[Frontiers in Bioscience, Landmark, 21, 502-513, January 1, 2016]

Phenylephrine promotes cardiac fibroblast proliferation through calcineurin-NFAT pathway

Jing Wang[12], Yibing Wang[3], Wei Zhang[2,4], Xi Zhao[2], Xiangfan Chen[2], Wenyan Xiao[2], Lingling Zhang[2], Yunxuan Chen[2], Weizhong Zhu[2,4]

[1]Department of Pharmacy, Nantong University Affiliated Hospital, Nantong, P. R. China, [2]Department of Pharmacology, Nantong University School of Pharmacy, Nantong, P. R. China, [3]Department of Pharmacy, Yan Cheng Institute of Health Science, [4]Key lab of inflammation and drug target, Jiangsu province, Nantong University, Nantong, P. R. China

TABLE OF CONTENTS

1. ABSTRACT

Ca^{2+}/calmodulin-dependent calcineurin (CaN) plays an important role in various Ca^{2+} signaling pathways, among which are those involved in cardiac diseases. It has also been shown that a heightened sympathetic tone accelerates the development of heart failure. The present study investigates whether the CaN-mediated nuclear factor of activated T-cells (NFAT) pathway is involved in cultured neonatal rat cardiac fibroblast proliferation induced by phenylephrine. CF proliferation was assessed by a cell survival assay and cell counts. Green fluorescent protein-tagged NFAT3 was used to determine the cellular location of NFAT3. CaN activity and protein levels were also determined by an activity assay kit and Western blotting, respectively. Results showed that phenylephrine promoted CF proliferation, which was abolished by α_1-adrenergic receptor antagonist (prazosin), a blocker of Ca^{2+} influx (nifedipine), an intracellular Ca^{2+} buffer (BAPTA-AM), CaN inhibitors (cyclosporin A and FK506), and over-expression of dominant negative CaN. Phenylephrine activated CaN and evoked NFAT3 nuclear translocation, both of which were blocked by cyclosporine A (CsA) or over-expression of dominant negative CaN. These results suggest that the Ca^{2+}/CaN/NFAT pathway mediates PE-induced CF proliferation, and this pathway might be a possible therapeutic target in cardiac fibrosis.

2. INTRODUCTION

Cardiac fibrosis, in response to hemodynamic or neurohormonal stress, is a hallmark feature of pathologic remodeling of the heart, leading to the progressive deterioration seen in heart failure. Fibrosis is marked by excessive accumulation of extracellular matrix (ECM) proteins and proliferation of cardiac fibroblasts (CFs), which are the major cardiac non-myocytes and are responsible for synthesis and excessive deposition of collagen (1,2). Therefore, limiting these two parameters may be a useful therapeutic approach to reducing pathological myocardial fibrosis.

Calcineurin (CaN), a serine-threonine phosphatase activated by Ca^{2+}-calmodulin, participates in signaling pathways which are important for gene regulation and external signal-mediated biological responses in various organisms and many types of cells (3). Accumulating evidence demonstrates that the signaling pathway mediated through CaN/NFAT (nuclear factor of activated T-cells) plays a pivotal role in cardiac and skeletal muscle hypertrophy and vascular development (4-6).

Heightened sympathetic tone is known to accelerate development of lethal arrhythmias after myocardial infarction (MI) (7) and congestive heart failure (HF) (8). Increased catecholamines induce myocardial hypertrophy, which is associated with increased protein synthesis by myocytes, induction of the fetal gene program, and development of interstitial fibrosis (9-12). In cultured cardiac myocytes, phenylephrine (PE, a synthetic catecholamine), causes cardiac myocyte hypertrophy and fetal gene expression primarily via alpha$_1$-adrenergic receptor (α_1-AR) activation (13). In cultured CFs, PE has been shown to induce cell proliferation (14), increase metalloproteinase-9 (MMP-9) activity and collagen production (15); however, the mechanism is not well studied. The function of α_1-AR subtypes in the vasculature has been reported - *in vitro* studies indicate that α_1-AR stimulation mediates cell growth and proliferation, as well as migration of vascular smooth muscle cells (16-18). Although the growth/proliferation effects of α_1-AR have been investigated in depth in vascular smooth muscle cells (19-21), there is new evidence that indicates α_1-ARs in surrounding fibroblasts may participate in cardiac remodeling events (22). Thus, we hypothesize that the proliferation of CFs via α_1-AR plays a role in the development of cardiac fibrosis, which strongly contributes to initiation and progression of heart failure. Thus, the present study aims to investigate whether the CaN/NFAT pathway is involved in the PE-induced CF proliferation. If so, the CaN/NFAT pathway may be a potential, attractive, therapeutic target for clinical treatment of myocardial fibrosis.

3. MATERIALS AND METHODS

3.1. Materials and reagents

Neonatal Sprague Dawley rats were obtained from the Animal Center of Nantong University (Nantong, China). All protocols were approved by The Board of Nantong University Animal Care and Use. Dulbecco's Modified Eagle's Medium (DMEM), methylthiazolyldiphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Calbiochem (LaJolla, CA, USA). PE, prazosin, nifedipine and BAPTA-AM were purchased from Sigma-Aldrich (St. Louis, MI, USA). Cyclosporin A (CsA) was obtained from Invitrogen Corp (Carlsbad, CA, USA). The hydroxyproline assay kit was purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The CaN assay kit was manufactured by Genmed Scientifics Inc. U.S.A (Boston, USA). Mouse anti-rat fibronectin, mouse anti-rat GAPDH and mouse polyclonal anti-CaN antibodies, streptavidin-biotin-peroxidase complex (SABC) immunocytochemistry kit, diaminobenzidine (DAB) kit, and BCA Protein Assay Kit were purchased from Boster Biological Technology (Wuhan, China).

3.2. Cell culture and adenoviral infection

Neonatal rat CFs were prepared from hearts of 3 to 5 day-old Sprague-Dawley rats by the differential attachment technique. Intact hearts were removed and immediately placed in ice-cold phosphate-buffered saline. Ventricles were minced, pooled, and digested in 0.25% trypsin. The cells obtained were centrifuged and re-suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cell mixture was plated for 90 min in a 5% CO_2 containing incubator at 37^{o}C to harvest CFs. After removal of the myocyte-enriched medium, fresh DMEM was added to the pre-plated CFs, which were subsequently cultured for 3-4 d before being passaged. Experiments were performed with cells in passage 3. Following 24 h of incubation with serum-free medium, fibroblasts were infected with adenovirus containing GFP-tagged NFAT3 and dominant negative rat CaN (CaN-DN), kindly provided by Dr. Xiongwen Chen, Temple University School of Medicine) at a multiplicity of infection of 100. After adenovirus infection for 24 h, culture medium was added with or without PE (10 μmol/L) in the presence or absence of CsA (0.5 μg/mL) for 48 h.

3.3. Immunohistochemistry

Paraformaldehyde-fixed CFs were analyzed by immunohistochemistry using an SABC kit according to the manufacturer's directions. CFs were incubated with primary antibody for 1 h at 37°C, followed by incubation with secondary antibodies (biotinylated goat anti-mouse immunoglobulin G) for 1 h at room temperature.

3.4. Cell proliferation assay

A colorimetric nonradioactive methyl thiazolyl tetrazolium (MTT) proliferation assay, as described in a previous study (23) was used. CFs (5×10^3 per well) were seeded in 96-well plates, after which they were incubated with serum-free DMEM for 24 h and then treated under various study conditions for 48 h. Thereafter, MTT (5 μg/mL) was added to each well, and plates were incubated at 37 °C for 4-6 h. The medium was then replaced with 150 μl DMSO and agitated for 10 min. Absorbance at 490 nm was measured using a microplate reader (Dynex Technology, Chantilly, VA, USA).

3.5. Cell counting

CFs (2.0×10^5 per well) were seeded in 12-well plates and treated under various study conditions for 48 h after starvation overnight. Thereafter, the cells were digested using 0.25% trypsin for 6-8 min at 37°C. Cell numbers were counted using a blood cell counting chamber.

3.6. Cell cycle analysis

To determine cell cycle distribution, cells (1×10^6/well) were plated in a 60-mm dish and then treated with or without PE in the presence or absence of CsA for 24 h. Cells were then harvested and fixed in 70% ethanol and stored at -20°C overnight. Following two washes with phosphate buffered saline (PBS), fixed cells were incubated in RNase (10 μg/mL) at 37°C for 30 min. The DNA was stained with propidium iodide (10 μg/mL) at 4°C for 30 min in the dark and then analyzed by a Coulter Epics XL-MCL (Becton Dickinson, USA).

3.7. Hydroxyproline assay

Cells were seeded in 12-well plates at 2.5×10^5 cells/well and allowed to attach for 24 h. After treatment with previously mentioned reagents for 24 h, the supernatant was collected. Hydroxyproline concentration in the culture medium was measured using a hydroxyproline assay kit (Nanjing, China) according to manufacturer instructions. Results were expressed as μg/mg total protein in the medium.

3.8. CaN phosphatase activity

CaN phosphatase activity was measured using a CaN cellular activity assay kit according to the manufacturer's directions. The RII phosphopeptide was used as a highly specific substrate for CaN. The detection of free inorganic phosphate released from RII by CaN was based on malachite green dye reaction. After incubation at 30°C for 20 min, the reactions were terminated, and the optical density at 660 nm was determined for each sample on a microplate reader. The amount of free phosphate was determined by using a phosphate standard curve. The activity was then corrected for protein concentration. A final value was expressed as nmol phosphate min/mg protein.

3.9. Western Blot Analysis of CaN

After various treatment conditions, cells were lysed with lysis buffer (mmol/L: 20 Tris-HCl, pH 7.5., 150 NaCl, 1 Na_2EDTA, 1 EGTA, 1% Triton, 2.5. sodium pyrophosphate, 1 beta glycerophosphate, 1 Na_3VO_4, 1 μg/ml leupeptin) and sonicated, followed by centrifugation at 14,000 rpm at 4 °C for 10 min to retrieve the supernatant. Protein concentration was measured using a BCA assay. Equal amounts of protein at 20 μg per lane were run on 10% SDS-PAGE and electrophoretically transferred on to a PDVF membrane. The membrane was incubated with mouse polyclonal anti-CaN or anti-GAPDH antibodies at a dilution of 1:500 in 5% skim milk in PBS at 4 °C overnight. After washing 3 times in PBS/Tween-20, the membranes were incubated with goat anti-mouse fluorescent antibody (1:5000) at room temperature for 60 min. Following four washes in PBS/Tween-20 and one wash in PBS alone, immunolabeled marker protein bands on the membranes were scanned and analyzed by a far infrared fluorescent image scanner and analyzer (Odyssey, U.S.A.).

3.10. Dynamic measurements of GFP-NFAT3

After infection with GFP-NFAT3 adenovirus for 24 h, cells were treated with or without PE (10 μmol/L) in the presence or absence of CsA (0.5 μg/mL). GFP-NFAT3 signal was measured by fluorescence microscopy (Leica, German) at the indicated time. Subcellular distribution of GFP-NFAT3 was quantized as the NFAT nuc/NFAT cyto using a region of interest (ROI) that covered the area of the nucleus (NFAT nuc) or cytoplasm (NFAT cyto) of the same size (number of pixels). Nuclear location of NFAT-GFP was confirmed only if the ratio of NFAT-GFP nuc to NFAT-GFP cyto was more than 3 fold. Similarly, cytosolic location of NFAT-GFP was defined only if the ratio of NFAT-GFP cyto to NFAT-GFP nuc pixels was greater than 3 fold. Image-J software was used for analysis, with the intensity of the regions of interest normalized to area.

3.11. Statistics

The results are expressed as means ± S.E.M. All data were analyzed with SPSS 11.5. One-way analysis of variance (ANOVA), repeated one-way ANOVA, post-hoc analysis and the Newman-Keuls test were used to compare differences among groups. Differences were considered statistically significant at $P < 0.05$.

4. RESULTS

4.1. α_1-adrenergic receptor mediated CF proliferation in a dose dependent manner

Figure 1A shows a representative image of cells stained with anti-fibronectin. Results indicate that 10 μmol/L of PE enhanced cell density. PE stimulated cell proliferation in a dose-dependent manner in cultured CFs, as shown by cell viability and cell number (Figure 1B, 1C). Although 0.1 μmol/L of PE had no

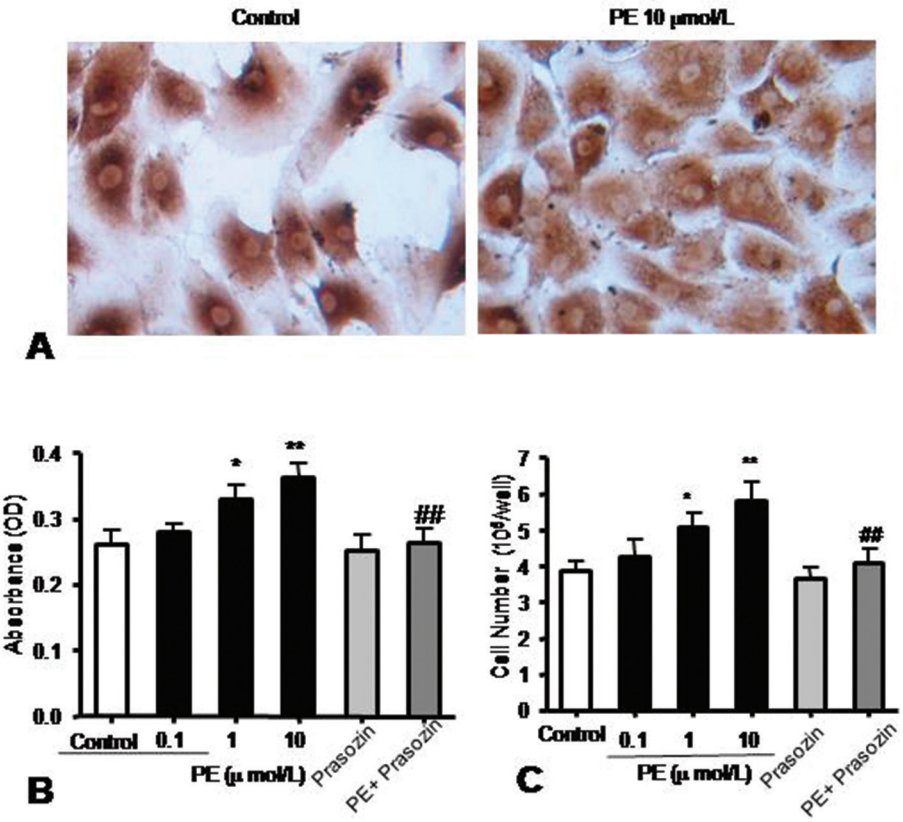

Figure 1. α_1-adrenergic receptor mediated the PE-induced proliferation of CFs. (A) Representative immunohistochemistry staining demonstrating CF purification and density. Cells were starved overnight and further incubated with or without 10 μmol/L of PE for 48 h. Cells were fixated and stained with anti-fibronectin. (B, C) Growth-arrested CFs were stimulated with various concentrations of PE (0.1., 1, 10 μmol/L) and 10 μmol/L of PE in the presence of 0.1. μmol/L prazosin for 48 h. Optical density with MTT labeling (B) and cell numbers (C) were measured. The average data were expressed as mean ± S.E.M. of 6 individual experiments. $^{*}P$ <0.0.5, $^{**}P$ <0.0.1 vs control, $^{\#\#}P$ <0.0.1 vs PE alone.

obvious effect on CF proliferation, 1 and 10 μmol/L of PE increased the absorbance of CFs by 27.5%, 40.8%, respectively, compared with the control group. Similarly, 1 and 10 μmol/L of PE increased CF numbers by 30.7% and 49.7%, respectively, compared with the control group. More importantly, PE-induced cell growth was blocked by 10 μmol/L of prazosin by 93.8 ± 9.7% and 95.1 ± 9.6%, respectively, while prazosin alone had no effect on cell growth (Figure 1B, 1C).

4.2. Blocking of Calcium influx and buffering of intracellular calcium suppressed PE-induced CF proliferation

To further demonstrate the role of calcium signaling in PE-induced cell proliferation, effects of the L-type voltage sensitive calcium-channel blocker nifedipine and the intracellular calcium-trapping drug BAPTA-AM on cellular proliferation were observed. Both nifedipine at 1 μmol/L and BAPTA-AM at 5 μmol/L exhibited significant inhibitory effects on PE-induced cell proliferation (Figure 2A, 2B) while neither nifedipine nor BAPTA-AM alone had an effect. These data indicate that both the influx of extracellular Ca^{2+} and release of Ca^{2+} from intracellular stores might be involved in PE-induced CF proliferation.

4.3. Inhibition of CaN by pharmacological approaches and mutation of CaN suppressed PE-induced CF proliferation

CsA, an efficient CaN inhibitor, was used to determine whether the CaN signaling pathway contributes to CF proliferation induced by PE. Cells were starved overnight and then incubated with 10 μmol/L of PE in the presence or absence of various doses of CsA for 48 h. It was seen that CsA decreased the PE-induced cell proliferation in a dose-dependent manner, as evidenced by MTT and cell number, while 0.5 μg/mL CsA alone had no significant effect (Figure 3A, 3B). To evaluate the relative magnitude of CF proliferation by PE and the inhibitory effects of CsA at different times point, growth-arrested CFs were stimulated with 10 μmol/L of PE in the presence or absence of CsA (0.5 μg/mL) for 12, 24, and 48 h. CFs exhibited significant growth at 24 and 48 h after exposure to PE. More importantly, CsA significantly inhibited PE-induced proliferation of CFs at the indicated time (Figure 3C). The conclusion that CaN is involved in

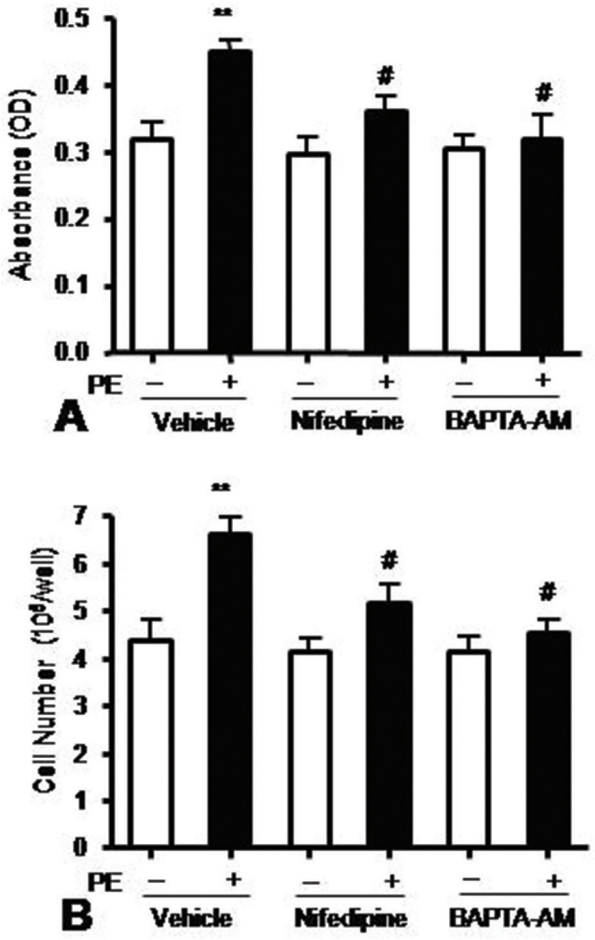

Figure 2. Intracellular and extracellular calcium were necessary for PE-induced CF proliferation. The starved cells were pre-incubated with nifedipine (1 μmol/L) or BAPTA-AM (5 μmol/L) for 30 min and further stimulated with PE (10 μmol/L) for 48 h. Optical density (A) and cell numbers (B) were measured. The average data were expressed as mean ± S.E.M. of 6 individual experiments. $^{**}P < 0.0.1$ vs control, $^{\#}P < 0.0.5$, vs PE alone.

CF proliferation was confirmed by use of another inhibitor of CaN, FK506 (1 μg/ml) (Figure 3D, 3E).

To confirm the pharmacological results above, enforced expression of dominant negative CaN (CaN-DN) was performed to manipulate the activation of CaN. The expression of CaN-DN was enhanced by 2.54 fold over the β-galactoside control after cells were infected with adenovirus containing CaN-DN (Figure 4A). Overexpression of CaN-DN markedly decreased the activation of CaN induced by PE stimulation for 30 min (Figure 4B), as well as significantly suppressed CF proliferation (Figure 4C, 4D).

4.4. Effects of CsA on the progression of CF cell cycle and hydroxyproline production induced by PE

To determine whether the growth-inhibitory effect of CsA was associated with perturbation of cell cycle, flow cytometric analysis was used. Flow cytometric analysis (Figure 5A, 5B) revealed that 24 h of serum deprivation caused an obvious suppression of cell-cycle progression, with approximately 81.6 ± 1.7% of the cells arrested in the G_0/G_1 phase. After stimulation with PE, the percentage of CFs in the G_0/G_1 phase was decreased to 68.1 ± 2.5% (P <0.0.5, n=4), whereas those entering the S phase increased from 6.9 ± 1.5% to 19.2. ± 2.0.% (P <0.0.5, n=4). In contrast, 0.5 μg/mL of CsA significantly blocked PE-induced cell-cycle progression in the S phase (P <0.05, n=4), with a concomitant arrest in the number of cells in G_0/G_1, suggesting that CsA inhibited the growth of CFs mainly through decreasing the number of cells in S phase, which is responsible for growth promotion.

Hydroxyproline content, an indicator of collagen deposition, was measured to investigate the influence of CsA on PE-induced collagen synthesis. Hydroxyproline content in the medium of cultured CFs significantly increased by 66.7 ± 3.9% in the PE group compared to non-treated cells. CsA pretreatment reduced PE-induced hydroxyproline production by 79.6 ± 8.9% (Figure 5C). These results indicate that PE treatment promotes the production and secretion of collagen through a CsA-sensitive pathway in CFs.

4.5. Activation and up-regulation of CaN induced by PE in CFs

We examined CaN activity after treatment with PE for 30 min, 2 h and 24 h. As Figure 6A shows, CaN was activated by PE, peaked at 30 min, and then decreased gradually. However, pretreatment of cells with CsA resulted in a significant reduction in CaN activation at 30 min after PE stimulation (Figure 6B). Interestingly, CaN protein levels were markedly increased (1.71 ± 0.15 fold over control) after treatment with PE for 24 h. Furthermore, this PE-evoked increase in CaN protein levels was markedly suppressed by pretreatment with CsA (Figure 6C, 6D). These results suggest that CaN signaling pathways are activated by PE and contribute to cell proliferation in CFs.

4.6. Inhibition of CaN by either pharmacological approach or mutant CaN blocked the PE-induced NFAT3 nuclear translocation

When cells were quiescent, NFAT3 was scattered inside the cytoplasm and absent from the nucleus. However, after treatment with PE (10 μmol/L) for 1 h, NFAT3 was present near the nucleus. NFAT3 was exclusively observed within the nucleus at 4 hours after treatment (Figure 7A). As shown in Figure 7B and 7C, PE increased nuclear NFAT3-GFP accumulation. The observed NFAT-GFP nuclear expression increased by 3 fold over the cytosolic expression. Inhibition of CaN with CsA (0.5. μg/mL) significantly reduced NFAT3 nuclear translocation compared with the PE group alone. Similarly, overexpression of CaN-DN blocked the PE-induced NFAT-GFP nuclear translocation (Figure 7D). These

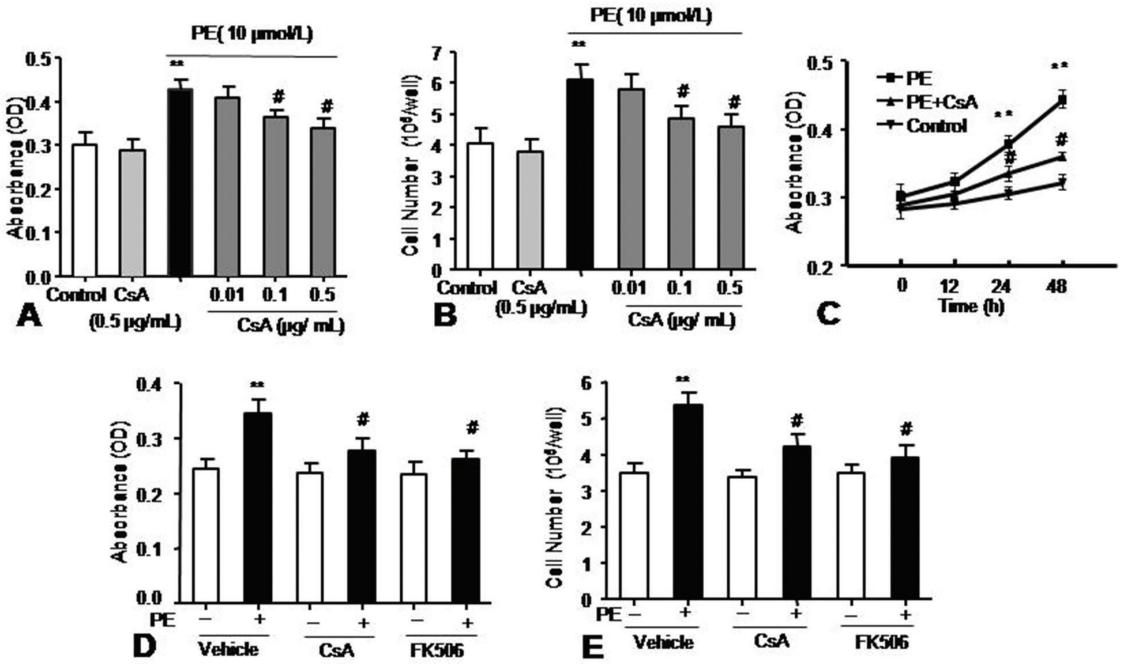

Figure 3. Inhibition of CaN by pharmacological approach suppressed PE-induced CF proliferation. (A, B) Quiescent CFs were treated with or without PE (10 μmol/L) in the presence or absence of CsA at different doses (0.0.1, 0.1., 0.5. μg/mL) for 48 h. CF proliferation was assayed by MTT (A) and cell numbers (B). The results are shown as the mean ± S.E.M. of 6 individual experiments; (C) PE-induced CF proliferation in a time-dependent manner. Arrested CFs were stimulated with 10 μmol/L PE in the absence or presence of CsA (0.5μg/mL) for 0, 12, 24, 48 h. Control group was not treated with PE and CsA; (D, E). Inhibitory effects of FK506 on the PE-induced cell proliferation. The average data were presented as mean ± S.E.M. of 6 individual experiments. $^{**}P$ <0.0.1 vs control cells; $^{\#}P$ <0.0.5 vs PE alone group.

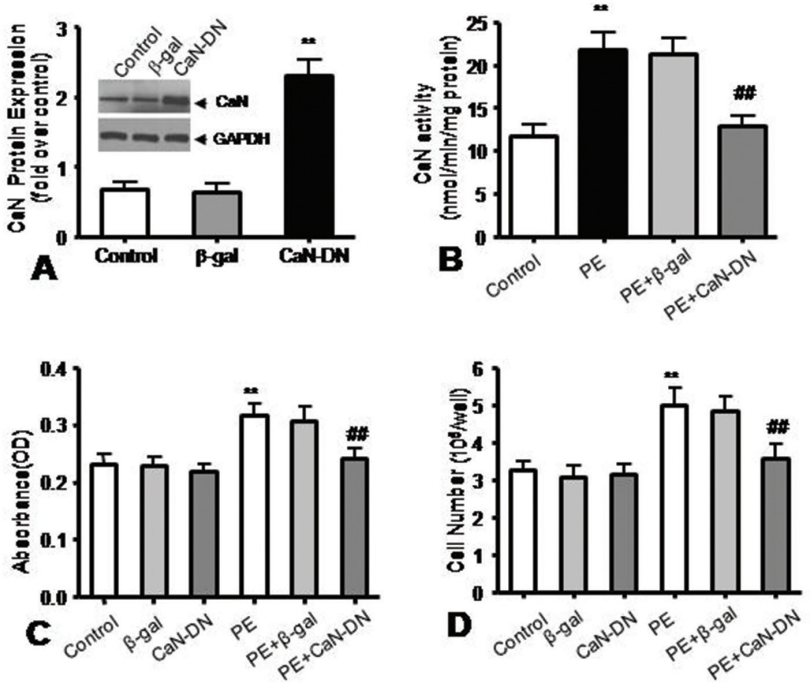

Figure 4. Inhibition of CaN by over-expression of dominant negative mutant CaN (CaN-DN) suppressed PE-induced CF proliferation. (A) Western blot confirmed the expression of dominant negative mutant CaN (CaN-DN) and average data. The data were expressed as mean ± S.E.M. from 3 independent experiments, **P <0.0.1 vs control or β-gal;(B) Over-expression of CaN-DN reduced the activation of CaN induced by PE; (C, D) Overexpression of CaN-DN suppressed the proliferative effect induced by PE. Optical density (C) and cell numbers (D) were measured. The average data were presented as mean ± S.E.M from 6 individual experiments. $^{**}P$ <0.0.1 vs control, $^{\#\#}P$ <0.0.1 vs PE+ β-gal.

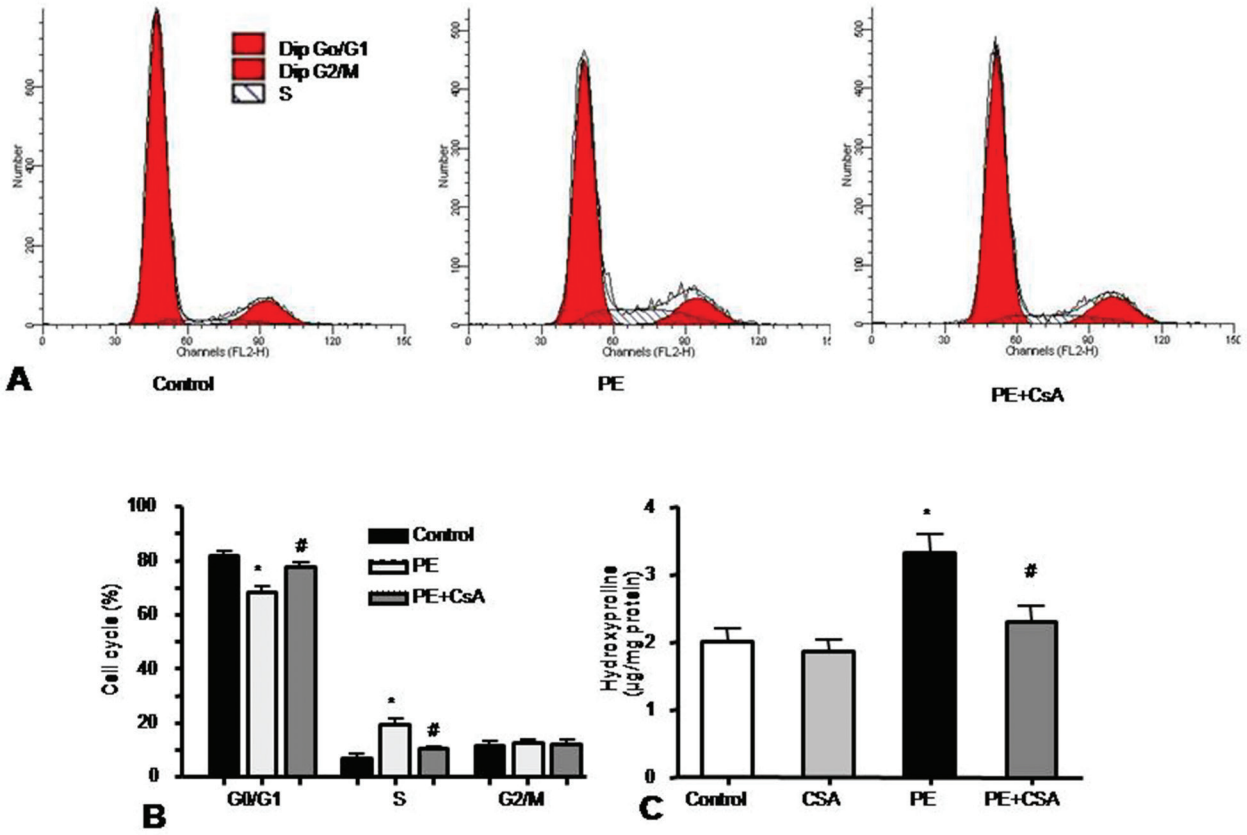

Figure 5. Effect of CsA on the progression of cell cycle and hydroxyproline production induced by PE. CFs were arrested by serum-deprivation for 24 h and then stimulated with PE (10 μmol/L) in the absence or presence of CsA(0.5 μg/ml) for 24 h. Cells were then subjected to flow cytometry assay for determinating cell-cycle distribution. (A) Representative cytometric profiles showing cells in the G_0/G_1, S, and G_2/M phases of the cell cycle. (B) The percentage of cells in the G_0/G_1, S, and G_2/M phases determined by flow cytometric analysis. (C) CsA inhibited hydroxyproline production. The average data were presented as mean ± S.E.M. from 4 independent experiments. **p*<0.0.5 vs control group, $^{\#}P$<0.0.5 vs PE group;

data suggest that the CaN/NFAT pathway is involved in PE-promoted CF proliferation.

5. DISCUSSION

Myocardial fibrosis is a common pathological change in a variety of cardiovascular diseases. Thus, the prevention and reversal of myocardial fibrosis have become main objectives in the treatment of cardiovascular diseases. In the present study, results indicate that: (1) CF proliferation was induced by PE in a dose dependent manner via prazosin-sensitive pathway; (2) PE-induced proliferation was inhibited by a blocker of Ca^{2+} influx, intracellular Ca^{2+} buffering, CaN inhibitors (CsA), and over-expression of CaN-DN; (3) CaN inhibitor, CsA, suppressed the PE-induced cell-cycle progression in the S phase, as well as production of hydroxyproline; (4) The activity and protein level of CaN evoked by PE was also inhibited by CsA; (5) PE induced NFAT nuclear translocation, which was subsequently blocked by either CsA or over-expression of CaN-DN. These results suggest that Ca^{2+}-CaN/NFAT pathway is involved in PE-induced CF proliferation.

Myocardial and circulating levels of adrenaline and noradrenalines are elevated in heart failure patients and are associated with deteriorating outcomes (24). An increase in catecholamine levels increases adrenergic drive and the force and rate of contraction of the heart to compensate for reduced pumping capacity. In addition to these cardiomyocyte-directed effects, catecholamines can directly modulate CF function.

Adrenaline and noradrenaline can both activate members of the α-AR and β-AR families of receptors. Although it is disputed whether the effects of catecholamines on CF are mediated predominantly via activation of the β_2-AR (25-28), recent studies have shown that there is a functional α_1-AR mediated CF proliferation (29,30). Infusion of PE induces cardiac hypertrophy and fibrosis in rat hearts (30). An *in vitro* study indicated that stimulation of α_1-ARs with PE induces the classic Gq-dependent activation of phospholipase C (PLC) and protein kinase C (PKC), leading to extracellular signal-regulated kinase (ERK) activation and nuclear translocation of ERK (29). Catecholamines stimulate collagen production (31), and interleukin-6 synthesis

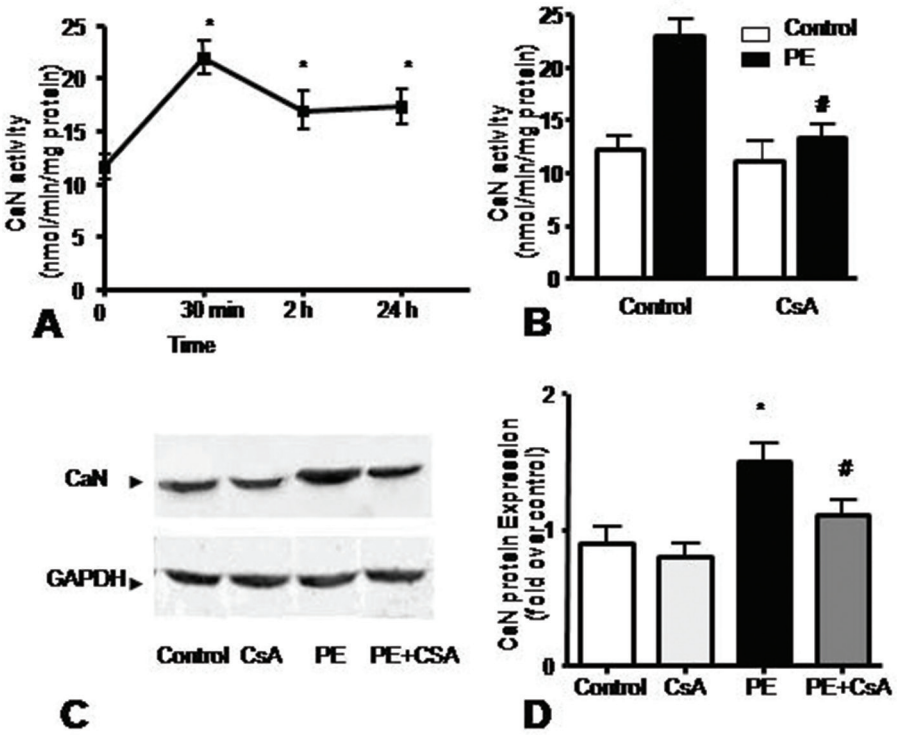

Figure 6. CsA inhibited the activation of CaN and protein level induced by PE. (A) PE induced activation of CaN in the time dependent manner in CFs. Quiescent cells were exposed to 10 μmol/L of PE for 30 min, 2 h, 24 h, and then CaN phosphatase activity was assayed as mentioned in materials and methods; (B) Effects of CsA on the activation evoked by PE. CFs were pretreated with CsA (0.5 μg/mL) for 30 min and then stimulated with PE for 30 min; (C, D) Up-regulation of CaN by PE stimulation for 24 h and inhibited by CsA. The average data were presented as mean ± S.E.M. of 5 individual experiments performed in duplicate.*P<0.0.5 vs control, #P < 0.0.5 vs PE group.

in cultured CFs is mediated by both α_1 and β-AR (32). In the present study, PE increased CF proliferation in a dose- and time-dependent manner. Prazosin completely suppressed the increase of CF proliferation induced by PE. Therefore, we conclude that PE stimulates CF proliferation via α_1-AR.

PE induction of an increase in free intracellular calcium content has been previously described in cardiomyocytes (33). Regardless of intracellular Ca^{2+} resources, it was demonstrated that the sustained increase in intracellular calcium is required for NFAT translocation and maintenance of intracellular Ca^{2+} through intracellular Ca^{2+} release and extracellular Ca^{2+} influx (34). Our experiments demonstrate that both the release of Ca^{2+} from intracellular Ca^{2+} stores and Ca^{2+} influx through the L-type Ca^{2+} channel are necessary for CF proliferation induced by PE.

Increased intracellular Ca^{2+} activates CaN, a calcium- and calmodulin-dependent phosphatase, which is required for diverse biological processes such as cell activation, development, hypertrophy and gene regulation in skeletal and cardiac muscle (6,35, 36). In this study, we observed the inhibitory effects of CsA, FK506 and over-expression of CaN-DN on PE-induced CF proliferation. CsA pretreatment was not able to decrease the basal CaN activity and protein levels, but CsA decreased CaN activity and protein levels evoked by PE in CFs. Therefore, we conclude that CaN is involved in PE-induced CF proliferation. And CsA inhibits PE-induced proliferation of CFs by regulating CaN expression and its activity.

To elucidate the underlying mechanism, we demonstrated that the inhibitory effect of CsA acts through inhibiting DNA synthesis, resulting in arrest of the cell-cycle progression from G_1 to S phase. In the regulation of cell proliferation, the G_1/S transition has been emphasized as a critical event in cell-cycle progression (37), which is coupled to DNA synthesis. The present study indicates that CsA significantly decreased the percentage of CFs entering S phase (the number of cells in G_2/M phase was not obviously altered), whereas those arrested in the G_0/G_1 phase were restored effectively. We hypothesized that the anti-proliferative characteristics of CsA are causally related to the modulation of signaling cascades involved in cell cycle progression and proliferation.

Hydroxyproline has been widely used as an indicator of both the presence and metabolism of collagen (38). The present study indicates that inhibition of CaN by a pharmacological approach abrogates the

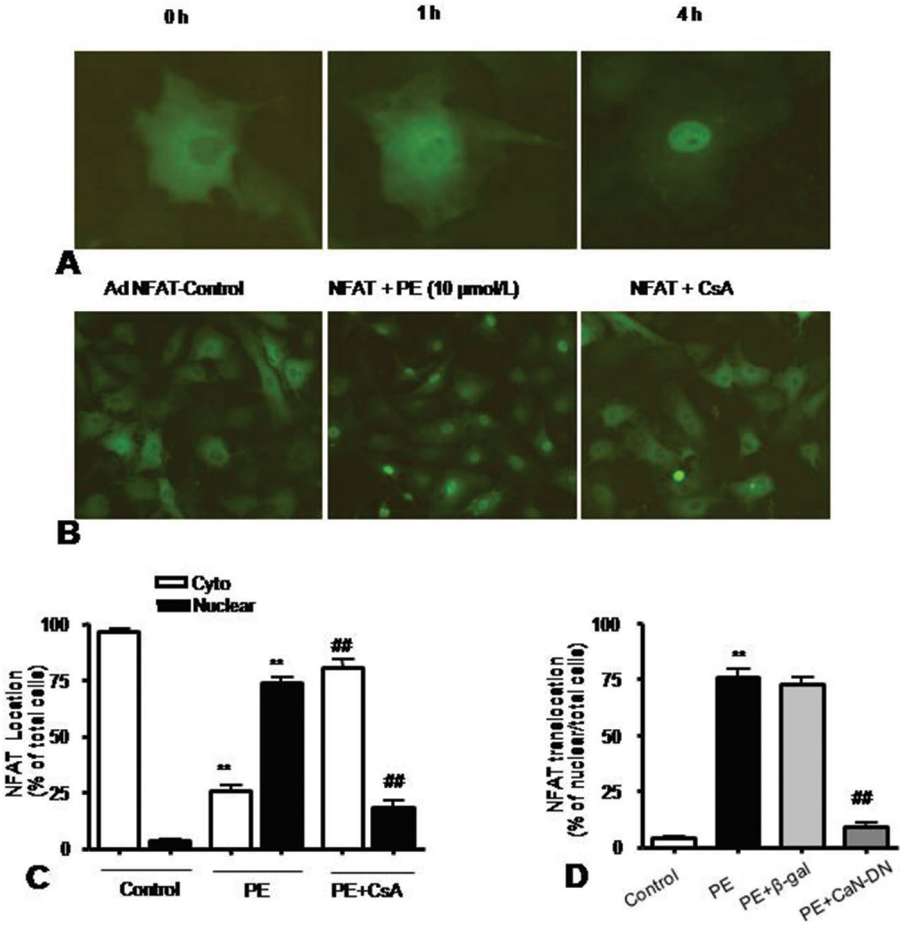

Figure 7. Inhibition of CaN by either pretreatment of CsA or overexpression of CaN-DN blocked the nuclear translocation of NFAT induced by PE treatment in cardiac fibroblast. Cells were transfected with adenovirus containing GFP-tagged NFAT3 and stimulated with PE after the cell were starved for 6 hours. (A) CFs were treated with PE (10 μmol/L) for 1 h and 4 h and then observed under fluorescence microscope (10x40). (B) Representative images were displayed (10x10). CFs were treated with or without PE (10 μmol/L) for 4 h in the presence or absence of CsA (0.5. μg/mL). (C) The average data were presented as mean ± S.E. from 5 individual experiments. $^{**}P$ <0.0.1 vs control, $^{\#\#}P$ <0.0.1 vs PE alone, respectively; (D) Expression of CaN-DN reduced nuclear NFAT accumulation induced by PE in cultured CFs. CFs was co-infected with GFP-tagged NFAT3 and CaN-DN adenoviruses. After 24 h infection, CFs were treated with or without PE for 4 h. Less nuclear distribution was observed in CFs expressed CaN-DN. NFAT nuclear distribution was quantified as nuclear/total cells. The average data were presented as mean ± S.E.M. of 5 individual experiments. $^{**}P$<0.0.1 vs control, $^{\#\#}P$<0.0.1 vs PE + β-gal group.

PE-induced hydroxyproline deposition, suggesting that CaN signaling plays a role in extracellular matrix regulation.

The CaN/NFAT signaling pathway plays a role in regulating growth differentiation and cell cycle progression in various cell types (39,40). CaN plays a role in promoting the nuclear retention of NFAT, which is the most well characterized substrate of CaN (41). Inactive NFAT is highly phosphorylated and localized in the cytoplasm but enters the nucleus (or has nuclear localization) after being activated. We detected subcellular localization of NFAT through GFP-tagged NFAT. Our results clearly show that NFAT was widely scattered in the cytoplasm in quiescent cells, but treatment with PE induced NFAT nuclear translocation, whereas CsA or CaN-DN prevented PE-induced NFAT nuclear translocation by modulating the activity of CaN. These results suggests that CaN activation is required for the hyperplastic growth of CFs induced by PE. Therefore, the CaN/NFAT pathway plays an important role in PE-induced CF proliferation. Our current study promotes the use of CaN/NFAT as a possible therapeutic target in α_1-AR induced fibrosis.

6. ACKNOWLEDGEMENTS

The authors thank Dr. Tomas LaRocca (Cardiovascular Research Center, Mount Sinai School of Medicine, New York, USA) and Valerie Myers (Cardiovascular Research Center, Temple University, Philadelphia, USA) for critical reading of manuscript. This project was supported by the Natural Science Foundation of Jiangsu Province (no. BK2007064) and National Science Foundation (No. 81370345).

7. REFERENCES

1. R. D. Brown, S. K. Ambler, M. D. Mitchell and C. S. Long: The cardiac fibroblast: therapeutic target in myocardial remodeling and failure. *Annu Rev Pharmacol Toxicol*, 45, 657-87 (2005) DOI: 10.1146/annurev.pharmtox.45.120403.095802 PMid:15822192
2. K. E. Porter and N. A. Turner: Cardiac fibroblasts: at the heart of myocardial remodeling. *Pharmacol Ther*, 123(2), 255-78 (2009) DOI: 10.1016/j.pharmthera.2009.05.002 PMid:19460403
3. G. R. Crabtree: Generic signals and specific outcomes: signaling through Ca2+, calcineurin, and NF-AT. *Cell*, 96(5), 611-4 (1999) DOI: 10.1016/S0092-8674(00)80571-1
4. I. A. Graef, F. Chen and G. R. Crabtree: NFAT signaling in vertebrate development. *Curr Opin Genet Dev*, 11(5), 505-12 (2001) DOI: 10.1016/S0959-437X(00)00225-2
5. J. D. Molkentin, J. R. Lu, C. L. Antos, B. Markham, J. Richardson, J. Robbins, S. R. Grant and E. N. Olson: A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell*, 93(2), 215-28 (1998) DOI: 10.1016/S0092-8674(00)81573-1
6. E. N. Olson and R. S. Williams: Remodeling muscles with calcineurin. *Bioessays*, 22(6), 510-9 (2000) DOI: 10.1002/(SICI)1521-1878(200006)22:6<510:AID-BIES4>3.0.CO;2-1
7. P. J. Schwartz, E. Vanoli, A. Zaza and G. Zuanetti: The effect of antiarrhythmic drugs on life-threatening arrhythmias induced by the interaction between acute myocardial ischemia and sympathetic hyperactivity. *Am Heart J*, 109(5 Pt 1), 937-48 (1985) DOI: 10.1016/0002-8703(85)90233-9
8. Y. Himura, S. Y. Felten, M. Kashiki, T. J. Lewandowski, J. M. Delehanty and C. S. Liang: Cardiac noradrenergic nerve terminal abnormalities in dogs with experimental congestive heart failure. *Circulation*, 88(3), 1299-309 (1993) DOI: 10.1161/01.CIR.88.3.1299 PMid:8102598
9. P. C. Simpson, B. J. Trudinger, A. Walker and P. J. Baird: The intrauterine treatment of fetal cardiac failure in a twin pregnancy with an acardiac, acephalic monster. *Am J Obstet Gynecol*, 147(7), 842-4 (1983) PMid:6650614
10. A. B. Sprenkle, S. F. Murray and C. C. Glembotski: Involvement of multiple cis elements in basal- and alpha-adrenergic agonist-inducible atrial natriuretic factor transcription. Roles for serum response elements and an SP-1-like element. *Circ Res*, 77(6), 1060-9 (1995) DOI: 10.1161/01.RES.77.6.1060 PMid:7586217
11. B. Bhambi and M. Eghbali: Effect of norepinephrine on myocardial collagen gene expression and response of cardiac fibroblasts after norepinephrine treatment. *Am J Pathol*, 139(5), 1131-42 (1991) PMid:1951630 PMCid: PMC1886331
12. N. Takahashi, A. Calderone, N. J. Izzo, Jr., T. M. Maki, J. D. Marsh and W. S. Colucci: Hypertrophic stimuli induce transforming growth factor-beta 1 expression in rat ventricular myocytes. *J Clin Invest*, 94(4), 1470-6 (1994) DOI: 10.1.172/JCI117485
13. D. M. Eble, M. Qi, S. Waldschmidt, P. A. Lucchesi, K. L. Byron and A. M. Samarel: Contractile activity is required for sarcomeric assembly in phenylephrine-induced cardiac myocyte hypertrophy. *Am J Physiol*, 274(5 Pt 1), C1226-37 (1998) PMid:9612209
14. M. Leicht, N. Greipel and H. Zimmer: Comitogenic effect of catecholamines on rat cardiac fibroblasts in culture. *Cardiovasc Res*, 48(2), 274-84 (2000) DOI: 10.1016/S0008-6363(00)00170-X
15. S. Goruppi, R. D. Patten, T. Force and J. M. Kyriakis: Helix-loop-helix protein p8, a transcriptional regulator required for cardiomyocyte hypertrophy and cardiac fibroblast matrix metalloprotease induction. *Mol Cell Biol*, 27(3), 993-1006 (2007) DOI:10.1.128/MCB.00996-06
16. B. B. Hoffman and Z. W. Hu: Alpha(1)-adrenoceptors (alpha(1)-AR) and vascular smooth muscle cell growth. *Prostate Suppl*, 9, 29-33 (2000) DOI: 10.1002/1097-0045(2000)45:9+<29:AID-PROS7>3.0.CO;2-Z

17. D. A. Siwik and R. D. Brown: Regulation of protein synthesis by alpha 1-adrenergic receptor subtypes in cultured rabbit aortic vascular smooth muscle cells. *J Cardiovasc Pharmacol*, 27(4), 508-18 (1996)
DOI: 10.1097/00005344-199604000-00009
PMid:8847867

18. H. Zhang, C. S. Facemire, A. J. Banes and J. E. Faber: Different alpha-adrenoceptors mediate migration of vascular smooth muscle cells and adventitial fibroblasts *in vitro*. *Am J Physiol Heart Circ Physiol*, 282(6), H2364-70 (2002)
DOI: 10.1152/ajpheart.00858.2001
PMid:12003847

19. Z. W. Hu, X. Y. Shi, R. Z. Lin and B. B. Hoffman: Alpha1 adrenergic receptors activate phosphatidylinositol 3-kinase in human vascular smooth muscle cells. Role in mitogenesis. *J Biol Chem*, 271(15), 8977-82 (1996)
DOI: 10.1074/jbc.271.15.8977
PMid:8621543

20. T. Nakaki, M. Nakayama, S. Yamamoto and R. Kato: Alpha 1-adrenergic stimulation and beta 2-adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol Pharmacol*, 37(1), 30-6 (1990)
PMid:2153907

21. R. Vashisht, M. Sian, P. J. Franks and M. K. O'Malley: Long-term reduction of intimal hyperplasia by the selective alpha-1 adrenergic antagonist doxazosin. *Br J Surg*, 79(12), 1285-8 (1992)
DOI: 10.1002/bjs.1800791212
PMid:1486418

22. J. E. Faber, N. Yang and X. Xin: Expression of alpha-adrenoceptor subtypes by smooth muscle cells and adventitial fibroblasts in rat aorta and in cell culture. *J Pharmacol Exp Ther*, 298(2), 441-52 (2001)
PMid:11454904

23. C. G. Brilla, C. Scheer and H. Rupp: Renin-angiotensin system and myocardial collagen matrix: modulation of cardiac fibroblast function by angiotensin II type 1 receptor antagonism. *J Hypertens Suppl*, 15(6), S13-9 (1997)
DOI: 10.1097/00004872-199715066-00004
PMid:9493122

24. B. Swynghedauw: Molecular mechanisms of myocardial remodeling. *Physiol Rev*, 79(1), 215-62 (1999)
PMid:9922372

25. A. B. Gustafsson and L. L. Brunton: beta-adrenergic stimulation of rat cardiac fibroblasts enhances induction of nitric-oxide synthase by interleukin-1beta via message stabilization. *Mol Pharmacol*, 58(6), 1470-8 (2000)
PMid:11093787

26. J. G. Meszaros, A. M. Gonzalez, Y. Endo-Mochizuki, S. Villegas, F. Villarreal and L. L. Brunton: Identification of G protein-coupled signaling pathways in cardiac fibroblasts: cross talk between G(q) and G(s). *Am J Physiol Cell Physiol*, 278(1), C154-62 (2000)
PMid:10644523

27. N. A. Turner, K. E. Porter, W. H. Smith, H. L. White, S. G. Ball and A. J. Balmforth: Chronic beta2-adrenergic receptor stimulation increases proliferation of human cardiac fibroblasts via an autocrine mechanism. *Cardiovasc Res*, 57(3), 784-92 (2003)
DOI: 10.1016/S0008-6363(02)00729-0

28. F. Yin, Z. Z. Lu, Q. D. Han and Y. Y. Zhang: (Expression of beta2-adrenergic receptor and its effect on the proliferation of neonatal rat cardiac fibroblasts). *Sheng Li Xue Bao*, 55(3), 251-4 (2003)
PMid:12817289

29. D. Cervantes, C. Crosby and Y. Xiang: Arrestin orchestrates crosstalk between G protein-coupled receptors to modulate the spatiotemporal activation of ERK MAPK. *Circ Res*, 106(1), 79-88
DOI: 10.1161/CIRCRESAHA.109.198580
PMid:19926878 PMCid: PMC2818802

30. R. S. Farivar, D. C. Crawford, A. V. Chobanian and P. Brecher: Effect of angiotensin II blockade on the fibroproliferative response to phenylephrine in the rat heart. *Hypertension*, 25(4 Pt 2), 809-13 (1995)
DOI: 10.1161/01.HYP.25.4.809
PMid:7536716

31. K. B. Lai, J. E. Sanderson and C. M. Yu: Suppression of collagen production in norepinephrine stimulated cardiac fibroblasts culture: differential effect of alpha and beta-adrenoreceptor antagonism. *Cardiovasc Drugs Ther*, 23(4), 271-80 (2009)
DOI: 10.1007/s10557-009-6183-6
PMid:19575289

32. A. Burger, M. Benicke, A. Deten and H. G. Zimmer: Catecholamines stimulate interleukin-6 synthesis in rat cardiac fibroblasts. *Am J Physiol Heart Circ Physiol*, 281(1), H14-21 (2001)
PMid:11406463

33. J. S. Karliner, T. Kagiya and P. C. Simpson: Effects of pertussis toxin on alpha 1-agonist-mediated phosphatidylinositide turnover and myocardial cell hypertrophy in neonatal rat ventricular myocytes. *Experientia*, 46(1), 81-4 (1990)
DOI: 10.1007/BF01955423
PMid:2153573

34. A. T. Serafini, R. S. Lewis, N. A. Clipstone, R. J. Bram, C. Fanger, S. Fiering, L. A. Herzenberg and G. R. Crabtree: Isolation of mutant T lymphocytes with defects in capacitative calcium entry. *Immunity*, 3(2), 239-50 (1995)
DOI: 10.1016/1074-7613(95)90093-4

35. G. R. Crabtree: Calcium, calcineurin, and the control of transcription. *J Biol Chem*, 276(4), 2313-6 (2001)
DOI: 10.1074/jbc.R000024200
PMid:11096121

36. F. Rusnak and P. Mertz: Calcineurin: form and function. *Physiol Rev*, 80(4), 1483-521 (2000)
PMid:11015619

37. C. F. Welsh, K. Roovers, J. Villanueva, Y. Liu, M. A. Schwartz and R. K. Assoian: Timing of cyclin D1 expression within G1 phase is controlled by Rho. *Nat Cell Biol*, 3(11), 950-7 (2001)
DOI: 10.1038/ncb1101-950
PMid:11715015

38. B. Peterkofsky and S. Udenfriend: Conversion of Proline to Collagen Hydroxyproline in a Cell-Free System from Chick Embryo. *J Biol Chem*, 238, 3966-77 (1963)
PMid:14086732

39. L. Lipskaia and A. M. Lompre: Alteration in temporal kinetics of Ca2+ signaling and control of growth and proliferation. *Biol Cell*, 96(1), 55-68 (2004)
DOI: 10.1.016/j.biolcel.2003.1.1.0.01

40. J. P. Viola, L. D. Carvalho, B. P. Fonseca and L. K. Teixeira: NFAT transcription factors: from cell cycle to tumor development. *Braz J Med Biol Res*, 38(3), 335-44 (2005)
DOI: /S0100-879X2005000300003

41. G. R. Crabtree and E. N. Olson: NFAT signaling: choreographing the social lives of cells. *Cell*, 109 Suppl, S67-79 (2002)
DOI: 10.1016/S0092-8674(02)00699-2

Key Words: Calcineurin; Phenylephrine; Nuclear factor of activated T cells; Proliferation; Fibroblast

Send correspondence to: Weizhong Zhu, Department of Pharmacology, Nantong University Medical College, 19 Qi Xiu Road, Nantong, 226001, People's Republic of China, Tel: 86051385051859, Fax:86051385051858, E-mail: zhuwz@ntu.edu.cn