneously hypertensive rat [44] and potentiates phenylephrine-induced MAPK activation in primary cardiomyocytes [23]. These findings suggest that Y1, Y2 and Y5 receptors are all involved in cardiac hypertrophy and Y1 and Y2 might play consistent or opposing roles in the hypertrophic process under different study conditions. There is no doubt that these *in vitro* results provide significant support for *in vivo* research. In the present study, we focused on the outcome of long-term NPY administration in the subcutaneous infusion on heart function and hypertrophy but did not identify which receptor might participate in this process. The potential role of NPY receptors in this model will be investigated in future studies.

Acknowledgements

The present work was supported by the project of Natural Science Foundation of China (no. 81271207, 81471115, 81102434, 81470523) and Science Foundation of Heilongjiang Province for youth (no. QC2012C061).

Disclosure Statement

The authors have declared that no competing interests exist.

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