Original Article

Ca\(^{2+}\)-calcineurin signaling is involved in norepinephrine-induced cardiac fibroblasts activation

Chun-Jing Tian, Xiao Pang

Department of Cardiology, First Affiliated Hospital of The Medical College of Shihezi University

Received March 14, 2015; Accepted April 26, 2015; Epub May 1, 2015; Published May 15, 2015

Abstract: Cardiac fibroblasts (CFs) activation plays a vital role in cardiac fibrosis. There are some studies demonstrate that norepinephrine (NE, an \(\alpha_1\)-adrenoceptor agonist) induced CFs proliferation. But whether Ca\(^{2+}\)-calcineurin, a signaling concerned with growth and differentiation in various cell types, is participated in NE-induced CFs activation is unclear. In present study, we determined NE-induced CFs proliferation and differentiation, synthesis of collagen, and calcineurin (CaN) activity, and the effects of phentolamine (Phen, an \(\alpha_1\)-adrenoceptor antagonist), verapamil (Ver, a calcium channel blocker) and cyclosporine A (CsA, an inhibitor of CaN) on NE-induced CFs activation. The results showed that NE induced CFs proliferation and differentiation, increased \(\alpha\)-SMA protein expression, increased collagen I, collagen III and fibronectin production, promoted ECM expression, activated CaN and increased CaN protein expression, which were inhibited by Phen, Ver and CsA. In vivo, more collagen deposition could be observed and total collagen volume fraction (CVF) was significantly increased in NE group. Phen, Ver and CsA decreased NE-induced collagen deposition, reduced cardiac fibrosis. Thus, our results demonstrate that Ca\(^{2+}\)/CaN is involved in NE-induced CFs proliferation and collagen synthesis.

Keywords: Calcineurin, norepinephrine, cardiac fibroblasts, activation

Introduction

Cardiac fibrosis is a scarring process, which is associated with cardiac remodeling, and characterized by CFs activation and excess extracellular matrix (ECM) accumulation. CFs comprise approximately two-thirds of the total number of cardiac cell types [1]. The activation of CFs comprises proliferation and differentiation into myofibroblasts (MyoFbs), which are specialized CFs formed by irreversible acquisition of \(\alpha\)-SMA. MyoFbs have observably higher capacity for collagen synthesis, then promoted collagen excessive deposition [2].

A lot of research confirmed that prolonged activation of sympathetic nervous system (SNS) is responsible for alteration in normal myocardial structure and function and promote cardiac fibrosis. NE, the primary transmitter of SNS, exerts its effect by binding to G-protein-coupled adrenergic receptors and then activating the phospholipase C pathway [3]. More recent studies find that NE-induced fibroblasts proliferation by p38MAPK and Akt phosphorylation [4]. In addition, NE increased collagen and fibronectin synthesis, promotes ECM deposition and enhance cardiac fibrosis [5-7].

CaN is a cytoplasmic protein serine/threonine phosphatase, consisted of the catalytic calcineurin A subunit (CnA) and the regulatory calcineurin B subunit (CnB), CaN plays a crucial role in development of cardiac fibrosis [8, 9]. CaN is located in the downstream of Ca\(^{2+}\), the catalytic regulating mechanism is complicated. When the cells were stimulated, elevating of intracellular Ca\(^{2+}\) concentration results in calcium-dependent binding of calmodulin to CaN, thus relieving the inhibitory activity of the auto-inhibitory domain (AID) on the catalytic domain, then CaN is activated [10]. Ca\(^{2+}\)/CaN signaling can induced CFs activation through Electrical field stimulation, mechanical stress, Pressure overload, Angiotensin II signaling [11-13]. Yet, whether the Ca\(^{2+}\)/CaN pathway mediates NE-induced CFs activation has not been well elucidated.

Our results demonstrate that NE increased CFs proliferation, induced CFs differentiation, promoted collagen synthesis, and activated CaN,
Ca\(^{2+}\)/CaN is involved in NE-induced CFs activation

which were inhibited by Phen, Ver and CsA. In vivo, NE increased total CVF and promoted collagen deposition, inhibition of Ca\(^{2+}\)/CaN pathway reduced NE-induced cardiac fibrosis. Based on these data, we believe that the Ca\(^{2+}\)/CaN pathway participated in NE-induced CFs activation, and this pathway may become an attractive therapeutic target for the clinical treatment of cardiac fibrosis.

Materials and methods

Reagents

DMEM (12800-058, Gibco), fetal bovine serum (FBS, 10099-141, Gibco), trypsin (27250018, Gibco), Collagenase II (C6885, Sigma), MTT (0793, Amresco), NE (N5785), Phen (111102), Ver (120701B), CsA (S0048), calcineurin assay kit (NJBI), anti-CaN Aα monoclonal antibody (ab109412, Abcam), anti-α-SMA polyclonal antibody (sc-32251, Santa Cruz), anti-GAPDH polyclonal antibody (sc-25778, Santa Cruz), ELISA kit (Wuhan ColorfulGene Biological Technology). Other chemicals and reagents were of analytical grade.

Experimental animals and protocol

Sprague Dawley (SD) rats, weighing 240-280 g, and 1-2-day-old SD rats were obtained from the Animal Center of Peking University (Beijing). All experiments were approved by the Committee on the Ethics of Animal Experiments of Peking University. All animal care and experimental protocols complied with the Animal Management Rule of the Ministry of Health, People's Republic of China (documentation no. 55, 2001) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication no. 85-23, revised 1996). SD Rats were randomly divided into two groups, saline (control) or NE was delivered by micro-osmotic pumps for 14 days, then NE group was divided into Phen group, Ver group and CsA group, and treated for 14 days.

Cell culture

CFs were enzymatically isolated from the hearts of 1-2-day-old SD rats. After disinfection, the hearts were removed and washed with PBS. Digestion the tissues with trypsin and collagenase II. The pellets were resuspended in DMEM supplemented with 10% FBS and seeded in 25 cm\(^2\) tissue culture flask. After 1.5 hours, cell supernatant was removed, and added the new DMEM medium with 10% FBS in the culture flask.

Cell proliferation assay

After CFs were treated with the medications for 48 hr. MTT (5 g/l) was added into cell cultures and incubated with the cells for 4 hr. Then the medium was completely removed without disturbing the formazan crystals. After DMSO was added to the wells, the plate was shaken for 10

Figure 1. NE-induced proliferation in CFs. Quiescent CFs were treated with NE (1 µmol/l), Ver (10 µmol/l), Phen (10 µmol/l) + NE (1 µmol/l), Ver (10 µmol/l) + NE (1 µmol/l), CsA (1 µmol/l) + NE (1 µmol/l) for 48 hr. (A) Optical density and (B) cell number were measured. Data were expressed as the means ± SD of 8 (MTT) or 6 (cell number) separate experiments. *P<0.05 compared with the control; #P<0.05 compared with NE.
Ca\textsuperscript{2+}/CaN is involved in NE-induced CFs activation

Immunoblotting analysis

Whole cell lysates were prepared using the cell lysis buffer. After determining the Protein concentration, equal samples were loaded and separated by electrophoresis. Proteins were transferred onto a nitrocellulose filter membrane. Nonspecific proteins were blocked with 5% nonfat dried milk for 1 hr. Membranes were incubated with primary antibodies and subsequently incubated with secondary antibodies. Protein expression was analyzed by NIH image software and normalized to that of GAPDH.

Calcineurin enzymatic activity

The activity of CaN was assessed using a calcineurin activity assay kit following the manufacturer’s instructions. Then the Protein concentration was determined, and the CaN activity was corrected for protein concentration. The

Figure 2. Effect of NE and the inhibitor Phen, Ver and CsA on expression levels of α-SMA and changes in collagen I, collagen III and fibronectin in CFs culture supernatant. (A) α-SMA protein expression was measured by Western blot (Mean ± SD, n=4 experiments). Data were expressed as (B) collagen I concentration (μg/l), (C) collagen III concentration (μg/l) and (D) fibronectin concentration (ng/ml), (Mean ± SD, n=7 experiment per point) *P<0.05 compared with the control; #P<0.05 compared with NE.
Ca\(^{2+}\)/CaN is involved in NE-induced CFs activation

CaN activity was expressed as a percentage compared with the control group.

**Content of collagen and fibronectin in cell cultures**

The culture supernatants were collected. After clearing the lysates by centrifugation, the content of collagen and fibronectin was measured using the ELISA Kit according to the manufacturer’s instructions.

**Morphological observation of myocardial collagen and quantitative analysis**

Freshly excised hearts were arrested in diastole, fixed in 4% formalin, paraffin-embedded myocardial blocks were sectioned by a conventional microtome, Van-Gieson (VG) method was used for staining. Image-Pro 6.0 software was used to analyze the obtained images. CVF was calculated as the following equation: CVF=the left ventricular collagen area/field area determined. Six fields were selected randomly and the mean values were used as the cardiac CVF.

**Statistical analysis**

The results are expressed as means ± SD. All data were analyzed with SPSS 17.0 ANOVA, post-hoc analysis and the Newman-Keuls test was used to compare differences among groups. P-values less than 0.05 were considered statistically significant.

**Results**

**NE induced CFs proliferation in vitro**

CFs were incubated in the various combinations of medicines for 48 hr. The optical density of cells was detected. NE increased the absorbance of CFs by 43.9% compared with the control group. Phen, Ver and CsA decreased NE-induced proliferation of CFs by 42.3%, 36.5%, and 32.8%, respectively (Figure 1A, all P<0.05). To confirm these findings, cell number was measured. NE increased CFs number by 40.5% compared with the control group. Phen, Ver and CsA decreased NE-induced CFs numbers by 48.8%, 37.6%, and 34.4%, respectively (Figure 1B, all P<0.05).

![Figure 3. Effect of NE and the inhibitors on expression levels and activity of CaN in CFs. (A) CaN protein expression was measured by Western blot (n=4), and (B) CaN activity was detected using a calcineurin assay kit (n=6). Mean ± SD, *P<0.05 compared with the control; #P<0.05 compared with NE.](image-url)
Ca\textsuperscript{2+}/CaN is involved in NE-induced CFs activation

**NE induced CFs differentiation into MyoFbs**

When CFs were stimulated by NE, CFs differentiate into MyoFbs which express the highly contractile protein α-SMA and promoted collagen synthesis as previously described. After treating with the various combinations of medications for 48 hr, whole cell lysates were prepared using the Cell lysis reagent. The α-SMA protein expression was assessed with immunoblotting. NE increased α-SMA level by 43.9%, however, Phen, Ver and CsA decreased α-SMA levels by 55.7%, 40.1%, 32.8%, respectively (Figure 2A, all \(P<0.05\)).

The concentration of collagen and fibronectin in culture supernatants was measured by ELISA. NE lead to increased synthesis of the collagen I, collagen III and fibronectin. The increment was from 3.21 ± 0.38 μg/l, 1.65 ± 0.18 μg/l, 3.02 ± 0.22 ng/ml in the control group to 4.98 ± 0.47 μg/l, 2.31 ± 0.28 μg/l, 4.33 ± 0.38 ng/ml, respectively. Whereas Phen, Ver and CsA decreased NE-induced collagen I synthesis to 2.63 ± 0.28 μg/l, 2.87 ± 0.44 μg/l, 3.06 ± 0.45 μg/l (Figure 2B, all \(P<0.05\)), decreased NE-induced collagen III synthesis to 1.37 ± 0.20 μg/l, 1.49 ± 0.22 μg/l, 1.58 ± 0.30 μg/l (Figure 2C, all \(P<0.05\)), decreased NE-induced fibronectin synthesis to 2.46 ± 0.26 ng/ml, 2.58 ± 0.14 ng/ml, 2.92 ± 0.15 ng/ml, respectively (Figure 2D, all \(P<0.05\)).

**NE increased CaN protein expression and activity**

After CFs were incubated at 37°C for 24 hr, cells were treated with the various combinations of medications for 48 hr. NE increased CaN level and activity by 61.9%, 32.6%, respectively, whereas Phen, Ver and CsA decreased CaN levels and activity by 53.3%, 43.5%, 39.5% and 41.2%, 32.4%, 25.2%, respectively (Figure 3A, 3B, all \(P<0.05\)). The relative expression levels of the protein were normalized with GAPDH.

**Inhibition of Ca\textsuperscript{2+}/CaN signaling reduced NE-induced cardiac fibrosis in vivo**

The myocardial tissues were treated and used for VG staining, and then the images were captured by the digital single lens reflex and analy-
Ca\textsuperscript{2+}/CaN is involved in NE-induced CFs activation

<table>
<thead>
<tr>
<th>Groups</th>
<th>CVF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>2.77 ± 0.73</td>
</tr>
<tr>
<td>NE group</td>
<td>6.58 ± 0.86**</td>
</tr>
<tr>
<td>NE + Phen group</td>
<td>4.09 ± 0.69***</td>
</tr>
<tr>
<td>NE + Ver group</td>
<td>5.02 ± 0.71**</td>
</tr>
<tr>
<td>NE + CsA group</td>
<td>5.67 ± 0.62**</td>
</tr>
</tbody>
</table>

**P<0.01 compared with the control; *P<0.01 compared with NE; #P<0.05 compared with NE.

Discussion

Cardiac remodeling is considered an independent danger factor of cardiovascular disease [1]. Cardiac remodeling involves changes in cardiac myocytes and in the ECM. In the ECM, collagens maintain the structural integrity and control the function of contraction in heart, increasing of ECM results in diastolic cardiac dysfunction and heart failure (HF). A lot of research confirmed that SNS activation plays an important role in the development of cardiac remodeling, SNS activation increases catecholamine release, the concentration of NE increased significantly [14].

Lots of research show that NE exerts its effect by binding to G protein-coupled receptor and induces a variety of cell proliferation. NE stimulation allows GTP association with Gq, dissociation of the trimeric G proteins and activation of PLC\textgreek{g} via direct interaction with Gq/GTP, when a phospholipase cleaves a membrane phospholipid phosphatidylinositol 4, 5 bisphosphate (PIP\textgreek{g}), soluble inositol triphosphate (IP\textgreek{g}) and membrane-bound diacyl glycerol (DAG) are created [15]. IP\textgreek{g} induces acute release of intracellular Ca\textsuperscript{2+} stores, which forces calmodulin (CaM) to interact with CaN causing a conformational shift, and α1D no longer blocks the active site [8, 16-19]. After activation, CaN modulates a variety of genes expression, which subsequently mediates various biological processes.

Briest [20] found that NE induced cardiac fibrosis and increased the mRNAs of collagen I and III, promoted collagen synthesis. In the left ventricles of wild-type mice, aortic banding resulted in elevated collagen I and collagen III, fibrinectin, α-SMA, all of which were reduced in fibroblasts after treatment with CaN/NFAT blocker, illustrating that CaN/NFAT pathway mediated stress-induced differentiation of CFs to MyoFbs and ECM production [12]. Chen [11] found that CFs proliferation, myocardial fibrosis, CN activity were enhanced by electrical field stimulation (EFS), these effects were abolished by CN inhibitors, suggesting that CaN/NFAT pathway mediates CFs proliferation induced by EFS.

In previously study, we found that NE induced left ventricular hypertrophy, cardiac fibrosis and promoted ECM remodeling in SD rats. Phen, an α\textgreek{g}-AR antagonist, reduced expression of matrix metalloproteinase 2 and collagen I, reduced hydroxyproline, CVF, then improved cardiac remodeling. In present study, we treated CFs with NE for 48 hr and found that NE induced CFs proliferation, increased CFs number, which were partly inhibited by pretreatment of cells with inhibitors of α\textgreek{g}-AR (Phen), calcium channel (Ver) and CaN (CsA). These results suggest that NE can induced CFs proliferation through α\textgreek{g}-AR, Ca\textsuperscript{2+}/CaN signaling is involved in NE induced CFs proliferation. The activation of CFs comprises proliferation and differentiation, then we explored whether Ca\textsuperscript{2+}/CaN signaling is involved in NE induced CFs differentiation. We treated CFs with NE for 48 hr and found that NE induced CFs differentiation into MyoFbs, increased α-SMA expression and promoted collagen I, collagen III and fibronectin synthesis, which were partly inhibited by Phen, Ver, CsA. These data shown that Ca\textsuperscript{2+}/CaN signaling is involved in NE induced CFs activation. To confirm these findings, we detected CaN protein expression and activity induced by NE, and found that NE also increased CaN protein expression and activity significantly, which were inhibited by Phen, Ver and CsA pretreatment. In vivo, more collagen deposition could be observed around the small blood vessels in the cardiac muscles and total CVF was significantly increased in the left ventricular myocardium in NE group. Myocardium collagen deposition in Phen group, Ver group and CsA group was less than those of NE group and CVF were significantly decreased (Figure 4; Table 1).
Ca\textsuperscript{2+} /CaN is involved in NE-induced CFs activation

than NE group; CVF also decreased compare with NE group. These results suggest that inhibition of Ca\textsuperscript{2+}/CaN pathway reduced NE-induced cardiac fibrosis.

In conclusion, our study demonstrates that Ca\textsuperscript{2+}/CaN pathway plays an important role in the regulation of NE-induced CFs activation. Phen, Ver and CsA suppresses NE-induced CFs activation by inhibiting \( \alpha_1 \)-adrenoceptor, Ca\textsuperscript{2+} channel and CaN activity, respectively. Thus, Ca\textsuperscript{2+}/CaN pathway participates in regulating NE-induced activation of cardiac fibroblasts. Then the Ca\textsuperscript{2+}/CaN pathway might be a possible therapeutic target for NE-induced cardiac fibrosis and heart failure.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiao Pang, Department of Cardiology, First Affiliated Hospital of The Medical College of Shihezi University, Shihezi, Xinjiang 832002, China. Tel: +86-13779209712; E-mail: px0993@163.com

References


