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# Neuropeptide Y induces cardiomyocyte hypertrophy via calcineurin signaling in rats

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**Abstract**

Neuropeptide Y (NPY) has been shown to participate in cardiac hypertrophy. However, the mechanisms by which NPY induces cardiomyocyte hypertrophy are poorly understood. This study tested the hypothesis that NPY induces cardiomyocyte hypertrophy through $Ca^{2+}$/CaM-dependent calcineurin (CaN) pathway in cultured neonatal rat cardiomyocytes. After 24-h treatment, NPY (100 nM) significantly increased $^{3}$H-leucine incorporation and c-Jun mRNA expression, concomitant with augment of CaN activity and protein level in cardiomyocytes compared to those cells without NPY treatment. The enhancement of $^{3}$H-leucine incorporation and c-Jun mRNA expression in cardiomyocytes treated with NPY were markedly inhibited by cyclosporine A (CsA), a selective inhibitor of CaN. We also investigated the effect of NPY on intracellular $Ca^{2+}$ level in cardiomyocytes. There were no obvious changes in intracellular $Ca^{2+}$ level of cytoplasm and nucleus in cardiomyocytes treated with NPY (100 nM) for 10 min. However, NPY significantly increased intracellular $Ca^{2+}$ level of cytoplasm and nucleus in cardiomyocytes after 24-h treatment. The result suggested that NPY could induce hypertrophy of cardiomyocytes via $Ca^{2+}$/CaM-dependent CaN signal pathway. The enhancement of $[Ca^{2+}]_i$ caused by NPY may activate CaN signal pathways to mediate cardiac hypertrophy.


*Keywords:* Neuropeptide Y; Calcineurin; Cardiac hypertrophy; $Ca^{2+}$ signaling

## 1. Introduction

Cardiac hypertrophy, a construction remodeling in response to many diseases, characterizes the increase in size and mass of individual cardiomyocyte. In the early stage, hypertrophy is beneficial to the adaptation of myocardium to the pathologic stimuli, but prolonged hypertrophy becomes deleterious and even results in heart failure and sudden death ultimately. Cardiac hypertrophy occurs in response to multi-stimuli including mechanical stress or various neurohumoral factors. These stimuli lead to hypertrophic gene expression and phenotypes [1,2].

Neuropeptide Y (NPY), a member of Neuropeptides family, is involved in the regulation of vascular tone and becomes a potent stimulus of hypertension [3]. NPY stimulates contractions of blood vessel significantly and promotes the contractive effects of other constrictor agents [4]. Recent studies suggest that NPY relates to cardiac hypertrophy. The enhancement of plasmas NPY exists in heart failure and cardiac hypertrophy [5]. In some studies, NPY has been shown to be effective on hypertrophy or proliferation in cells like growth factors [6,7].

Hypertrophic stimuli cause hypertrophic gene expression via a number of specific intracellular signaling cascades. Some previous researches have showed that NPY activated important signal tranductors inducing cardiac hypertrophy such as Gi, MAPK and PKC [8,9]. NPY also stimulated cellular L-type calcium channel and resulted in the increase of cytoplasmic $[Ca^{2+}]$ [10]. In addition, the hypertrophic

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effect of NPY is inhibited by L-type calcium channel blockade [11]. It means that the signaling pathways depending on $Ca^{2+}$ signal play important roles on it.

The calcium/calmodulin-dependent protein phosphatase CaN is an important inducer of myocardial hypertrophy. CaN dephosphorylates nuclear factor of activated T cell (NFAT) proteins, a family of calcium-regulated transcription factors, and then NFAT translocates into nuclear to regulate hypertrophic gene repression [12]. Numerous studies demonstrated that CaN activation is required for hypertrophy, and the CaN inhibitors can prevent or diminish cardiac hypertrophy induced by pressure overload, hypertension, myocardial infarction, or contractile abnormalities [13–16].

In this study, we investigated the effect of NPY on the hypertrophy of rat cardiomyocytes and the role of $Ca^{2+}$/CaM-dependent CaN signaling pathway in this process. In addition, we observed the effect of NPY on $Ca^{2+}$ signal in cardiomyocytes primarily. Our aim is to further understand the mechanism of cardiac hypertrophy and find new approaches to block cardiac hypertrophy.

## 2. Materials and methods

### 2.1. Cell culture

Ventricular cardiomyocytes were isolated from neonatal Wistar rats (1–3 days old, provided by Chinese military medical scientific institute) by 0.25% trypsin (GIBCO) digestion and purified as described previously [17]. The cells were resuspended in DMEM (GIBCO) containing 20% fetal calf serum and 1% penicillin–streptomycin and plated on tissue culture plastic for 2 h to remove non-cardiomyocyte cells. Cardiomyocytes were plated at low density ($2\times10^4$ cells/ml) for $[Ca^{2+}]_i$ determination and at high density ($1.5\times10^5$ or $1\times10^6$ cells/ml) for other studies. Following serum starvation for 24 h, cardiomyocytes in good condition were incubated with agents and prepared for assays as follows.

### 2.2. Incorporation of $^3$H-Leu in cardiomyocytes

Cells were incubated with 10 mN NPY (Sigma), 100 nM NPY or 100 nM NPY together with 5 μg/ml cyclosporine A (CsA) (Sandoz) for 24 h, respectively. One microcurie per milliliter $^3$H-leucine (Chinese Atomic Research Institute, Beijing, China) was added and incubated for 12 h. The radioactivity incorporated into the trichloroacetic acid-precipitable material was determined by β-scintillation counting.

### 2.3. RT-PCR for c-Jun mRNA expression in cardiomyocytes

The cells were incubated with 100 nM NPY or 100 nM NPY together with 5 μg/ml CsA for 24 h. Total RNA was isolated with total RNA isolation system (Promega) and subjected to RT-PCR with Access RT-PCR system (Promega). The PCR primers were designed on the basis of published cDNA sequence for rat c-Jun and β-actin as follows: sense c-Jun for, 5′AGCAATGGGCACATCACC 3′; antisense c-Jun for, 5′TCTGCGGCTCTTCCTTCA 3′; sense for β-actin, 5′CTCTTTGATGTCACGCAGGATTTC 3′; anti-sense for β-actin, 5′GTGGGCCGCTCTAGGCACCAA 3′. The reverse transcription with a first strand cDNA synthesis and amplification (30 cycles, annealing of c-Jun at 54 °C, annealing of β-actin at 60 °C) was carried out as technical bulletin. The PCR products were subjected to electrophoresis on a 2% agarose gel and stained with ethidium bromide.

### 2.4. CaN activity assay [18]

Cells were incubated with 10 mN NPY, 100 nM NPY or 100 nM NPY together with 5 ug/ml CsA for 24 h, respectively. Then cells were washed and lysed in extraction buffer [50 mmol/l Tris (PH 7.5), 0.1 mmol/l EGTA, 1 mmol/l EDTA, 0.5 mmol/l dithiothreitol (DTT, Sigma), 50 μg/ml phenylmethysulfonyl fluoride (PMSF, Sigma), 50 μg/ml soybean trypsin inhibitor (STI, Sigma), 5 μg/ml Leupeptin (Sigma), 5 μg/ml Aprotinin (Sigma)]. After centrifugation for 10 min at $12{,}000\times g$, the supernatant was preserved for CaN activity assay. 20 μl supernatant was incubated with 380 μl substrate buffer I [50 mmol/l Tris (PH7.4), 0.5 mmol/l DTT, 0.2 mg/ml BSA, 10 mmol/l *p*-nitrophenyl phosphate (PNPP, Sigma), 0.5 mmol/l $MnCl_2$, 0.2 mmol/l $CaCl_2$, 0.3 umol/l CaM(Sigma)] or 380 μl substrate buffer (50 mmol/l Tris PH7.4, 0.5 mmol/l DTT, 0.2 mg/ml BSA, 10 mmol/l PNPP, 0.5 mmol/l $MnCl_2$, 3 mmol/l EGTA) for 10 min at 30 °C. Then 40 μl buffer (0.5 mmol/l $Na_2CO_3$, 0.4 mmol/l EGTA) was added to terminate the reaction immediately. Absorbance in 390 nm wavelength ($A_{390\ nm}$) was determinate by spectrophotometer. CaN activity ratio=$A_{390\ nm}$ in substrate buffer I−$A_{390\ nm}$ in substrate buffer II/protein concentration. The protein concentration in supernatant was measured with Lowry method.

### 2.5. Western blotting for CaN-α expression in cardiomyocytes

Cells were incubated with 100 nM NPY for 24 h, and then washed and lysed in cellular lysis buffer [50 mmol/l Tris.Cl (pH=8.0), 150 mmol/l NaCl, 0.02%$NaN_3$, 100 μg/ml PMSF, 1 μg/ml Aproptinin, 1% Triton X-100]. After centrifugation for 2 min at $12{,}000\times g$, the protein concentration in supernatant was measured with Lowry method. 20-μl samples were resolved by SDS-polyacrylamide gel electrophoresis (Bio-Rad), and the proteins were transferred to PVDF membranes. The membranes were blocked for 1.5 h by 3% BSA in TBST buffer (10 mM Tris, 0.1 M NaCl, 0.1% Tween-20, pH 7.4). Following the incubation with monoclonal anti-CaN-α antibody (Sigma), blots were washed six times for 10 min each with TBST buffer and then incubated with appropriate horseradish peroxidase-labeled secondary antibodies (Santa Cruz) in TBST buffer

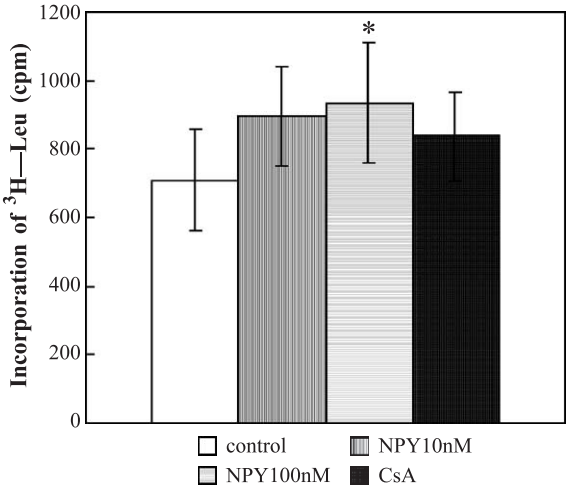


Fig. 1. Incorporation of $^3$H-Leu in cardiomyocytes. Cardiomyocytes were cultured in 24-well plates and in serum-free medium for 24 h prior to the stimulation with NPY (10 nM), NPY (100 nM) or CsA (5 μg/ml) together with NPY (100 nM) for 24 h. $^3$H-Leucine was added at 1 μCi per well, and incubated for 12 h. The radioactivity incorporated into the cells was determined. The data are mean±S.D. of six independent studies. *$P$<0.05 versus control.

for 1 h at room temperature. Following six washes of 10-min duration each with TBST, the proteins were detected by chemiluminescence (Santa Cruz).

### 2.6. Assay of [Ca2+]I

(1) The cells were incubated with 10 μmol/l fluo3-AM (Sigma) for 30 min at 37 °C. Following three washes by Hank's buffer, the cells simulated by 100 nM NPY were immediately observed by Leica fluorescence microscope connected with the computer in 488 nm exciting wavelength and the images were recorded at 1-min interval. The fluorescence intensity in the cell was analyzed with TILL Vision software.

(2) The cells simulated by 100 nM NPY for 24 h were washed by Hank's buffer and incubated with 10 μmol/l fluo3-AM (Sigma) for 30 min at 37 °C. The steps as stated above were used to observed and analyzed images.

### 2.7. Data analysis

Results were expressed as means±S.D. One-way ANOVA followed by Dunnett's test were used for the statistical comparison among multiple groups. Differences among means were considered significant at $P$<0.05. Data were analyzed using SPSS statistical software.

## 3. Result

### 3.1. The cardiomyocytes hypertrophy was induced by NPY

To investigate the role of NPY on cardiomyocytes hypertrophy, protein synthesis was measured by incorporation of $^3$H-leucine into the myocytes. As shown in Fig. 1, $^3$H-Leu incorporation in cells exposed to 10 nM NPY for 24 h was slightly higher than the control group, but there was no significant change between the two groups. However, compared with the control group, $^3$H-Leu incorporation in 100 nM NPY group increased significantly ($P$<0.05). C-Jun is one of the early hypertrophic genes, so we further tested c-Jun mRNA expression in cardiomyocytes by RT-PCR. Consistent with the increase of protein synthesis, c-Jun mRNA in cardiomyocytes was enhanced considerably by stimulation of 100 nM NPY ($P$<0.001) (as shown in Fig. 2). These results suggested that NPY played an important role in cardiac hypertrophy.

### 3.2. Cardiomyocytes hypertrophy induced by NPY was blunted by CaN inhibition (CsA)

CsA, an efficient CaN inhibitor, was used to interfere with the effect of NPY and to clarify whether CaN signal pathway contributes to the cardiac hypertrophy induced by NPY. Contrary to cells incubated with only 100 nm NPY, incorporation of $^3$H-leucine into the cells incubated with 100 nm NPY and 5 μg/ml CsA together was unchanged

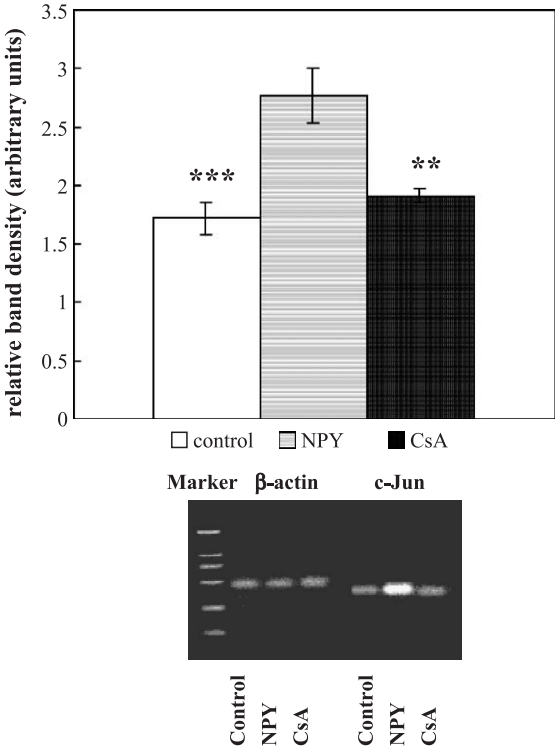


Fig. 2. Expression of c-Jun mRNA in cardiomyocytes. The cardiomyocytes incubated by NPY (100 nM), NPY (100 nM) together with CsA (5 μg/ml) for 24 h were prepared for isolating total RNA of cells by total RNA isolation system. One microgram of total RNA/per each sample was subjected to RT-PCR using the primers specific for c-Jun. As an internal control, β-actin was amplified synchronously. The data are mean±S.D. of three independent studies. **$P$<0.01, ***$P$<0.001 versus control. Ethidium bromide staining of the RT-PCR products is shown.

compared with the control. C-Jun mRNA expression in cells incubated with 100 nm NPY and 5 μg/ml CsA together was distinctly lower than that stimulated by only 100 nm NPY, and was unchanged compared with the control (as shown in Figs. 1 and 2). It is suggested that hypertrophic effect of NPY was CaN-dependent and was weakened by CsA.

### 3.3. NPY activated CaN signal pathways in cardiomyocytes

Having demonstrated that CaN signaling mediated cardiomyocyte hypertrophy induced by NPY, we investigated the activity of CaN and CaN-α protein expression in cardiomyocytes stimulated by NPY. Although 10 nM NPY did not affect the activity of CaN, 100 nm NPY induced a distinct enhancement in the activity of CaN ($P<0.05$) (as shown in Fig. 3). CsA (5 μg/ml) counteracted this effect of NPY. Moreover, CaN-α protein expression in cells was measured by Western blot. CaN-α protein expression was promoted considerably in cardiomyocytes simulated by NPY (100 nM) ($P<0.05$) (as shown in Fig. 4). The results suggested that CaN signal pathways in cardiomyocytes were activated by NPY and contributed to cardiac hypertrophy induced by NPY.

### 3.4. NPY enhanced $[Ca^{2+}]_i$ of cardiomyocytes

CaN is $Ca^{2+}$/CaM-dependent phosphatase, and the lasting increase in $[Ca^{2+}]$ of cytoplasm was necessary for the activation of CaN. The $[Ca^{2+}]$ in plasmas and nuclear of cardiomyocytes were reflected by fluorescence intensity of fluo3-AM combined with intracellular $Ca^{2+}$. 24 h after incubation with 100 nm NPY, both $[Ca^{2+}]$ in plasmas and in nuclear was significantly elevated compared with control cells ($P<0.001$, 0.001, respectively) (as shown in Fig. 5). Contrarily, we did not observe the changes of $[Ca^{2+}]$ in plasmas and nuclear of cardiomyocytes stimulated by NPY within 10 min (Table 1).

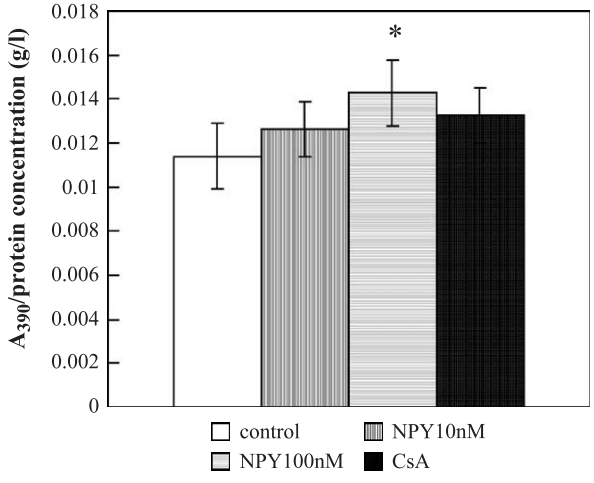


Fig. 3. Assay of Calcineurin activation. Cells were incubated with 10 nM NPY, 100 nM NPY, 100 nM NPY together with 5 μg/ml CsA for 24 h, respectively. Cells were washed and lysed in extraction buffer. After centrifugation, 20 μl supernatant reacted with 380 μl substrate buffer I for 10 min at 30 °C, and another 20 μl supernatant reacted with 380 μl substrate buffer II synchronously. Calcineurin activity ratio=$A_{390\ nm}$ in substrate buffer I−$A_{390\ nm}$ in substrate buffer II/protein concentration. The data are mean±S.D. of four independent studies. *$P<0.05$ versus control.

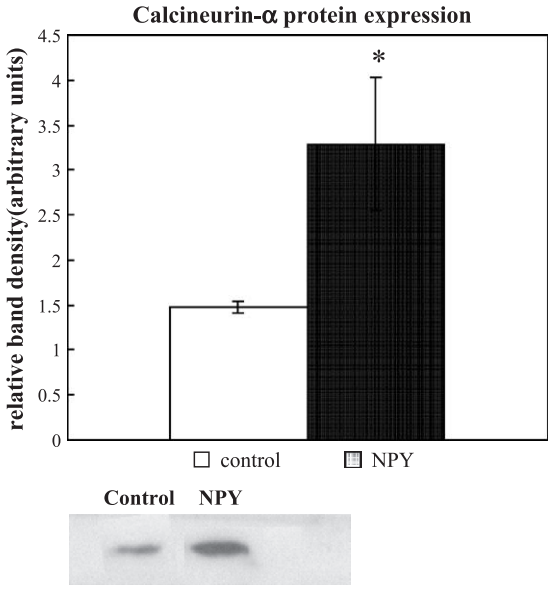


Fig. 4. Western blotting for calcineurin-α expression in cardiomyocytes. Cells were incubated with 100 nM NPY for 24 h, and then washed and lysed. Twenty-microliter samples were resolved by SDS-polyacrylamide gel electrophoresis, and the proteins were transferred to PVDF membranes. Monoclonal anti-Calcineurin-α antibody was used to blot Calcineurin proteins. The result was detected by chemiluminescence. The data are mean±S.D. of four independent studies. *$P<0.05$ versus control.

## 4. Discussion

In this study, we found that NPY in higher concentration (100 nM) significantly increased the incorporation of $^{3}$H-Leu into cardiomyocytes, although NPY in lower concentration (10 nM) did not result in distinct changes. It means that NPY enhanced protein synthesis of cardiomyocytes and higher concentration was required (Fig. 1). Moreover, early reaction hypertrophic gene, c-Jun mRNA expression in cardiomyocytes was promoted by NPY (Fig. 2). In previous studies, NPY was identified to relate to the cardiac hypertrophy in vivo. The enhanced NPY level in plasmas that were observed in human and animal suffering from cardiac hypertrophy or heart failure was co-relational with the degrees of hypertrophy [5]. Millar et al. [6] reported that NPY enhanced protein/DNA, RNA/DNA and incorporation of $^{14}$C-phenylalanine into cardiomyocytes in vitro. All the evidence mentioned above suggested that NPY could induce cardiac hypertrophy. As we know, NPY is an important factor contributing to hypertension by regulating blood vessel tone [3,4]. In hypertension, long-duration mechanical

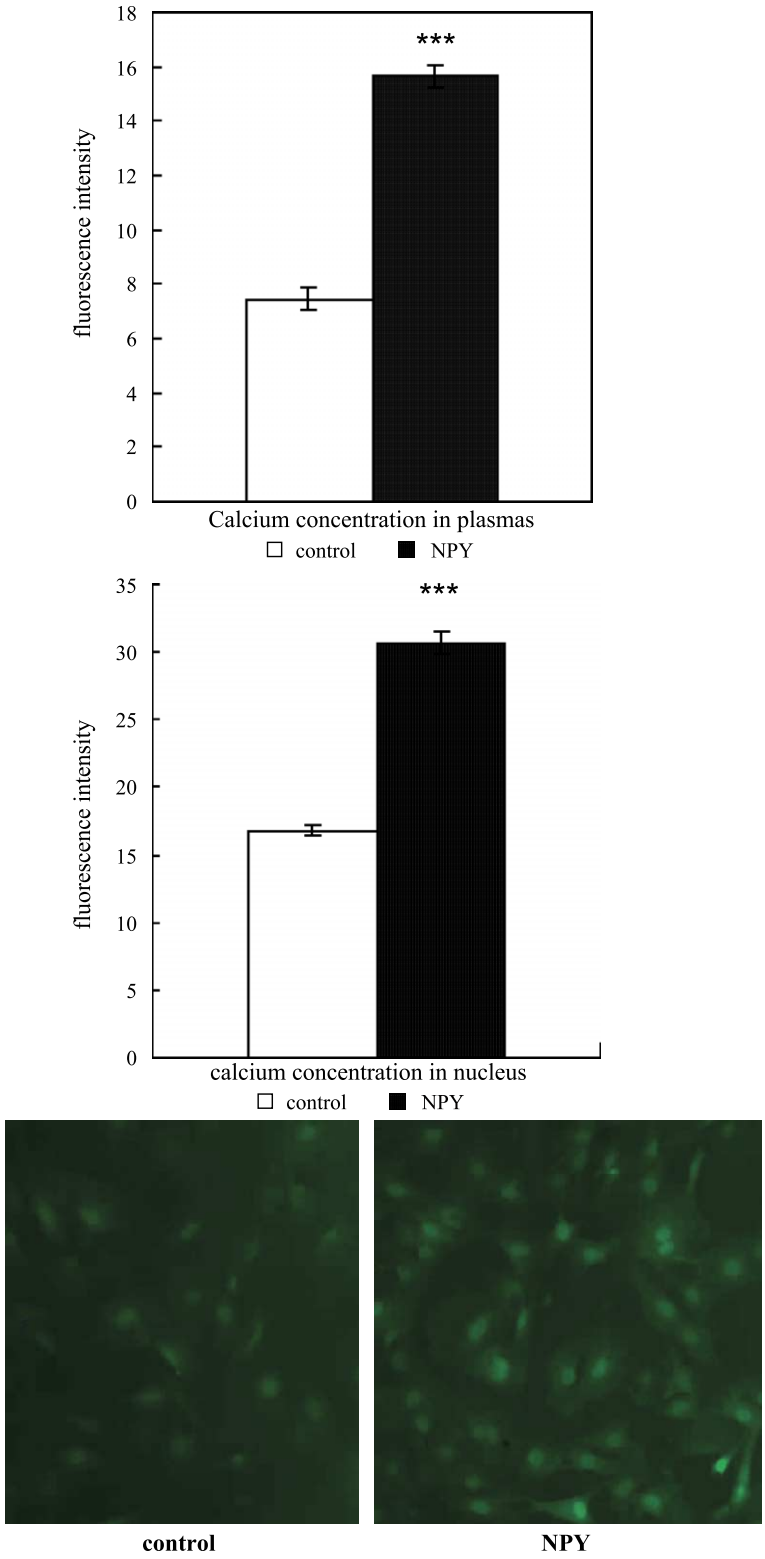


Fig. 5. The [$Ca^{2+}$] in plasma and nuclear of cardiomyocytes. The cells simulated by 100 nM NPY for 24 h were incubated with 10 umol/l fluo3-AM (Sigma) for 30 min at 37 °C. The [$Ca^{2+}$] in plasmas and nuclear of cardiomyocytes was measured by the fluorescence intensity in cells observed by a fluorescence microscope in 488 nm exciting wavelength and was analyzed with TILL Vision software. The data are mean±S.D. of four independent studies. *$P$<0.001 versus control.

Table 1
0, 5, 10 min after incubation with 100 nM NPY, the fluorescence intensity in cells reflecting the $[Ca^{2+}]$ in plasmas and nuclear of cardiomyocytes ($n$=4)

| | 0 min | 5 min | 10 min |
|---|---|---|---|
| The fluorescence intensity in plasmas of cardiomyocytes | 7.7775±0.859 | 7.5365±2.834 | 7.82225±2.233 |
| The fluorescence intensity in nuclear of cardiomyocytes | 16.4085±2.730 | 15.60825±3.460 | 14.39875±2.704 |

stress and neurohormonal factors including NPY, catecholamine, endothelin, angiotensin II and so on cause cardiac hypertrophy which is a beneficially adaptive response initially but lead to decompensation ultimately [1]. In this regard, NPY accompanies with hypertension all along as an important stimulating factor. Therefore, it is necessary to further study the mechanisms of NPY-induced hypertrophy in order to find new approaches treating cardiac hypertrophy.

CaN is uniquely activated by sustained elevation of $[Ca^{2+}]_i$. Nuclear factor of activated T cells (NFAT), a family of transcriptional regulators and an established target of the CaN signaling pathway, is dephosphorylated by CaN and subsequently translocates to nucleus [12]. In downstream of this signaling pathway, NFAT together with AP-1, C-MAF, $GATA_4$ and other nuclear transcription factors induce a wide variety of biological responses including lymphocyte activation, neuronal and muscle development, neurite outgrowth, and morphogenesis of vertebrate heart valves [19,20]. It was proved recently that CaN was sufficient to promote cardiac hypertrophy both in vivo and in vitro. Cardiac hypertrophy occurred in transgenic mice overexpressing a truncated constitutively active CaN-α and truncated NFATc4. Furthermore, inhibition of CaN prevents the development of cardiac hypertrophy in response to certain stimuli [13–15]. But different opinions on the role of the CaN pathway were also presented [21–23]. However, in this study, the data support previous studies showing CaN as a positive regulator of cardiomyocyte hypertrophy. CsA, a specific inhibitor of CaN, ameliorated significantly the enhancement in $^3$H-Leu incorporation into cardiomyocytes and c-Jun mRNA expression induced by NPY. It means that CaN signal pathway contributes to cardiac hypertrophy caused by NPY. Moreover, NPY activated effectively the CaN signal pathway in cardiomyocytes. CaN activity and CaN-α protein expression in cardiomyocytes was promoted significantly by NPY (100 nM) (Figs. 3 and 4). In addition, since CaN signaling associates with multiple signaling cascades such as PKC, ERK and JNK [2], other signal pathways may also be mediated due to activation of CaN by NPY. Indeed, we found the JNK target-c–JUN mRNA expression was promoted in cardiomyocytes stimulated by NPY, and significantly attenuated by CsA. JNKs are known regulators of cardiac hypertrophy in vivo [24], but it remains unclear whether JNK can be activated by NPY and interacts with CaN signal pathway to cardiac hypertrophy induced by NPY. Since high level of NPY in blood plasma and cardiac muscle are accompanied with hypertension all along, and NPY has been shown to have a significant effect on cardiac hypertrophy via CaN signal pathway in this study, we assumed that CaN is an important signal pathway to cardiac hypertrophy following hypertension.

Calcium is a fundamental regulator of various cellular functions. Calcium signaling in different location causes different pathways of gene transcription [25]. In previous studies, NPY stimulated the opening of L-type $Ca^{2+}$ channel and caused enhancement of cytosolic $Ca^{2+}$ concentration [10]. Furthermore, cardiac hypertrophy induced by NPY was blocked by L-type $Ca^{2+}$ channel inhibition [11]. In this work, lasting simulation of NPY elevated $Ca^{2+}$ concentration in cytoplasm and nucleus. It is indicated that stimulation of L-type $Ca^{2+}$ channel by NPY may contribute to enhancement of cytosolic $Ca^{2+}$ concentration and subsequent activation of CaN. The other $Ca^{2+}$ signaling in cytoplasm that affects cardiac hypertrophy due to NPY remains unknown.

Another vital signal pathways mediating cardiac hypertrophy is calcium-calmodulin-dependent protein kinases, which is controlled by calcium signaling in nucleus and appears to be a parallel pathway depending on calcium with CaN signaling [26–28]. Some studies showed that the δ isoform of CaM kinase II, the predominant form of CaM kinase in the heart, was necessary and sufficient to cause hypertrophy in vitro [27]. In addition, CaM kinase IV is shown to be sufficient to induce cardiac hypertrophy in transgenic mice overexpressing a constitutively active form of CaM kinase IV specifically in the heart [28]. It remains unclear whether CaM kinase pathway is activated by enhancement of nuclear $Ca^{2+}$ concentration due to NPY.

In summary, we directly identified that NPY was an important factor inducing cardiac hypertrophy in vitro. CaN signaling is necessary for cardiac hypertrophy in response to the stimulation of NPY. The increase of $[Ca^{2+}]$ in cytoplasm may be causative in activation of CaN. The increase of $[Ca^{2+}]$ in nuclear may activate other signal pathways to cardiac hypertrophy.

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