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Nifedipine inhibits angiotensin II-induced cardiac fibrosis via downregulating Nox4-derived ROS generation and suppressing ERK1/2, JNK signaling pathways

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Nifedipine, a classic L-type dihydropyridine calcium channel blocker (CCB), has been reported to possess multiple cardioprotective properties. However, little is known about the effects of nifedipine on cardiac fibrosis induced by angiotensinII (AngII) and the detailed molecular mechanisms. In this study, we found that nifedipine attenuated AngII-induced cardiac fibrosis *in vitro via* inhibiting the proliferation, differentiation of cardiac fibroblasts and antagonizing the upregulation of extracellular matrix (ECM) protein fibronectin (FN) and the pro-fibrotic cytokine connective tissue growth factor (CTGF). Furthermore, nifedipine suppressed the upregulation of NAD(P)H oxidase 4 (Nox4) and the production of reactive oxygen species (ROS) induced by AngII. In addition, it markedly inhibited the phosphorylation of extracellular signal-regulate kinases 1/2 (ERK1/2) and c-Jun NH(2)-terminal kinase (JNK) stimulated by AngII. However, nifedipine exhibited no effect on the variation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). These results suggested that (1) nifedipine inhibited cardiac fibrosis induced by AngII; (2) the anti-fibrotic effects of nifedipine may be mediated by interfering with the production of ROS and the activation of ERK1/2 and JNK signaling pathways; (3) the classic calcium channel blocking action of nifedipine may not be involved in the anti-fibrotic activities.

1. Introduction

Cardiac fibrosis is a pathological process characterized by fibroblast accumulation and excess deposition of extracellular matrix (ECM) proteins in the myocardium (Fan et al. 2012), which results in abnormalities in cardiac conduction and mechanical function, unremitted cardiac fibrosis can lead to cardiac hypertrophy, arrhythmias and hear failure (Berk et al. 2007). Thus, to rationally clarify the underlying mechanisms of cardiac fibrosis and explore anti-fibrotic drugs are likely to be invaluable in curbing cardiovascular disease.

During cardiac remodeling, cardiac fibroblasts undergo some important phenotypic changes such as exaggerated proliferation, differentiation into myofibroblasts (α-smooth muscle actin positive cells) and excessive deposition of ECM components (Bouzegrhane and Thibault 2002). A plethora of studies have demonstrated that angiotensinII (AngII) is an important factor that contributes to cardiac fibrosis *via* binding to AngII type 1 receptor (AT1) activating several intracellular signaling cascades (H et al. 2010; Kawano et al. 2000; Singh and Mehta 2003). Accumulating evidence confirmed that ROS play a pivotal role in mediating cardiac fibrosis (Chen and Mehta 2006; Poli and Parola 1997). It has been reported that AngII induces ROS generation in cardiac fibroblasts *via* stimulating nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases (Griendling et al. 2000; Lijnen et al. 2012; Wang et al. 2012), genetic depletion or pharmacological blockade of NAD(P)H oxidases blunts cardiac fibrosis induced by AngII (Rocic and Lucchesi 2005). Furthermore, NAD(P)H oxidase 4 (Nox4) is abundantly expressed in cardiac fibroblasts and is considered to be the predominant Nox isoform contributing to ROS production. Previous studies demonstrated that downregulation of Nox4 inhibited myofibroblast formation, CTGF expression and the secretion of ECM in cardiac fibroblasts (Barnes and Gorin 2011; Colston et al. 2005; Cucoranu et al. 2005). Moreover, it is well established that AngII mediates cardiac fibrosis through provoking the activation of mitogen activated protein kinases (MAPKs) including ERK1/2, p38 MAPK and JNK (Gu et al. 2012; Tharaux et al. 2000). MAPK signaling pathway has been demonstrated to be involved in the proliferation and differentiation as well as other related fibrotic markers expression of cardiac fibroblasts (Schnee and Hsueh 2000), targeting MAPKs may be a promising therapeutic approach for cardiac fibrosis (Chen et al. 2012).

Abbreviations: AngII, angiotensin II; ECM, extracellular matrix; FN, fibronectin; CTGF, connective tissue growth factor; Nox4, NAD(P)H oxidase 4; ERK1/2, extracellular signal-regulate kinases 1/2; JNK, c-Jun NH(2)-terminal kinase; ROS, reactive oxygen species; MAPKs, mitogen activated protein kinases; α-SMA, α-smooth muscle actin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

Nifedipine, one of the dihydropyridine calcium channel blockers (CCBs), is traditionally used for the treatment of hypertension and coronary heart disease. However, recent studies have demonstrated the pleiotropic effects of nifedipine on diabetic nephropathy and atherosclerosis *via* anti-oxidant or anti-inflammatory mechanisms (Hashimoto et al. 2010; Matsui et al. 2009; Yamagishi et al. 2006). A current study demonstrated that nifedipine prevented hepatic fibrosis by increasing peroxisome proliferator-activated receptor γ (PPARγ) activity (Nakagami et al. 2012). Nevertheless, whether nifedipine has a direct anti-fibrotic effect on AngII-induced cardiac fibrosis has not been investigated.

In this study, we evaluated the effects of nifedipine on AngII-induced cardiac fibrosis and explored the intrinsic molecular mechanisms. Our data demonstrated that nifedipine significantly inhibited Ang II-induced cardiac fibrosis by interfering with the production of Nox4-derived ROS and the activation of the MAPK signaling pathway.

2. Investigations and results

2.1. Nifedipine inhibits cardiac fibrolasts proliferation induced by AngII

Cardiac fibroblast proliferation is known as a critical factor in cardiac fibrosis. 5-Ethynyl-2′-deoxyuridine (EdU) assay kit was employed to investigate whether nifedipine affects AngII-induced proliferation of cardiac fibroblasts. As shown in Fig. 1, the new proliferating cells (red color) in AngII (10^{-7} M) group were markedly increased as compared to control group ($P < 0.05$). However, coincubation with nifedipine (10 μM) significantly inhibited the proliferation of cardiac fibroblasts induced by AngII ($P < 0.05$). Nifedipine alone had no effect on normal proliferation of cardiac fibroblasts.

2.2. Nifedipine inhibits cardiac fibroblasts phenotypic differentiation induced by AngII

To determine the role of nifedipine in AngII-induced phenotypic modulation of fibroblasts, the expression of α-SMA was investigated, which is considered as an indicator of fibroblast differentiation into myofibroblast. As shown in Fig. 2A, after stimulation with AngII (10^{-7} M) for 24 h, there was a significant increase in α-SMA expression, compared with the control group ($p < 0.05$), while the upregulation was markedly inhibited by coincubation with nifedipine (10 μM) ($p < 0.05$). These results were also validated by Western blot analysis (Fig. 2B). Nifedipine alone had no effect on the expression of α-SMA.

2.3. Effects of nifedipine on FN and CTGF expression induced by AngII

Upregulation of CTGF in fibrotic tissue appears to correlate closely with the severity of fibrosis, which is considered to be a critical marker gene of cardiac fibrosis (M et al. 2005; Roestenberg et al. 2004). FN is one of the important ECM proteins, which is indispensable for directing cell attachment and migration (KL et al. 2012). To determine the effects of nifedipine on pro-fibrosis factors and ECM accumulation, the expression of CTGF and FN were determined by Western blot analysis. The protein levels of FN and CTGF were greatly increased after AngII (10^{-7} M) stimulation for 24 h ($p < 0.05$). However, AngII-induced upregulation of FN and CTGF were significantly suppressed by nifedipine (10 μM) ($p < 0.05$). Nifedipine alone has no effects on the basal expression levels of FN, CTGF (Fig. 3A and B).

2.4. Effect of nifedipine on AngII-induced variation of $[Ca^{2+}]_i$

It is demonstrated that AngII stimulation evoked the rapid increases of $[Ca^{2+}]_i$ (Fu et al. 1999), and subsequently induced a pathophysiologic cascade in cardiac fibrosis (Chen et al. 2010). The selective fluorescent probe fluo-4/AM was adopted to detected the variations of $[Ca^{2+}]_i$. As shown in Fig. 4A, The $[Ca^{2+}]_i$ fluorescence intensity was markedly increased in response to AngII (10^{-7} M) stimulation, while nifedipine (10 μM) treatment has no effect on the $[Ca^{2+}]_i$ fluorescence intensity elevation induced by AngII ($p > 0.05$) (Fig. 4B and C).

2.5. Effects of nifedipine on the expression of Nox4 and the production of ROS

It is well established that AngII activates NAD(P)H oxidase resultant ROS generation in cardiac fibroblasts and Nox4 is the primary Nox subunit in cardiac fibroblasts (Rocic and Lucchesi 2005; Wang et al. 2012). In our present study, Nox4 expression was significantly increased after AngII (10^{-7} M) stimulation ($p < 0.05$). However, the upregulation of Nox4 was markedly inhibited by coincubation with nifedipine (10 μM) ($p < 0.05$) (Fig. 5A). Furthermore, the production of ROS was obviously increased after the stimulation of AngII ($p < 0.05$), while nifedipine treatment effectively restrained the generation of ROS ($p < 0.05$) (Fig. 5B). Nifedipine alone has no effects on the production of ROS and the basal expression levels of Nox4.

2.6. Nifedipine suppressed the phosphorylation of ERK 1/2, JNK induced by AngII

It is demonstrated that the activation of MAPKs plays an important role in cardiac fibrosis formation (Goette et al. 2002; Muslin 2008). As shown in Fig. 6, AngII (10^{-7} M) stimulation elicited a rapid and robust phosphorylation of ERK1/2, JNK and p38 MAPK ($p < 0.05$). However, nifedipine treatment greatly prevented the phosphorylation of ERK1/2 and JNK induced by AngII (Fig. 6A and B) ($p < 0.05$), but has no effect on phosphorylation of p38 MAPK (Fig. 6C). Nifedipine alone had no effects on the phosphorylation levels of ERK1/2, JNK and p38 MAPK.

2.7. Effects of ERK and JNK inhibition on the expression of FN, CTGF, a-SMA

To validate the roles of ERK1/2 and JNK activation in mediating Ang II-induced cardiac fibrosis, we investigated the effects of pharmacological ERK and JNK inhibitors on a-SMA, FN and CTGF expression. As shown in Fig. 7, inhibiting ERK1/2 activity with PD98059 and JNK activity with SP600125 blocked AngII-induced increases in a-SMA, FN, CTGF expression ($p < 0.05$).

3. Discussion

Cardiac fibrosis, a common complication of hypertension, is demonstrated as a critical risk factor for the development of congestive heart failure (Fedak et al. 2005). Proliferation and differentiation of cardiac fibroblasts into myofibroblasts are pivotal events in the initiation of cardiac fibrosis, which are also responsible for the production of the increased amounts of ECM (Souders et al. 2009). Nifedipine, a dihydropyridine calcium channel blocker (CCB), is widely used for the treatment of hypertension and angina and has been reported to possess mul-

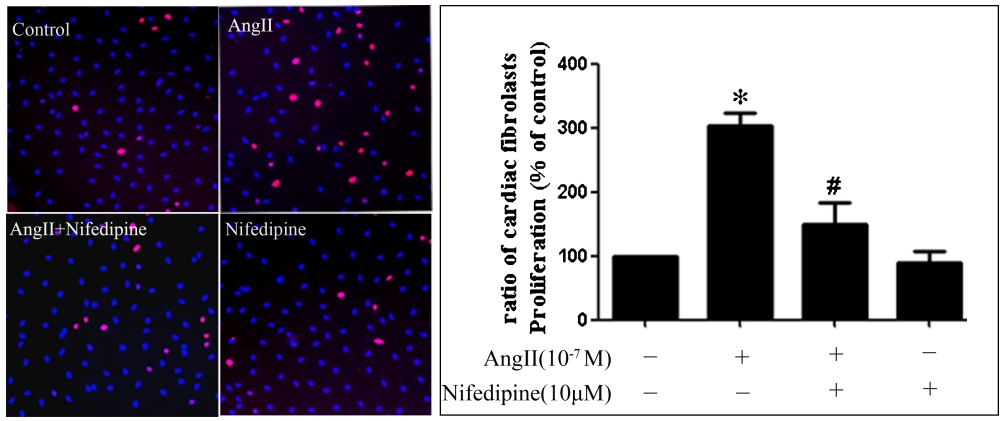

Fig. 1: Effect of nifedipine on cardiac fibroblasts proliferation induced by AngII. Each group was added into EdU (10 μM), respectively, and then cells were treated with AngII (10^{-7} M) and/or nifedipine (10 μM, 1 h prior to AngII stimulation) for 24 h. Similar results were obtained from 3 independent experiments. Data are expressed as mean ± SEM. *$P < 0.05$ vs. control; #$P < 0.05$ vs. AngII treated group

tiple pharmacological actions. Published studies provide new insight into the use of nifedipine for treatment of atherosclerosis, cirrhosis, diabetes and pneumonia independent of its blood-pressure lowering effect (Deshwal et al. 2012; Nakagami et al. 2012; Wang et al. 2011). Nevertheless, whether nifedipine inhibits AngII-induced cardiac fibrosis is still unknown. Thus, the present studies were performed to evaluate the effect of nifedipine on AngII-induced cardiac fibrosis and further explore the underlying mechanisms.

We found that AngII stimulation significantly increased the incorporation of EdU and the expression of a-SMA, CTGF and FN, which were significantly depressed by the treatment of nifedipine. Therefore, our findings revealed that nifedipine may ameliorate AngII-induced cardiac fibrosis by suppressing the proliferation of activated cardiac fibroblasts, inhibiting the differentiation of cardiac fibroblasts as evaluated by expression of a-SMA and reducing pro-fibrotic cytokine and ECM accumulation as assessed by CTGF and FN expression. Since the etiology of cardiac fibrosis is multifactorial, the pleiotropic actions emphasize the potential evaluation of nifedipine as an effective therapeutic agent to the treatment of cardiac fibrosis.

It is well established that Ca^{2+} signals are essential for the development of cardiac fibrosis (Colston et al. 2002; Kumaran and Shivakumar 2002), and in the present study we found that the $[Ca^{2+}]_i$ were significantly increased after stimulation with AngII, these results were consistent with a previous report (Olson et al. 2008). However, our data demonstrated that the anti-fibrotic effects of nifedipine may not be related to its classic calcium channel block activity, since 1) present studies revealed that nifedipine has no inhibitory effect on the increase of intracellular Ca^{2+} induced by AngII, there is no significant difference between the two groups either on initial transient Ca^{2+} peak or the sustained plateau phase induced by AngII in cardiac fibroblasts; 2) another L-type calcium channel blocker verapamil did not exert any anti-fibrotic effect under the same experimental conditions (data not shown), 3) cardiac fibroblasts are non-excitable cells, which are reported to have no functional voltage-dependent calcium channels (Chen et al. 2010; Kohl and Noble 1996). These observations prompted us to investigate the possible mechanisms of the anti-fibrotic effects of nifedipine.

Recently, increasing attention has been given to the role of ROS induced by AngII in cardiac fibroblasts. ROS are derived from a variety of enzyme sources, of which the NAD(P)H oxidase family is considered as the predominant source for cardiac ROS production (Lijnen et al. 2012; Mohazzab et al. 1997; Zhao et al. 2009). Furthermore, previous studies have confirmed that Nox4 is the primary NAD(P)H oxidase subunit in cardiac fibroblasts (Colston et al. 2005; Rocic and Lucchesi 2005), and it is well established that Nox4 plays a critical role in the pathological activation of cardiac fibroblasts in cardiac fibrosis (Cucoranu et al. 2005). Moreover, several Ca^{2+} channel blockers have been demonstrated to possess antioxidant action

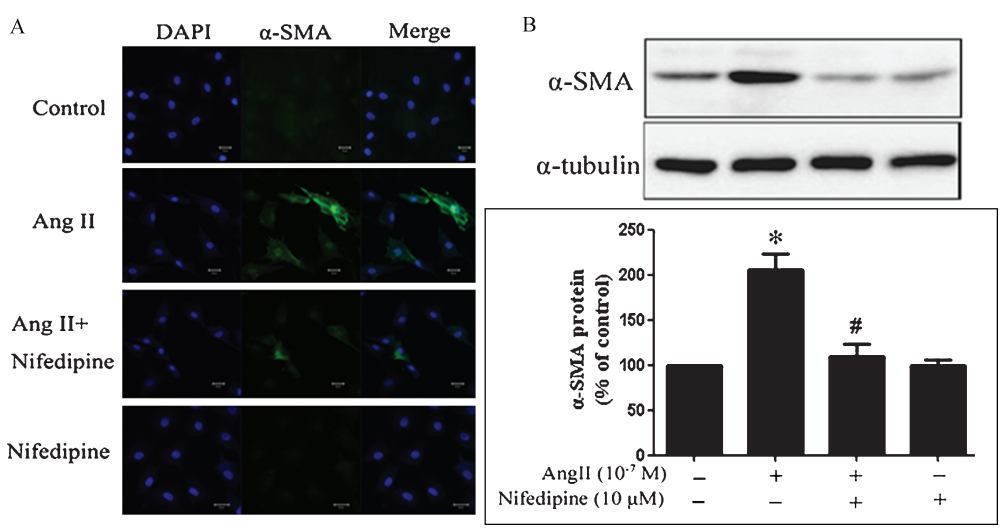

Fig. 2: Effect of nifedipine on cardiac fibroblasts differentiation induced by AngII. Cells were treated with AngII (10^{-7} M) and/or nifedipine (10 μM, 1 h prior to AngII stimulation) for 24 h. (A) The expression of α-SMA (green color) was detected by immunostaining; (B) Protein expression of α-SMA was also identified by Western blot. Similar results were obtained from 3 independent experiments. Data are expressed as mean ± SEM. *$P < 0.05$ vs. control; #$P < 0.05$ vs. AngII treated group

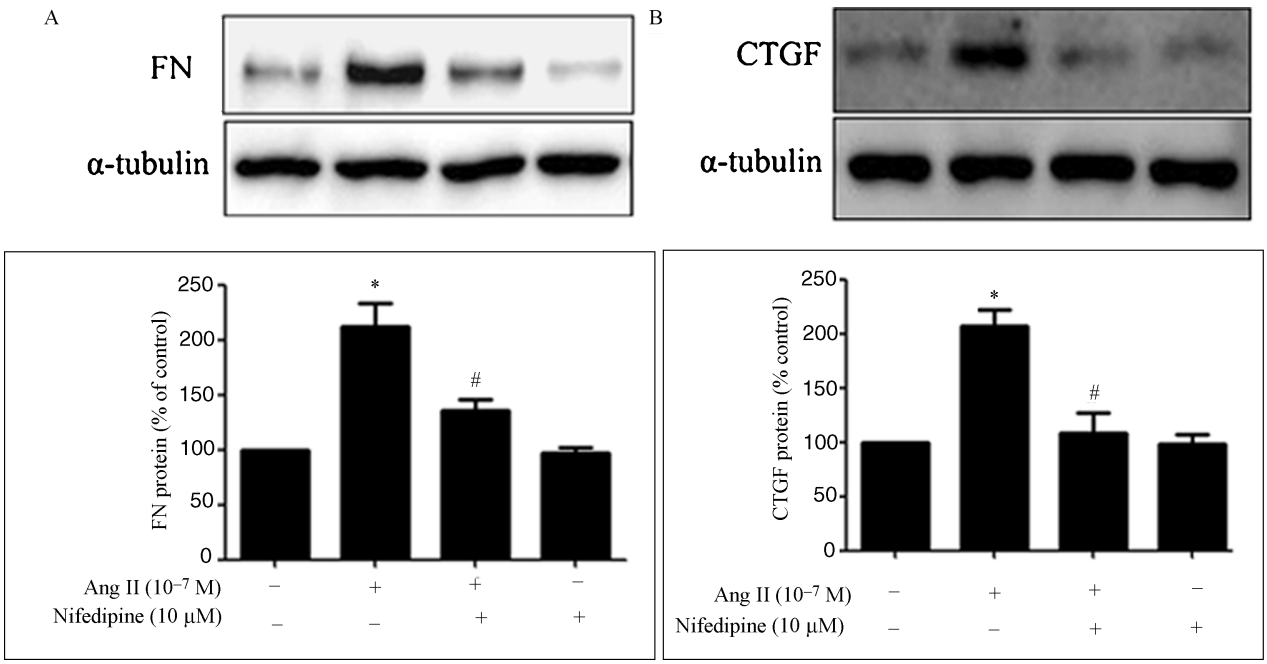

Fig. 3: Effects of nifedipine on AngII-induced upregulation of FN, CTGF in cardiac fibroblasts. Cells were treated with AngII (10^{-7} M) and/or nifedipine (10 μM, 1 h prior to AngII stimulation) for 24 h. The expression of FN (A) and CTGF (B) were identified by Western blot analysis. Similar results were obtained from 3 independent experiments. Data are expressed as mean ± SEM. *$P < 0.05$ vs. control; #$P < 0.05$ vs. AngII treated group

(Cominacini et al. 2003; Sung and Choi 2012), while little is known about whether nifedipine-mediated anti-fibrotic effects are related to the antioxidant action. Our present study indicated that nifedipine effectively suppressed the ROS formation induced by AngII in cardiac fibroblasts and nifedipine treatment significantly inhibited the Nox4 upregulation. This study demonstrated that nifedipine may act as an antioxidant against the oxidative stress response induced by AngII *via* attenuating the Nox4 upregulation and subsequent ROS generation in cardiac fibroblasts.

Accumulated evidence indicated that the activation of MAPK signaling pathway stimulated by AngII greatly contributes to cardiac fibrosis (Fu 2001; Gu et al. 2012). In the present study, we observed that the activation of ERK1/2, JNK and p38 MAPK was significantly increased after AngII stimulation, while nifedipine treatment effectively restrained the phosphorylation of ERK1/2 and JNK, but not p38 MAPK. Of importance, different antagonistic strategies were adopted to assess the function of the ERK and JNK in regulating AngII induced expression of a-SMA, CTGF and FN, and the results indicated that ERK1/2 inhibitor PD98059 and JNK inhibitor SP600125 markedly suppressed the upregulation of a-SMA, CTGF and FN in fibroblasts. The results revealed that nifedipine prevented cardiac fibrosis partly by selectively inhibiting the ERK1/2 and JNK signaling pathways.

In conclusion, the present study shed light on novel molecular mechanisms of nifedipine in attenuating AngII-induced cardiac fibrosis by inhibiting the generation of Nox4-derived ROS and the activation of ERK1/2 and JNK signaling pathways. The anti-fibrotic effects of nifedipine may be independent of its classic calcium channel blocking action. Our work supports the application of nifedipine for the prevention and treatment of cardiac fibrosis.

4. Experimental

4.1. Materials

Antibodies against α-SMA, α-tubulin, and nifedipine were purchased from Sigma–Aldrich (St. Louis, MO). PD98059 (ERK1/2 inhibitor), SP600125 (JNK inhibitor) were purchased from Calbiochem (San Diego, CA, USA).

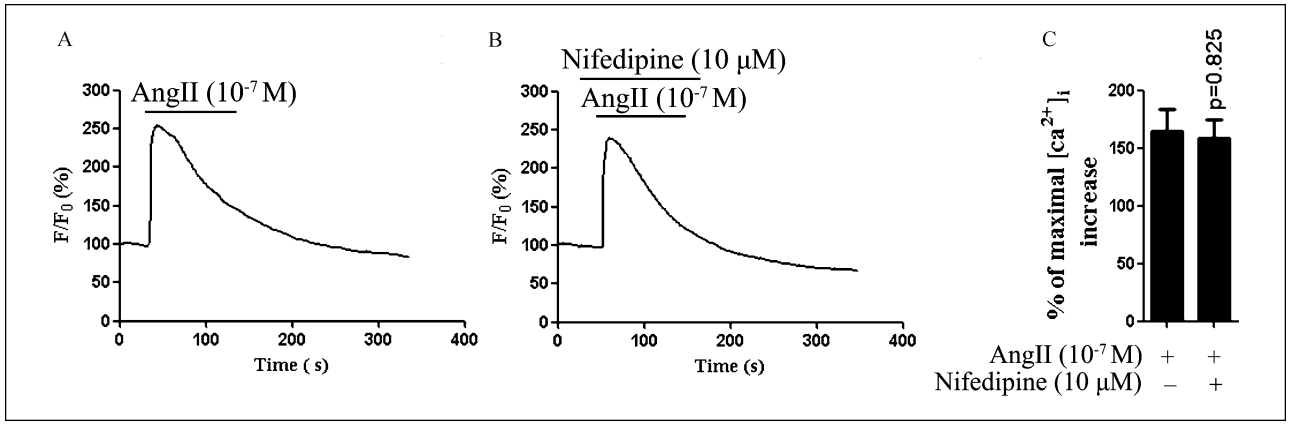

Fig. 4: Effects of nifedipine on AngII-induced $[Ca^{2+}]_i$ increase in cardiac fibroblasts. (A) Cells were stimulated with Ang II (10^{-7} M), and then the variation of $[Ca^{2+}]_i$ was detected (n = 7, the trace is representative mean of seven cells). (B) Cells were preincubated with nifedipine (10 μM) for 30 min, AngII (10^{-7} M) was applied in the continued presence of nifedipine (n = 8, the trace is representative mean of eight cells). (C) The bars represent the maximal $[Ca^{2+}]_i$ changes obtained by Ang II in controls and in the nifedipine-treated cells, P = 0.825

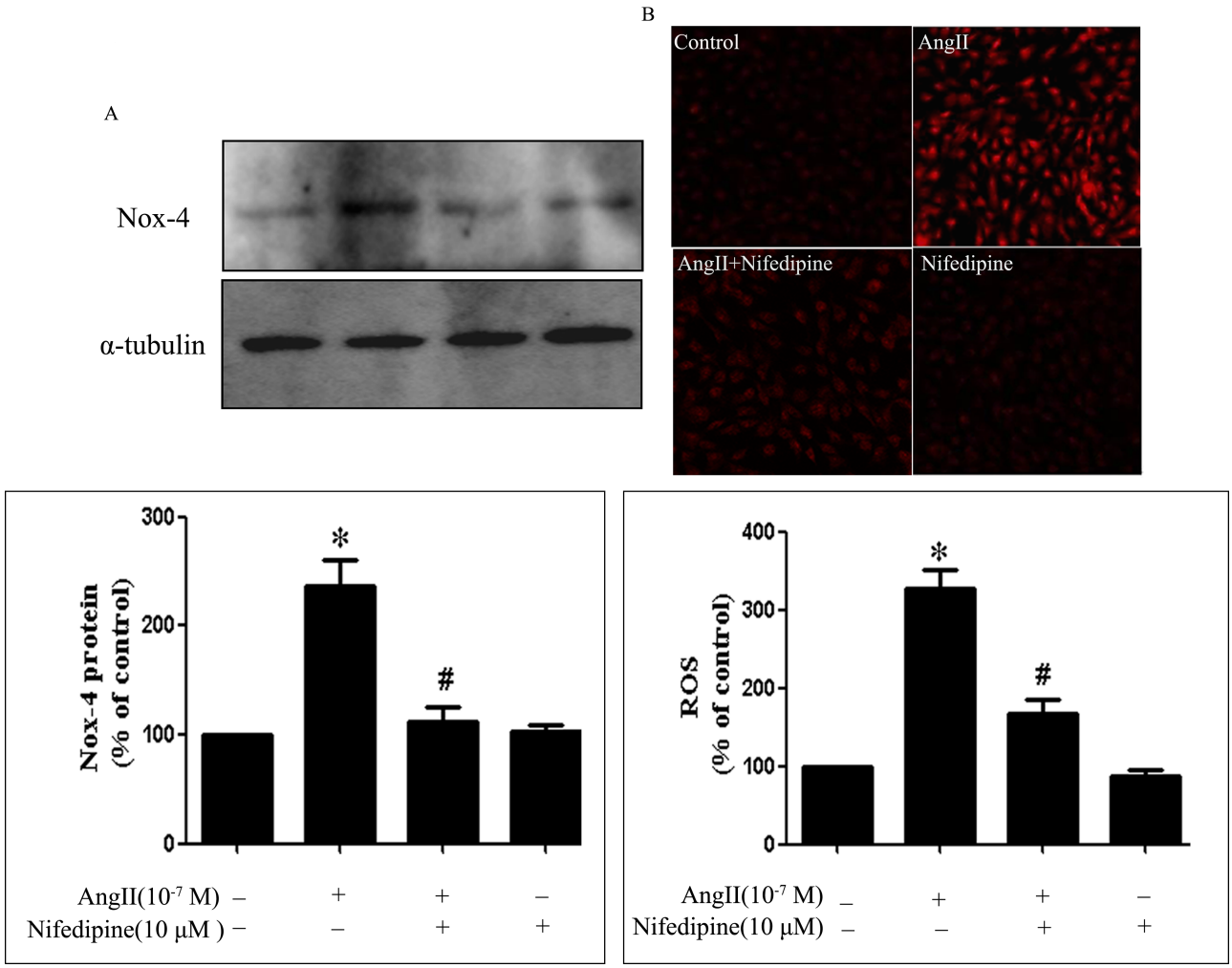

Fig. 5: Effect of nifedipine on AngII-induced expression of Nox4 and the production of ROS. (A) Cells were treated with Angll (10^{-7}M) and/or nifedipine (10 μM), 1h prior to Angll stimulation for 24 h, the expression of Nox4 was analyzed by Western blot. (B) The productions of ROS were determined by DHE (red fluorescence). Similar results were obtained from 3 independet experiments. Data are expressed as mean ± SEM. *P<0.05 vs. control; #P<0.05 vs. Angll treated group.

Antibodies against FN, Nox4 and CTGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit monoclonal antibody against phospho-JNK, phospho-ERK1/2, phospho-p38 MAPK and ERK1/2 were purchased from Cell Signaling technology (CST, Beverly, USA). Other chemicals and reagents were from Sigma–Aldrich unless otherwise indicated.

4.2. Cell isolation and culture

The experimental protocol complied with the Guidelines of Animal Experiments from Ethical Committee for Animal Research of Sun Yat-sen University. Cardiac fibroblasts were isolated from adult male Sprague-Dawley rats (200–250 g) and cultured as described previously (Chen et al. 2004; Zhang et al. 2007). Passages 2–4 were used for experiments.

4.3. Analysis of cell proliferation

Cardiac fibroblasts (1×10^4 cells/well) were grown on a 48-wells plate in DMEM with 10% fetal bovine serum (FBS), 12 h after incubation, the culture medium was replaced with serum-free DMEM, cells were labeled with 5-ethynyl-2′-deoxyuridine (EdU) (10 μM) and treated with AngII (10^{-7} M) and/or nifedipine (10 μM) for 24 h. Cell proliferation was detected by EdU

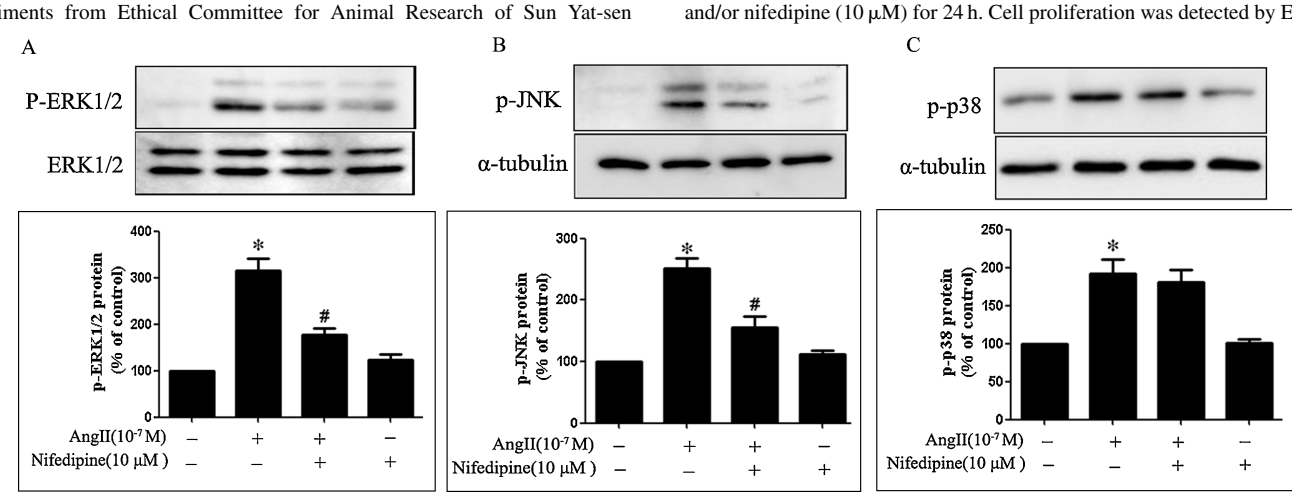

Fig. 6: Effects of nifedipine on the proteins levels of phosphorylation of ERK1/2, JNK and p38 MAPK in cardiac fibroblasts. (A) Nifedipine inhibited AngII-induced phosphorylation of ERK1/2. (B) Nifedipine inhibited AngII-induced phosphorylation of JNK. (C) Nifedipine has no effect on AngII-induced phosphorylation of p38 MAPK. Similar results were obtained from 3 independent experiments. Data are expressed as mean ± SEM. *P < 0.05 vs. control; #P < 0.05 vs. AngII treated group

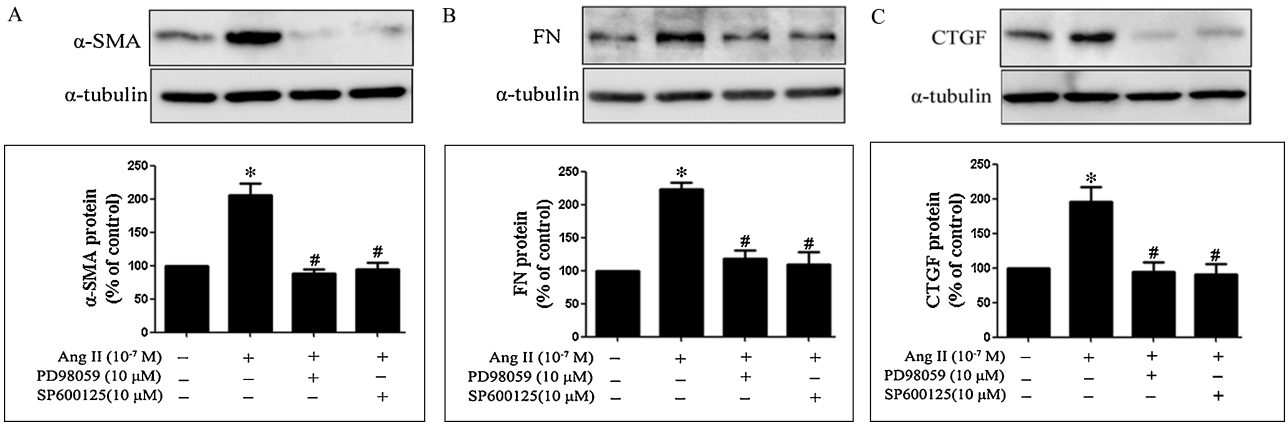

Fig. 7: Effects of PD98059 and SP6000125 on AngII-induced upregulation of a-SMA, FN, CTGF. Cardiac fibroblasts were exposed to PD98059 (10 μM) and SP6000125 (10 μM) for 30 min and then stimulated with AngII (10^{-7} M) for 24 h, the expression of a-SMA (A), FN (B) and CTGF (C) were identified by Western blot, respectively. Similar results were obtained from 3 independent experiments. Data are expressed as mean ± SEM. *$P < 0.05$ vs. control; #$P < 0.05$ vs. AngII treated group

assay kit (Ribobio, China) according to the manufacturer's recommendations. The fluorescence was detected by High Content Analysis (Array Scan VTI, Thermo Fisher).

4.4. Immunofluorescent staining

Cardiac fibroblasts (4×10^4 cells/well) were grown on the coverslips and then treated with AngII (10^{-7} M) and/or nifedipine (10 μM) for 24 h. The fibroblasts were fixed in 4% paraformaldehyde and blocked with 10% normal goat serum for 30 min at room temperature, and then immunoreacted with anti-α-SMA antibody (1:200) overnight at 4 °C, followed by incubation with Alexa 488 labeled anti-mouse IgG (1:1000) for 2 h in the dark at room temperature. The slips were stained with DAPI (Molecular Probes, 10 μg/ml) for 10 min in the dark at room temperature and then were visualized and analyzed with a confocal microscope (Zeiss LSM 710).

4.5. Western blotting

Cardiac fibroblasts were harvested after treatment with AngII (10^{-7} M) and/or nifedipine (10 μM). Total protein was determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein samples were separated by 8% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in Tris buffered saline-Tween 20 (TBS-T) with 5% (w/v) skimmed milk or 2% BSA (w/v) for 1 h at room temperature and then were probed with primary antibodies of α-tubulin (1:10000), FN (1:1000), CTGF (1:1000), α-SMA(1:1000), Nox4 (1:1000), ERK1/2 (1:1000), phosphorylated ERK1/2 (1:1000), phosphorylated p38 (1:1000) or phosphorylated JNK (1:1000) overnight at 4 °C. The blots were washed with TBS-T three times and then incubated with the corresponding secondary antibody for 1 h at room temperature (Promega; 1:5000). Bound antibodies were visualized with enhanced chemiluminescence (Las4000 GE Healthcare). The band of α-tubulin protein was used as the internal control to normalize for protein loading.

4.6. Measurement of $[Ca^{2+}]_i$

Cardiac fibroblasts (4×10^4 cells/well) were grown on confocal dishes, cultured in DMEM medium with 10% FBS for 24 h. The fibroblasts were washed with Tyrode solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 10 HEPES, 10 glucose (PH 7.4 adjusted with NaOH) for three times, then incubated with Fluo-4/AM (5 μM) at 37 °C in the dark for 30 min. After washing out the Fluo-4/AM with Tyrode solution the cells were first treated with nifedipine (10 μM) for 30 min and then were perfused with AngII (10^{-7} M) for 5 min. The fluorescence intensities were acquired on a confocal microscope with 40 × UV fluor oil-immersion objective lens. Fluo-4 fluorescence was excited with the 488-nm line of an argon ion laser. Changes in $[Ca^{2+}]_i$ are reported as the fluo-4 ratio F/F0, F stands for fluorescence intensity and F0 is the Fluo-4 fluorescence intensity recorded under steady-state conditions at the beginning of the experiment.

4.7. Quantification of intracellular ROS levels

Dihydroethidium (DHE, Sigma-Aldrich) was used to evaluate the superoxide levels in the cardiac fibroblasts. Cells were first loaded with DHE (5 μM) at 37 °C in the dark for 30 min and then washed with PBS. After replaced with the fresh medium, they were treated with nifedipine (10 μM) for 30 min and then stimulated with AngII (10^{-7} M) for 30 min. Cells were then harvested, washed with PBS, the fluorescence intensities were acquired with a confocal microscope (Zeiss LSM 710).

4.8. Statistical analysis

All of the data were indicated as means ± SEM. Mean difference among the various treatment groups was tested by One-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. The analyses were performed using the SPSS statistic software 13.0. Difference between groups were considered to be statistically significant when a value of $P < 0.05$.

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