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BNIP3 induces IL6 and calcineurin/NFAT3 hypertrophic-related pathways in H9c2 cardiomyoblast cells

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Abstract Ischemia/reperfusion injury causes cardiomyocyte apoptosis, ventricular remodeling, leading to a dilated heart. Hypoxia is one of the causes involved in ischemia damage, and BNIP3 is a hypoxia-inducible marker and also a sensor to induce mitochondria-dependent apoptosis. Recent reports discussed ablating BNIP3 can restrain cardiomyocytes apoptosis and post-infarction remodeling. BNIP3 is a crucial therapeutic target. However, the BNIP3-induced hypertrophy aspect is rarely investigated. Here, we transiently transfected BNIP3 plasmids into H9c2 cardiomyoblast cells to evaluate the molecular signaling and hypertrophy markers using Western blot. We measured the cell size change using actin staining. We disclose that BNIP3 overexpression induced an increase in cell size, activated the pathological-related hypertrophy signaling pathways, such as IL6-MEK5-ERK5, IL6-JAK2-STAT1/3, calcineurin/NFAT3 and p38β MAPK resulting in the fetal genes, ANP and BNP expressing. Concluding above, BNIP3 acts as a pathological hypertrophy inducer, which might be a potential therapeutic target for heart damage prevention.

Keywords BNIP3 · Hypertrophy · NFAT3 · IL6 · H9c2 cardiomyoblast cells

Abbreviations

ANP	Atrial natriuretic peptide
BNP	B-type natriuretic peptide
ERK5	Extracellular-regulated kinase 5
IL6	Interleukin-6
JAK2	Janus kinase 2

Dennis Jine-Yuan Hsieh, Chih-Yang Huang, and Jin-Ming Hwang share equal contribution.

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β-MHC	β-Myosin heavy chain
MAPK	Mitogen-activated protein kinase
NFAT3	Nuclear factor of activated T cells 3
STAT1/3	Signal transducers and activators of transcription-1,-3

Introduction

Ischemic heart diseases are complicated by many pathological progressions, such as cardiomyocytes slippage, hypertrophy, cardiac architecture change which compensate for the loss of systolic function, and decompensate to the dilated ventricular, concluding in a term ventricular remodeling, and finally resulting in heart failure [1]. Hypertrophy is an epidemiological independent risk for cardiac morbidity [2] therefore investigating hypertrophy progression is important in hypoxic or ischemic heart disease.

Cardiac hypertrophy is an adaptive effect for increased heart work load under stimulation. The cells appear to increase cell size and sarcomere reorganization increases contraction function [3]. Multiple stimuli from mechanical, hemodynamic, hormonal, and pathological stimuli cause hypertrophy. Cardiac hypertrophy includes two subtypes: growth induced by physiological stimulations (e.g., IGF-1 and growth hormone), called physiological hypertrophy and growth induced by pathologic stimuli, called pathological hypertrophy [4]. Several hormones such as norepinephrine and phenylephrine bind to the α1-adrenergic receptor, angiotensin II binds to the AT-1 receptor, endothelin-1 binds to the ET-1 receptor [5], and IGF-2 binds to the IGF-2R [6] coupled to Gαq to stimulate pathological hypertrophy. They can activate the Gαq/Ca^{2+}-mediated signaling to the calcineurin/nuclear factor of activated T cells (calcineurin/NFAT3) pathway, which in turn activates the NFAT3 to translocate into the nucleus and coactivate with phosphorylated GATA4 to transcribe hypertrophic response genes such as ANP and BNP [7, 8]. IL6 was reported to cause cardiac hypertrophy through the IL6 signal transducing receptor component, glycoprotein 130 (gp130) [9]. IL6 triggers gp130 dimerizing, leading to phosphorylation and activation of JAK2 and consequent recruitment of STAT3. The activated STAT3 forms homodimer or heterodimer with STAT1 to transcribe the target genes [10, 11]. In addition, chronically phosphorylated STAT1/3 was presented in the dilated cardiomyopathy. The STAT3-dependent pathway was reported to promote cardiac myocyte hypertrophy [12, 13]. The ERK5 pathway was also induced by gp130 and played a crucial role in eccentric hypertrophy. MEK5-ERK5 signaling was triggered by IL6 induced rapidly decompensating eccentric cardiac hypertrophy that progressed to dilated cardiomyopathy and sudden death [14]. The MAPK p38β was found to mediate cardiac hypertrophy in pressure overload model [15], evidenced in the increase of myocardial cell growth and ANP expression [16].

The hypoxia marker, BNIP3 (Bcl-2/adenovirus E1B nineteen-kDa interacting protein 3), is transcriptionally upregulated by HIF-1α during hypoxia. It is a member of pro-apoptotic BH3-only subfamily of Bcl-2 family [17, 18]. BNIP3 has a BH3 domain which functions to bind with Bcl-2 or Bcl-X_L, and possesses a C-terminal transmembrane domain for targeting to mitochondria and promoting cell apoptosis [19–21]. BNIP3 insertion results in the depolarization and opening of mitochondrial permeability transition pores (MPTP), consequently, pathological cell death occurs via apoptosis [22], necrosis [23, 24], and autophagic cell death [25, 26]. Evidences reveal that post-infarction remodeling after ischemia/reperfusion causes cardiomyocytes slippage, cardiac dilating, and in turn cardiac dropout [1]. The hypoxia-induced marker, BNIP3, was defined as the major factor to cause cardiomyocyte apoptosis and ventricular remodeling in various ischemic heart diseases [22]. BNIP3 abolishment is an efficient strategy to salvage heart from remodeling [27]. However, the BNIP3-induced molecular regulations related to hypertrophy are still unclear. In the present study, we showed that the activation of pathological hypertrophic IL6-MEK5-ERK5, IL6-JAK2-STAT3 and calcineurin/NFAT3, p38MAPK pathways, increased fetal gene ANP and BNP expression, and myocardial cell enlargement revealed that BNIP3 is a strong pathological hypertrophy inducer.

Materials and methods

Cell culture and transfection

H9c2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) and 1.5 g/l sodium bicarbonate, incubated at 37°C in 5% CO_2 incubator. Full lengths of BNIP3 open reading frame were cloned and inserted into *Bam*HI site of pcDNA3-HA for BNIP3 protein expression. BNIP3 plasmids were prepared using AxyPrep™ Plasmid Maxiprep kit (Axygen biosciences, CA, USA) and transfected into the cells using GeneJuice® transfection reagent (Novagen, WI, USA) according to the manufacturer's guidelines. After 6 h, the cells were fed with fresh medium and harvested at experimental time point as indicated.

Western blot

The cell lysate proteins were separated using 12% SDS-PAGE and transferred to PVDF membranes. Residual protein sites were blocked in Tween/Tris-buffer saline (TBS) containing 5% skim milk. The filters were incubated with 1:1000 diluted primary antibody p38β, IL6, p-JAK2, STAT-1, STAT-3, BNP, p-GATA4, NFATc3, Gαq, α-tubulin (Santa Cruz, CA, USA), p-ERK5, ERK5 (Upstate, MA, USA), ANP (Abcam, Cambridge, UK), BNIP3 (Cell signaling, MA, USA) in TBS plus 2.5% skim milk at the recommended concentrations at 4°C overnight and incubated with secondary antibodies for 1 h at room temperature. Antibody reaction was visualized with enhanced chemiluminescence (ECL) reagent (Millipore, MA, USA).

Actin stain

H9c2 cells were fixed by 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 1% BSA in phosphate buffer saline (PBS) for 10 min, respectively. Actin filaments were stained by using rhodamine-labeled phalloidin (red) for 40 min and washed by PBS trice between each step. Then cell nuclei were stained by DAPI (blue). Cells were examined and photographed using fluorescence microscope, the cells' area was measured using Carl Zesis Axio vision LE software. More than 10 fields for each condition were quantified, triple independent experiments were performed.

Statistical analysis

Each experiment was triple repeated and results were presented as the mean ± SEM, and statistical comparisons were made using the Student's t test. Significance was defined at the $P < 0.05$ level.

Results

Expression of BNIP3 induces hypertrophic growth in H9c2 myocardiac cells

Hypertrophy is an independent risk involved in cardiac remodeling and heart failure. To investigate whether

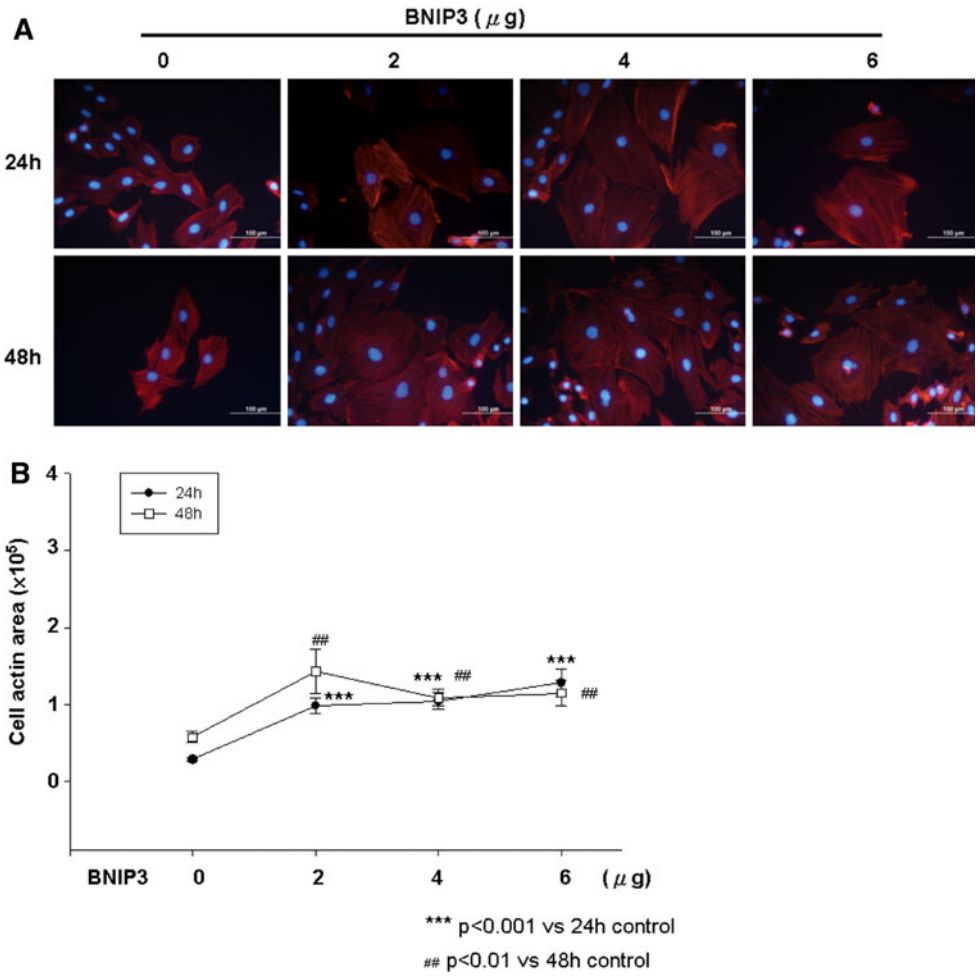

Fig. 1 Effect of BNIP3 on actin organization in H9c2 myocardial cells. **a** Hypertrophic growth and actin organization. BNIP3 were transfected into cells in dosage- and time-dependent manners as indicated. The cells were then fixed and stained with rhodamine-conjugated phalloidin to detect the actin filaments. **b** Quantitative results of cell area. The values represent the mean ± SE from at least 10 fields in each group. The increase in size induced by BNIP3 is highly significant ($P < 0.001$)

BNIP3 induces the hypertrophy effect, we transfected wild-type BNIP3 into H9c2 cells using a dose- and time-dependent manners and stained actin filaments to observe the changes in cell size. Results showed that the cells became larger with the increase in BNIP3 dosage after 24 h transfection. The cells became even larger after 48 h with 2 μg BNIP3 transfection (Fig. 1).

BNIP3 overexpression induces hypertrophic signaling activation and ANP/BNP protein induction

To explore whether BNIP3 activates the hypertrophy signaling pathways, BNIP3 was transfected into cells at a time-dependent manner and cells were harvested for Western blot analysis. The transcription factors, NFAT3, regulating some hypertrophy genes were activated with the increase of BNIP3 expression. Following that, hypertrophy markers, ANP and BNP, were upregulated after NFAT3 activation, but the upstream Gαq has no change (Fig. 2). It suggests that BNIP3-induced calcineurin/NFAT3 activation and enhanced the downstream target genes, ANP and BNP expressions, to perform the hypertrophy effect. As we know p38 MAPK is another hypertrophic mediator to activate GATA4, here we showed the increase of p38β and phosphorylated GATA4 with BNIP3 treatment, explaining the participation of BNIP3 in hypertrophy also through p38β MAPK signaling pathway (Fig. 3). Additionally, we further examine the IL6-related pathways. The IL6 highly expressed with BNIP3 stimulation, and the downstream kinase, JAK2, was in turn activated, and the transcription factor STAT1/3 protein levels were upregulated as well (Fig. 4b). It suggests that BNIP3 upregulated the IL6-JAK2-STAT3 signaling to enhance hypertrophy. As the eccentric hypertrophy signaling, we observed phosphorylated ERK5 were elevated with BNIP3 increase. The tendency of p-ERK5 were synchronizing with IL6 expression (Fig. 4a). This result suggests the eccentric hypertrophy signal, IL6-MEK5-ERK5, was also participated in BNIP3-induced hypertrophy effect. Taken together, we suggest BNIP3 induces hypertrophy via calcineurin/NFAT3, p38-GATA4, IL6-MEK5-ERK5, and IL6-JAK2-STAT3 signal pathways

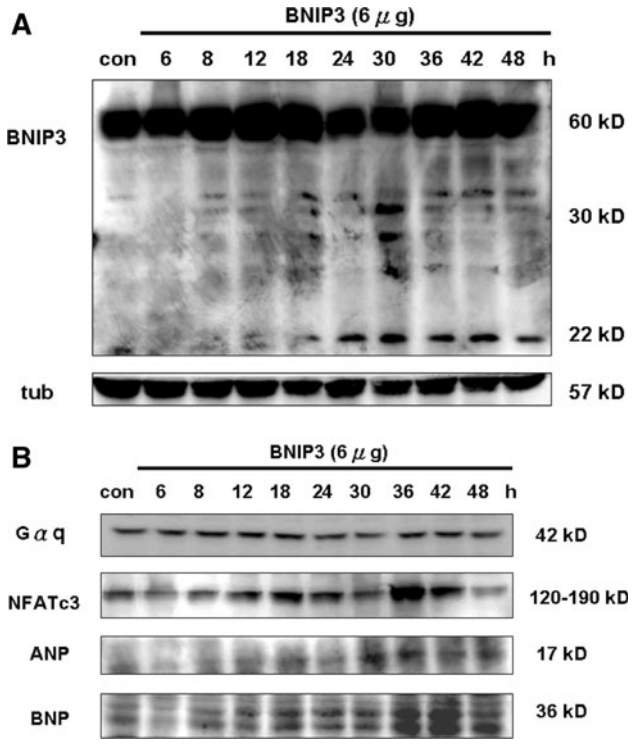

Fig. 2 Activation of calcineurin/NFAT3 signaling pathway in BNIP3 overexpression. BNIP3 (6 μg) were transfected into cells and harvested in time-dependent manner for Western blot analysis. **a** Change of BNIP3 protein level. **b** Changes of NFAT3, ANP and BNP protein levels

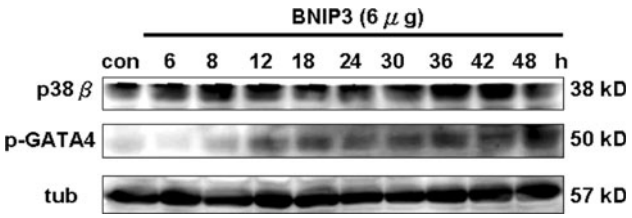

Fig. 3 Effect of BNIP3 on p38β-GATA4 signaling pathway in H9c2 myocardial cells. BNIP3 (6 μg) were transfected into cells and harvested in time-dependent manner for Western blot analysis. Changes of p38β and phosphorylated GATA4 protein levels

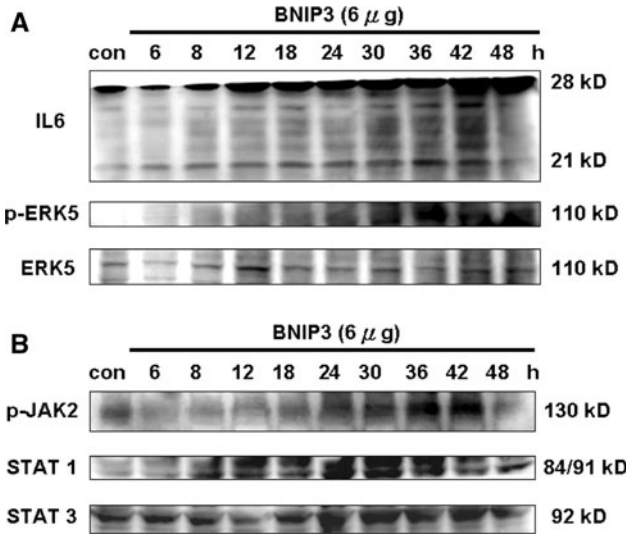

Fig. 4 Induction of IL6-related signaling pathway in BNIP3 overexpression. BNIP3 (6 μg) were transfected into cells and harvested in time-dependent manner for Western blot analysis. **a** Changes of IL6, phosphorylated ERK5 and ERK5 protein levels. **b** Changes of phosphorylated JAK2, STAT1, and STAT3 protein levels

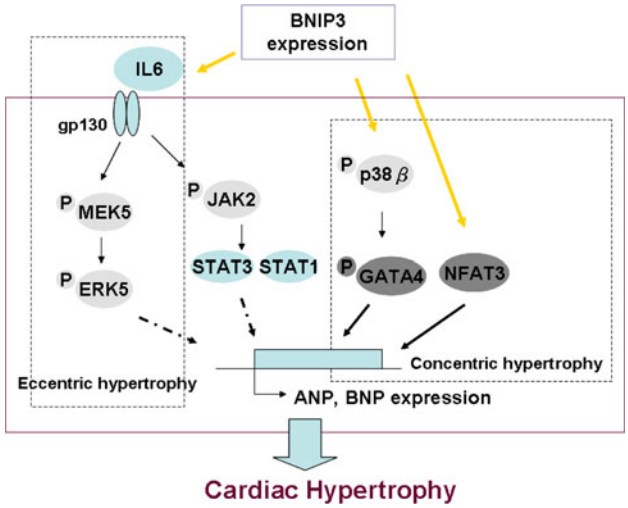

Fig. 5 Working hypothesis. Myocardial cell hypertrophy caused by BNIP3 expression is associated with the increase of calcineurin/NFAT3, p38-GATA4, IL6-MEK5-ERK5, and IL6-JAK2-STAT3 signaling pathways. At the same time, the cardiac hypertrophic markers, ANP and BNP, were highly expressed

to further elevate ANP and BNP expression and cell enlargement (Fig. 5).

Discussion

Major findings

The data presented here showed that BNIP3 plays a role in stimulating hypertrophy in cultured H9c2 myocardial cells. The specific evidences are: first, expression of BNIP3-induced actin filaments and hypertrophic growth (Fig. 1). Second, BNIP3-induced activation of pathological hypertrophic signallings such as IL6-MEK5-ERK5, JAK2-STAT1/3, calcineurin/NFAT, p38-GATA4 and resulted in hypertrophic markers, ANP and BNP increase. All of these data indicated that BNIP3 might play multiple roles in the pathological hypertrophy of post-infarction remodeling.

In the researches of ischemic heart disease, BNIP3 could be the most dominant therapeutic target. Recently, reports showed that abolition of BNIP3 restrains post-infarctional remodeling, BNIP3 obviously a major determinant of ventricular remodeling [27] and could be an apoptosis inducer. However, BNIP3 has not yet been found as the cardiac hypertrophy inducer. Although hypoxia was shown to be potential inducer of cardiac hypertrophy and BNIP3 is a hypoxic marker, there is still no linkage between BNIP3 and hypertrophy. However, we found out that BNIP3 induced the related hypertrophic signallings and enhanced hypertrophic growth. All these evidences link up BNIP3-induced cardiac hypertrophy and remodeling.

ANP, BNP, cell enlargement

The specific changes in cardiac hypertrophy in cardiomyocytes include quantitative and qualitative changes of gene expression. Several sarcomeric genes are expressed as the fetal isoforms such as skeletal α-actin, β-MHC [28] and re-expressed the fetal genes, ANP and BNP [29, 30]. In the present study, high ANP and BNP expressions were observed following BNIP3 increase. The cell size increased with the increase in BNIP3 dosage in 24 h and at 48 h showed significant enlargement. We even observed that some cells shrank and became fragile after 48 h of BNIP3 expression. Thus, we speculate that apoptosis or necrosis induced by BNIP3 is accompanied with hypertrophy occurring after long time treatment.

Calcineurin/NFAT signaling activation

Phosphatase 2B, calcineurin, is among the best established mediators of hypertrophy [31], and the activation of NFAT3 was also examined. We showed here that NFAT3 increased with BNIP3 expression. This suggests that BNIP3 induces hypertrophy through activating the calcium-dependent signaling, calcineurin/NFAT3. BNIP3 targets the mitochondria and also endoplasmic reticulum (ER), which is a primary calcium intracellular storage site. Once BNIP3 targets the ER, calcium will be released into the cytosol and be taken up by juxtaposed mitochondria [32]. We speculate that BNIP3 induces calcium release to make cytosolic calcium increase, therefore the calcineurin was activated in this situation, without a significant change in the Gαq protein level.

P38β-GATA4 signaling activation

P38 has also been mentioned involved in pathogen-induced hypertrophy of cardiomyocyte [33]. Constitutive activation of p38 stimulated the expression of ANP [34] and p38 imparts BNP gene induction through direct modification of GATA4 [35]. We showed that p38β expression and phosphorylated GATA4 increase within BNIP3 expression, and these results also reveal the occurrence of hypertrophy.

IL6-related pathways: MEK5-ERK5 and JAK2-STAT3

Cytokine IL6, a potent hypertrophic effecter, is produced by immune cells and also by cardiovascular components, such as endothelial cells, vascular smooth muscle cells, and ischemic myocytes [36–39], stimulating heart by autocrine and paracrine fashions [9]. We showed that IL6 highly expressed with BNIP3 increasing, indicating that BNIP3 might induce IL6 secretion by myocardial cells and affect themselves to perform hypertrophy. Long-term intermittent

hypoxia causes decompensating to eccentric cardiac hypertrophy via activating the IL6-related MEK5-ERK5 signaling [40]. We further showed that the ERK5 activation indicated the morphological change toward eccentric hypertrophy. Another IL6-induced pathway, JAK2-STAT3, was also activated simultaneously with IL6 expression. These hypertrophy-related molecular signals confirmed the morphological changes.

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