Calcium Signal Pathway is Involved in Prostaglandin E_2 Induced Cardiac Fibrosis in Cardiac Fibroblasts

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ABSTRACT - Prostaglandin E_2 (PGE_2), one of the arachidonic acid metabolites synthetized from arachidonic acid through cyclooxygenase (COX) catalysis, demonstrates multiple physiological and pathological actions through different subtypes of EP receptors. **Purpose:** The present study was designed to explore the effects of PGE_2 on cardiac fibrosis and the involved mechanism. **Methods:** We used Western blot analysis, real-time quantitative PCR and immunostaining to assess the mechanism. **Results:** Our data showed that in cultured adult rat cardiac fibroblasts (CFs), PGE_2 effectively promoted the expression of α-smooth muscle actin (α-SMA), connective tissue growth factor (CTGF), fibronectin (FN)and collagen I, and induced $[Ca^{2+}]_i$ increase. Besides, calcium increase evoked by PGE_2 is mediated by virtue of EP_1 activation. Instead of EP_3 or EP_4, inhibition of EP_1 attenuated PGE_2-stimulated upregulation of α-SMA， CTGF, FN, collagen I and $[Ca^{2+}]_i$, as well as the nuclear factor of activated T cell cytoplasmic 4 protein (NFATc4) translocation. **Conclusions:** PGE_2 appears to promote cardiac fibrosis *via* EP_1 receptor and calcium signal pathway.

INTRODUCTION

As one of the pathological changes in cardiac remodeling, cardiac fibrosis is the key element of cardiac dysfunction development among cardiovascular diseases. Cardiac fibrosis is a complex pathological process, the pathologic features of which include the phenotypic change and excessive proliferation of cardiac fibroblasts (CFs), disordered arrangement and disproportionate increase of myocardial collagen, and excessive deposition of extracellular matrix (ECM) protein. It is considered that long-term pressure overload, neurohumoral factors stimulation, ischemia and oxidative stress trigger the inflammatory reactions in heart diseases [2]. Inflammation is widely accepted as a component of pathogenesis and progression of cardiac fibrosis and in return, increased inflammatory cytokines and acute-phase reactants unambiguously promote the development of cardiac fibroblasts [3, 4]. As the key enzyme mediating inflammatory response, cyclooxygenase-2 (COX-2) plays an important role in cardiovascular diseases. Our previous research found that angiotensin II (Ang II) upregulated cardiac COX-2 expression in cardiac fibroblasts and inhibition of COX-2 could attenuate Ang II-induced cardiac fibrosis [5]. In addition, in ischemic myocardium, induction of COX-2 resulted in myocardial dysfunction and remodeling linked with chronic heart failure [6]. However, until now, there is no evidence showing that COX-2 could act as signaling molecules, so it is believed that COX-2 may provoke cardiac fibrosis reaction through its downstream products -- prostaglandins (PGs).

Prostaglandins (PGs) are synthetized from arachidonic acid *via* COX catalysis. As the substrates of COX, prostaglandins are a kind of arachidonic acid metabolites, which are ubiquitously distributed and participate in a variety of inflammatory responses [7]. Among prostaglandins, PGE_2 is the most widely produced prostaglandin in the body and acts versatilely. PGE_2 is also regarded as a key factor to the pathogenesis of cardiac remodeling and the related studies have

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become more extensive in breadth and depth. In ventricular cardiomyocytes, PGE_2 induces the activation of Stat3, which plays an essential role in PGE_2-induced increase in cell size and protein synthesis [8]. In contrast, Hardling et al. indicated that PGE_2 stimulated proliferation of CFs *via* p42/44 MAPK signaling and Akt-regulation of cyclin D3 [9]. It has been revealed that PGE_2 could limit myofibroblast proliferation, transformation, and collagen secretion *via* EP_2 receptor in pulmonary fibrosis [10]. Nakagawa N et al. reported that PGE_2-EP_4 is an endogenous renoprotective system that limits the renal fibrosis by acting on multiple cellular targets [11]. PGE_2 is functionally versatile but the specific mechanism by which PGE_2 works on fibrosis is not fully understood. In present study, we investigated the role of PGE_2 in cardiac fibrosis and further experiments were carried out to unravel its mechanism.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), new born calf serum (NBCS), collagenase type II, penicillin/streptomycin, Lipofectamine 2000 and fluo-4/AM were purchased from Life technologies/ Invitrogen (Carlsbad, CA., USA). Anti-NFATc4, anti-FN, anti-α-SMA antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-CTGF, anti-Collagen I antibodies were purchased from Cell Signaling Technology (Boston, MA., USA). Trypsin, 3-(4, 5-dimethylthiazol-2-y1)-2, 5-dipheny-ltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), Anti-α-tubulin antibody, PGE_2, NS-398 (specific antagonist of COX-2), SC19220 (specific antagonist of EP_1) and L-161,982 (specific inhibitor of EP_4) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Ang II was purchased from Merck Millipore (Billerica, MA, USA).

Cell culture

The handling of animals and the experimental procedures for the experiments were strictly abided to the Guidelines of Animal Experiments from Animal Ethical and Welfare Committee of Sun Yat-sen University (Guangdong, Guangzhou, China). Cardiac fibroblasts were isolated and cultured as described previously [12, 13]. Briefly, adult male Sprague-Dawley rats (220-240g) were anaesthetized with 3% sodium pentobarbital (dissolved in physiologic buffer, 30 mg/kg of body weight, i.p.). After induction of deep anesthesia, rat hearts were excised, minced, and digested with 0.1% collagenase type II solution (37°C, 30 min), 0.25% trypsin for three 5-min periods and 0.1% collagenase type II for two or three 30-min periods. After digestion, the cells were pelleted and suspended in $75mm^2$ culture flasks (Corning Inc., NY., USA) with DMEM supplemented with 1% penicillin and streptomycin and 10% New Born Calf Serum. After a 60 min incubation period, unattached or weakly attached cells were removed, and well-attached cells were further cultured at 37 °C in a humidified atmosphere of 5% CO_2. On the following day, cells were washed three times with PBS and fresh DMEM was replenished. After 2 to 3 days, the confluent cells were detached by 0.25% trypsin and seeded at a density of 10^5 cells/ml in new 35mm*10mm dishes or 96-well plates (Corning Inc., NY., USA). Passages from second to fourth of cardiac fibroblasts were used for all experiments.

Measurement of PGE_2 release

Cardiac fibroblasts were incubated in 24-well plate with a density of 10^5 cells/ml overnight. After deprivation of serum for 12 h, the cells were treated with NS-398 at 10 μM for 1 h followed by treatment with Ang II (100nM) for 8 h. Then the supernatant of cardiac fibroblasts were collected and detected with an ELISA kit following the manufacturer's instruction (Enzo Life Sciences, Farmingdale, USA).

Western blot analysis

After treatment, total cell proteins were harvested using RIPA lysis buffer with protease inhibitor cocktail (Beyotime, Nantong, Jiangsu, China) on ice and protein concentrations were determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts of whole-cell lysate samples were separated by 8% or 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (PVDF, Bio-Rad, Hercules, CA, USA). The membranes were blocked in Tris buffered saline-Tween 20 (TBS-T) with 5% (w/v) skimmed milk for 1.5 h at room temperature and then were probed with anti-α-SMA (42 kDa, 1:1000 dilution), anti-CTGF (37 kDa, 1:1000 dilution), anti-FN (220 kDa, 1:500 dilution), anti-Collagen I (170kDa, 1:500 dilution) and anti-α-tubulin (55 kDa, 1:5000 dilution) at 4 ℃ overnight. The blots were washed three times with TBS-T and then incubated with corresponding horseradish peroxidases-labeled secondary antibodies (1:2500 dilution, Santa Cruz

Biotechnology, CA., USA) for 1 h at room temperature. Then the blots were visualized with enhanced chemiluminescence (ImageQuant LAS 4000, GE Healthcare Life Science, USA). The relative intensities of protein bands were analyzed by using Quantity-One software (Bio-Rad, Hercules, CA., USA) and the intensity of each protein band was normalized by that of α-tubulin.

RNA isolation and real-time quantitative PCR

CFs total RNA were isolated using TRI-zol reagent (Invitrogen, Carlsbad, CA., USA), and reverse-transcribed to cDNA using reverse transcription Kit (Thermo Scientific, Waltham, MA., USA) according to the instructions. Real-time quantitative PCR was performed with the above prepared cDNA and SYBR Green Master Mix (Thermo Scientific, Waltham, MA., USA) in real-time PCR amplifier (Thermo Scientific, Waltham, MA., USA). The amplification conditions were 7 min at 95 ℃, followed by 40 cycles of 10 s at 95 ℃, 30 s at 60 ℃ and 10 s at 20 ℃. Primer sequences were as follows EP_1: forward primer 5'-CCCCTACGGGCTTAACCTGA-3', reverse primer 5'-TTGGCAGCACCACAGATGTAT-3'; EP_2: forward primer 5'-GCTCCCTGCCTTTCACAATCT-3', reverse primer 5'-GTCCGACAACAGAGGACTGA-3'; EP_3: forward primer 5'-CTGCCAGATGATGAACAACCTG-3', reverse primer 5'-CCCTGGGGGAAGAAATTCTCA-3'; EP_4: forward primer 5'-CTTCGGTGGCCTGTCGG-3', reverse primer 5'-GATGAACACTCGCACCACGA-3'; β-actin: forward primer 5'-TCGTGCGTGACATTAAAGAG-3', reverse primer 5'-ATTGCCGATAGTGATGACCT-3' (synthesized by Sangon Biotech Co., Ltd, Shanghai, China). β-actin was used to normalize the mRNA level of each sample. Data were showed as fold changes over control group.

Cytotoxicity assay

Cardiac fibroblasts were seeded in 96-wells plate with a density of 5×10^4 cells/ml overnight. Then the cells were treated with different concentrations (5, 10, 15, and 20 μM) of NS-398, SC19220 or L-161,982 for 24 h. After that, the supernatants were removed and MTT (5 mg/ml in serum-free medium, 20 μl/well) was added to each well. The cells were cultured for an additional 4 h at 37 °C. Finally, the MTT medium was aspirated, 150 μl DMSO was added and the optical density was measured at 490 nm with an automated micro-plate reader (Bio-Tek, Winooski, VT., USA).

RNA interference

Cardiac fibroblasts were transiently transfected with selected 100 nM of siRNA targeting to EP_1 (The oligo sequence was designed by GenePharma Co., Ltd Shanghai, China) by using 5 μl of the siRNA transfection reagent. The RT-qPCR was employed to compare silencing efficiency of different duplex siRNAs. For a negative control, cells were transfected with a control siRNA duplex. After 48 h of transfection, Western blot assay, immunostaining, and calcium imaging were used to detect the corresponding effects.

Immunostaining

CFs were placed in special-made confocal dishes with a density of 2×10^4 cells/ml overnight. After pretreatments with different methods, CFs were fixed with 4% paraformaldehyde (Boster, Wuhan, Hubei, China) for 30 min, and permeabilized with 1% Triton X-100 (Boster, Wuhan, Hubei, China) for 10 min, then blocked with 10% goat serum for 1 h, and incubated with rabbit anti-NFATc4 antibody (1:150 dilution) at 4 ℃ overnight, then followed by fluorescent Alexa 488 goat anti-rabbit secondary antibody (1:1000 dilution, Thermo Scientific, Waltham, MA., USA) for 1 h at room temperature. After washing in PBS, CFs were stained with Hoechst 33342 (DNA dye, 1:1000 dilution, Boster, Wuhan, Hubei, China) for 10 min. Finally, the immunostained CFs were determined by Zeiss LSM 710 confocal microscope.

Measurement of $[Ca^{2+}]_i$ in CFs

Fluo-4/AM, the selective fluorescent probe, was employed to measure $[Ca^{2+}]_i$ in cardiac fibroblasts. CFs were seeded in special-made confocal dishes with a density of 10^4 cells/ml overnight. After different treatments, CFs were loaded with 5 μM Fluo-4/AM at 37°C for 30 min in standard Tyrode solution (in mmol/L: 145 NaCl, 4 KCl, 10 glucose, 1 $MgCl_2$, 1.8 $CaCl_2$ and 10 HEPES [pH 7.4]) or Ca^{2+}-free Tyrode solution (in mmol/L: 80 NaCl, 10 KCl, 10 glucose, 1 KH_2PO_4, 5 $MgSO_4$, 50 ethylamine sulfonic acid, 5 HEPES), and CFs were detected by using Laser Scanning Confocal Microscope (Zeiss LSM 710) under 488 nm line of an argon ion laser. The result for each group was the mean value of 10 cells which were randomly observed. Changes in $[Ca^{2+}]_i$ were showed as F/F_0 ratio, where F was fluorescence intensity and F_0 was fluorescence intensity of the fluo-4 baseline

recorded at the beginning of the experiment.

STATISTICAL ANALYSIS

All data were expressed as means±SEM. The statistical significance of mean differences was determined by one-way ANOVA with Tukey *post hoc* test for multiple comparisons involved using GraphPad Prism Software (GraphPad Inc. La Jolla, CA., USA). The summary result data were from at least three independent experiments.

RESULTS

1. NS-398 attenuated the production of PGE_2 induced by Ang II

MTT assay in Fig.S1 showed that 5 to 20 μM of NS-398 exhibited no obvious cytotoxic effect and 10 μM was used in the following experiments. It has been reported that PGE_2 could be largely produced when COX-2 was activated [14]. The culture medium of CFs was collected after Ang II-induced treatment to measure the concentration of PGE_2. As shown in Fig.1, PGE_2 release was increased after Ang II (100 nM) stimulation for 8 h ($P<0.05$), but 10 μM of NS-398 significantly suppressed the upregulation of PGE_2 secretion ($P<0.05$).

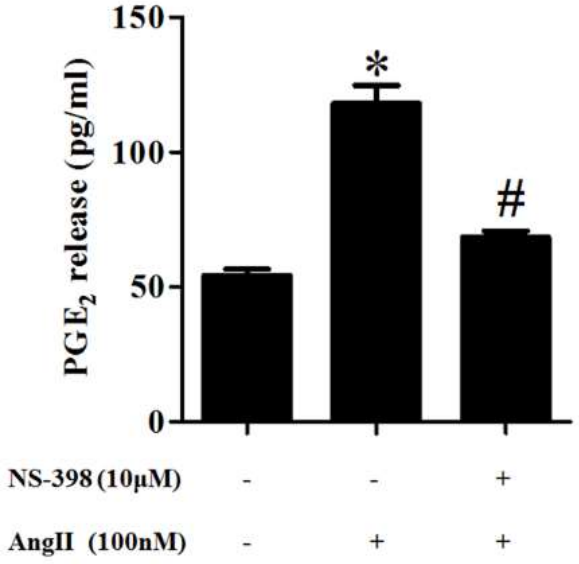

Figure 1. Effect of NS-398 on the production of PGE_2 in CFs. The culture medium was subjected to ELISA for the determination of PGE_2 secretion. The results are expressed as mean±SEM of three independent experiments. $^{*}P<0.05$ vs. control, $^{\#}P<0.05$ vs. Ang II treated group. CFs: cardiac fibroblasts; NS-398: specific inhibitor of COX-2; PGE_2: Prostaglandin E_2.

2. PGE_2 induced the expression of α-SMA，CTGF, FN and Collagen I in CFs

In the development of myocardial fibrosis, upregulation of α-SMA and CTGF expression, which are excellent surrogate markers for activated fibroblasts in fibrosis, devote to myofibroblast differentiation and persistence [15]. Increased collagen I synthesis is another characteristic of fibroblast activation. FN is an omnipresent extracellular matrix glycoprotein which is integrant for regulating cell migration and attachment [16]. To investigate whether PGE_2 could induce cardiac fibrosis, Western blot assay was used. 10^{-8}, 10^{-7} and 10^{-6} M of PGE_2 were used to stimulate CFs for 24 h. It is observed that 10^{-7} and 10^{-6} M of PGE_2 could increase the protein levels of α-SMA，CTGF, FN and Collagen I ($P<0.05$, Fig. 2A-D).

3. PGE_2 could promote calcium influx and intracellular calcium release.

It is well known that the increase of $[Ca^{2+}]_i$ and subsequently induced pathophysiologic cascade are essential for the development of cardiac fibrosis [17, 18].To explore if PGE_2 could induce $[Ca^{2+}]_i$ elevation in CFs, Ca^{2+} in CFs was stained with Fluo-4/AM. CFs were incubated with standard Tyrode solution during the measurement. Ca^{2+} peak occurred transiently after added with 10^{-6} M of PGE_2 and was gradually back to the baseline (Fig. 3A). As shown in Fig. 3B, CFs were first treated with thapsigargin (TG, 2 μM, Sigma-Aldrich, St. Louis, MO., USA), and then stimulated with 10^{-6} M of PGE_2 when the increase of $[Ca^{2+}]_i$ was back to base level. Evidently, Ca^{2+} peak occurred again and gradually fell back to the baseline. To further investigate whether PGE_2 could induce intracellular calcium release, Ca^{2+}-free Tyrode solution was applied. CFs in Ca^{2+}-free Tyrode solution were treated with 10^{-6} M of PGE_2, Ca^{2+} peak occurred transiently and dropped back to the baseline instantly (Fig. 3C). Taken together, these results revealed that PGE_2 could significantly induce calcium influx and promote intracellular calcium release in CFs.

4. Effect of SC19220 and L-161,982 on the expression of FN and CTGF induced by PGE_2

To investgate the mechanism of PGE_2 effect, RT-PCR was performed to detect the expression of EP in CFs. As shown in Fig.4A, in resting state, EP_1 and EP_4 mRNA were expressed much more abundant than EP_2 in CFs ($P<0.05$), and EP_3 expression had no difference compared with EP_2. To affirm the involvement of EP_1 and EP_4 receptor,

specific inhibitors were used in the following experiments.

After 24 h treatment of various concentrations of SC19220 (specific antagonist of EP_1) or L-161,982 (specific antagonist of EP_4), MTT assay was used to determine cell viability. As shown in Fig.4B and C, no obvious cytotoxic effect was observed with 5 to 20 μM of SC19220 or L-161,982. Therefore, 10 μM was used as the concentrations of compounds in the following experiments. To investigate whether inhibition of EP_1 and EP_4 affects the expression of FN and CTGF, CFs was pre-incubated with SC19220 or L-161,982 for 1 h. Our data showed that 10 μM of SC19220 diminished the protein levels of FN and CTGF stimulated by PGE_2 (1 μM, 24 h, $P<0.05$), but 10 μM of L-161,982 had no obvious effect on the upregulation of FN and CTGF (Fig. 4D and E), manifesting initially that EP_1 other than EP_4 participated in PGE_2-induced cardiac fibrosis.

5. Knockdown of EP_1 affected PGE_2-induced $[Ca^{2+}]_i$ and NFATc4 translocation

To farther corroborate if EP_1 affected PGE_2-induced $[Ca^{2+}]_i$ increase in CFs, RNA interference was used. A transfection experiment of CFs with siRNA was performed and RT-qPCR was employed to compare the efficiency of three independent siRNAs, marked si001, si002, si003. As shown in Fig. 5A, si003 decreased the mRNA level of EP_1 by 80% ($P<0.05$) and it was used in the following experiments (named siEP_1). After transfection with siRNA-EP_1, CFs in standard Tyrode solution were stimulated with TG (2 μM) first, and then with PGE_2 (Fig. 5C) or directly with 1 μM of PGE_2 (Fig.5B). $[Ca^{2+}]_i$ of siRNA-EP_1 treated group was obviously decreased compared with $[Ca^{2+}]_i$ of negative control group. To further investigate whether EP_1 could affect PGE_2-induced intracellular calcium release, siRNA-EP_1- transfected CFs in Ca^{2+}-free Tyrode solution were treated with 10^{-6} M of PGE_2. As shown in Fig. 5D, silencing EP_1 significantly decreased $[Ca^{2+}]_i$ evoked by PGE_2. Taken together, these results revealed that EP_1 could attenuate PGE_2-induced calcium influx and intracellular calcium release.

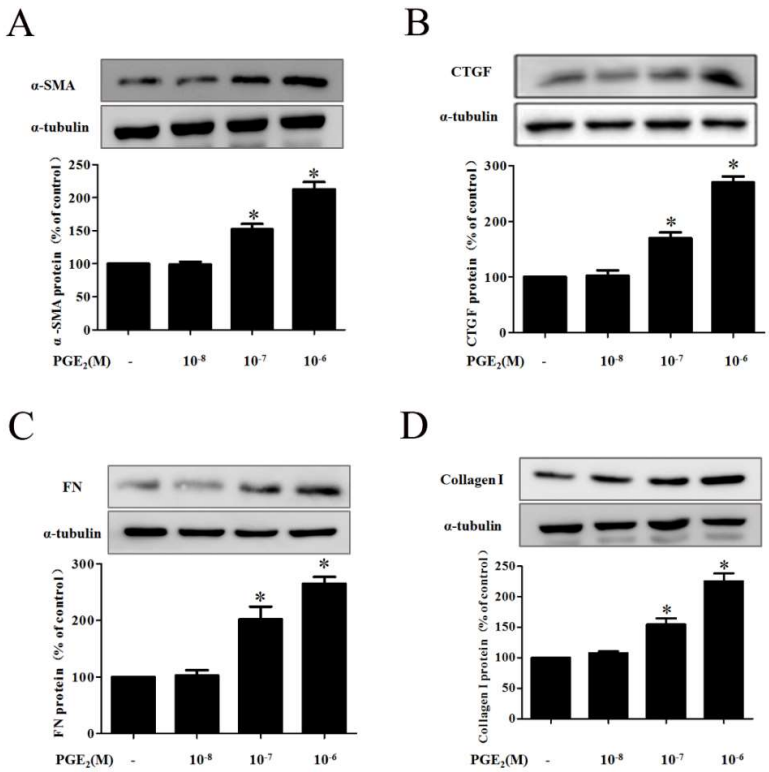

Figure 2. PGE_2 induced the expression of α-SMA, CTGF, FN and Collagen I in CFs. Cells were treated with different concentration of PGE_2 for 24 h. Protein expression of α-SMA (A), CTGF (B), FN (C) and Collagen I (D) was examined by Western blot. The results are expressed as mean±SEM of three independent experiments. $^*P<0.05$ vs. control. α-SMA: α-smooth muscle actin; CTGF: connective tissue growth factor; FN: fibronectin; PGE_2: Prostaglandin E_2; TG: thapsigargin.

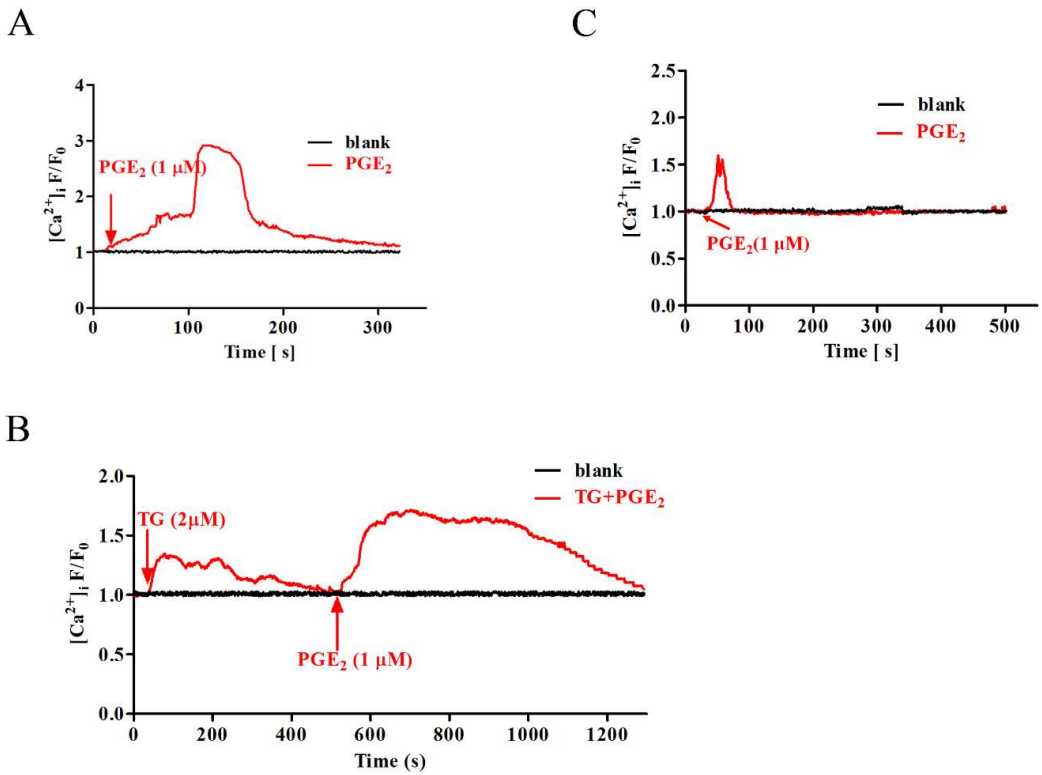

Figure 3. PGE_2 induced $[Ca^{2+}]_i$ increasing in cardiac fibroblasts. (A) Cells in Tyrode's solution were stimulated with PGE_2 (1 μM), and then the variation of $[Ca^{2+}]_i$ was detected (n = 10, the trace is representative mean of ten cells). (B) Cells in Tyrode's solution were stimulated with TG (2 μM) first, and then were stimulated with PGE_2 (1 μM) when the increase of $[Ca^{2+}]_i$ was back to base value. The variation of $[Ca^{2+}]_i$ was detected (n = 10, the trace is representative mean of ten cells). (C) Cells in Ca^{2+}-free Tyrode's solution were stimulated with PGE_2 (1 μM), and then the variation of $[Ca^{2+}]_i$ was detected (n = 10, the trace is representative mean of ten cells). PGE_2: Prostaglandin E_2; TG: thapsigargin.

NFATc4 is an important transcription factor in regulating cardiac fibrosis. Cellular calcium signals might activate the calcineurin pathway, resulting in rapid dephosphorylation of NFATc4 and then NFATc4 was translocated into the nucleus, which could produce a synergistic effect in regulating gene expression. [19, 20]. Hence, NFATc4 translocation was detected to determine whether the downstream of calcium signals were influenced by EP_1 silencing. In our study, the induction with PGE_2 (1 μM) for 4 h led to a shift of NFATc4 localization from cytosol to nucleus in CFs, but translocation did not happen in siRNA-EP_1-treated CFs (Fig. 6).

6. Knockdown of EP_1 affected PGE_2-induced α-SMA, CTGF, FN and Collagen I expression

To further verify the effects of EP_1 in PGE_2-induced cardiac fibrosis, we took approach of RNA interference to detect the protein level of fibrosis markers. Our results showed that knockdown of EP_1 could apparently suppress the upregulation of α-SMA, CTGF, FN and Collagen I stimulated by PGE_2 (1 μM, 24 h, $P<0.05$) and negative control sequence had no such effect (Fig. 7A-D).

DISCUSSION

PGE_2 has been extensively studied in the cardiovascular system. Pamela Harding et al. found that the functional subtypes of EP receptors could stimulate growth of neonatal cardiac fibroblasts [9]. However, adults are more heart disease-susceptible. The phenotype and function may be different in growth stage and developmental epochs. In our study, adult cardiac fibroblasts were used to better simulate the pathogenesis process in vitro model. And we found that PGE_2 promoted expression of pro-fibrotic proteins including α-SMA, CTGF, FN, and Collagen I (Fig.2A-D), indicating that PGE_2 may induce cardiac fibrosis by increasing pro-fibrotic genes and ECM accumulation. Interestingly, some researches showed that PGE_2 exerted antifibrotic effect on pulmonary and kidney fibrosis [11, 21, 22]. We speculate that the diverse effects of PGE_2 on fibrosis are due to particular receptors.

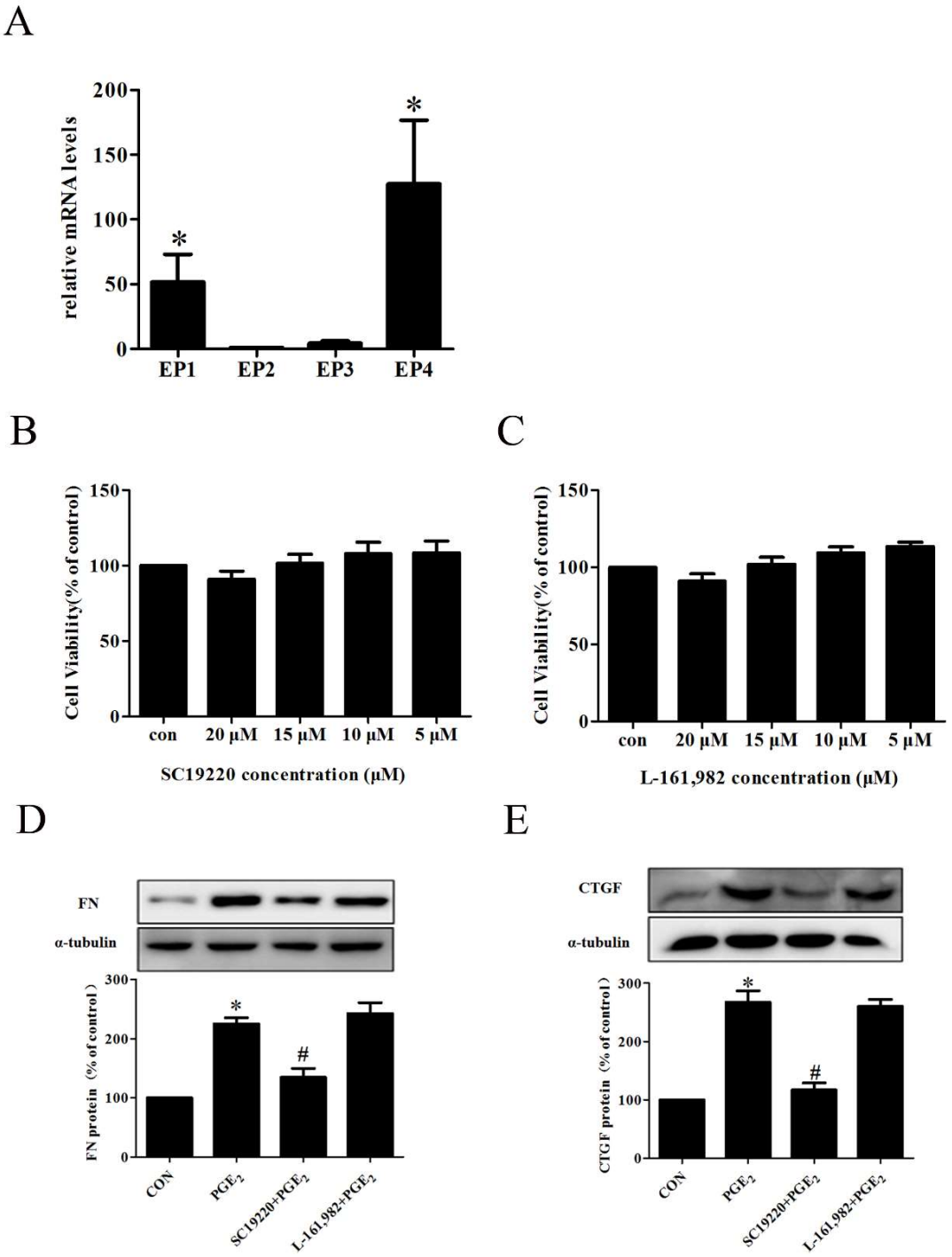

Figure 4. Effect of Selective EP1 or EP4 inhibitors on PGE_2-induced FN, CTGF upregulation in CFs. (A) The mRNA level of each gene was evaluated by real time PCR analysis using gene-specific primer. β-actin was used to normalize the mRNA level of each sample. Data shown are the fold increase in signal intensity compared with those of EP2 group and are presented as the means ± SD of values obtained from three independent experiments. *P＜0.05 vs. EP2 group. (B) Effect of SC19220 on cell viability of cardiac fibroblasts. Data are presented as mean±SEM of three independent experiments. P>0.05. (C) Effect of L-161,982 on cell viability of cardiac fibroblasts. Data are presented as mean±SEM of three independent experiments. P >0.05. Cells were treated with PGE_2 (1 μM) or vehicle control in the presence of SC19220 or L-161,982 (10 μM, 1 h prior to PGE_2 stimulation) for 24 h. Protein expression of FN (D) and CTGF (E) was examined by Western blot. The results are expressed as mean±SEM of three independent experiments.*P＜0.05 vs. control, #P ＜ 0.05 vs. PGE_2 treated group. CTGF: connective tissue growth factor; FN: fibronectin; PGE_2: Prostaglandin E_2.

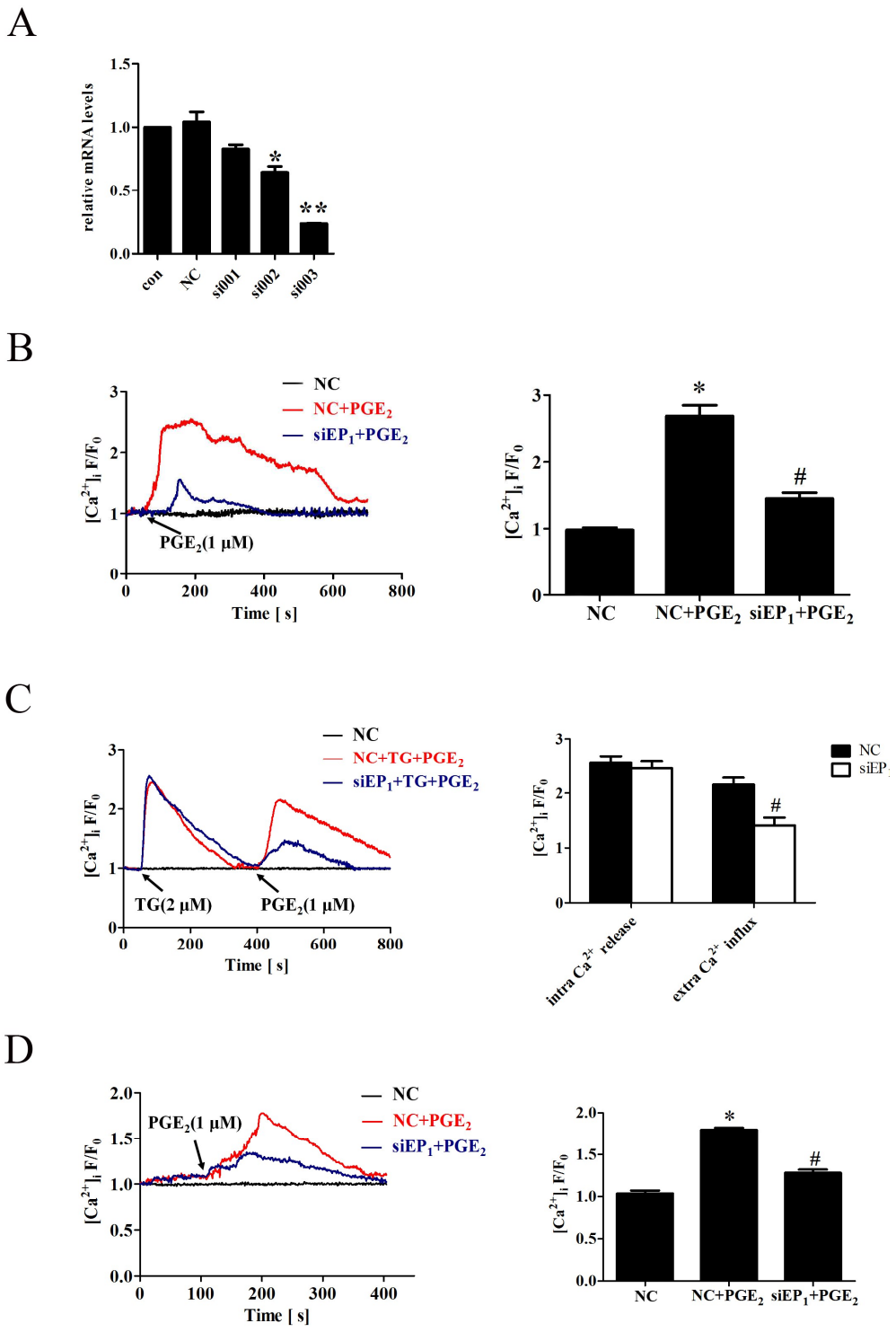

Figure 5. Change of $[Ca^{2+}]_i$ in siRNA-EP1 and negative control cells. (A) The efficiency for EP1 knockdown was determined by RT-qPCR. Data shown are the fold increase in signal intensity compared with those of control cells and are presented as the means ± SD of values obtained from three independent experiments. *P＜0.05 vs. control, **P＜0.01 vs. control. Negative control cells or siRNA-EP1 cells in Tyrode's solution were stimulated with TG (2 μM) first, and then were stimulated with PGE_2 (C) or directly with PGE_2 (1 μM) (B), and then the variation of $[Ca^{2+}]_i$ was detected (n = 10, the trace is representative mean of ten cells). (D) Negative control cells or siRNA-EP1 cells in Ca^{2+}-free Tyrode's solution were stimulated with PGE_2 (1 μM), and then the variation of $[Ca^{2+}]_i$ was detected (n = 10, the trace is representative mean of ten cells). *P＜0.05 vs. blank, #P＜0.05 vs. PGE_2 treated negative control group. PGE_2: Prostaglandin E_2; TG: thapsigargin.

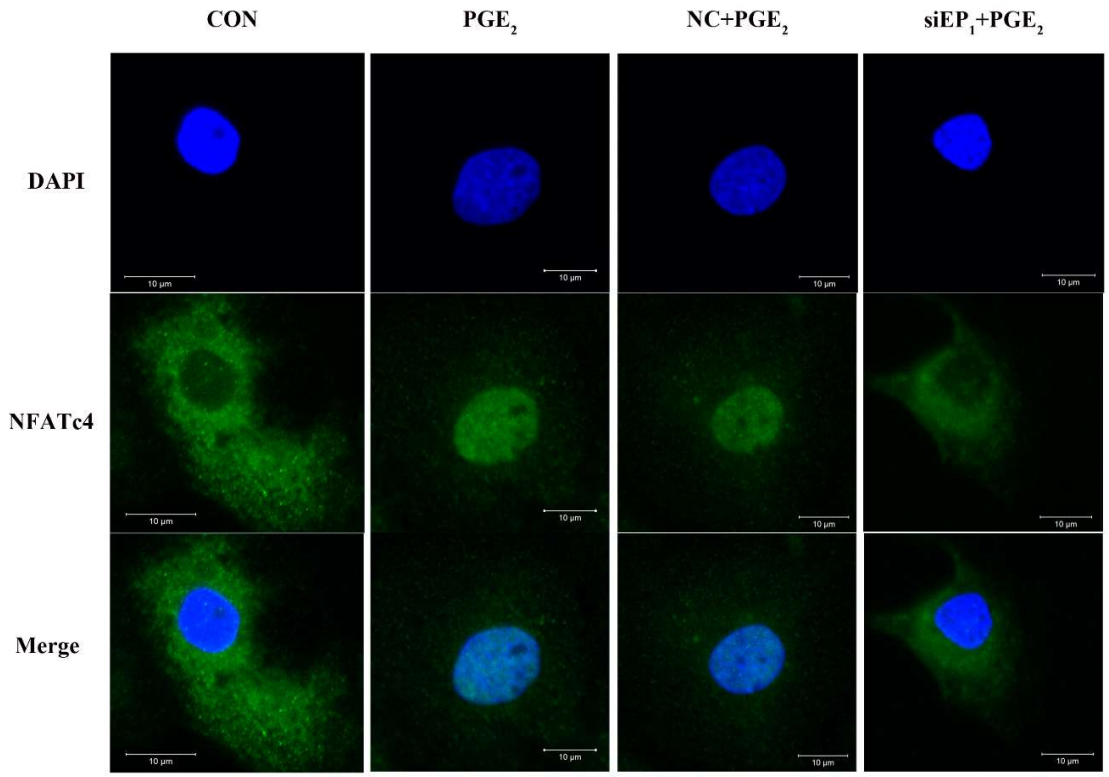

Figure 6. NFATc4 translocation in siRNA-EP1 and negative control cells. Immunocytochemical NFATc4 translocation in siRNA-EP1 and negative control cells (magnification 1000×). NFATc4: nuclear factor of activated T cell cytoplasmic 4 protein.

It is reported that all four EP-receptor subtypes are expressed in heart and involved in different signaling pathways [23]. It is believed that the specific cardiovascular effects of the subtype receptors play critical roles in pathogenesis of diseases. We also detected all four EP receptors expressed in adult cardiac fibroblasts (Fig.4A). EP receptors belong to the large family of seven transmembrane domain receptors coupled to specific G proteins with different second messenger signaling pathways [24]. EP_3 could couple to $G\alpha_i$ for signaling and inhibit adenylyl cyclase (AC) activation resulting in decreased cAMP concentrations [23]. And there are additional signaling mechanisms of EP_3 including Ca^{2+} release [25]. Because EP_3 was involved in cell calcium elevation, the measurement of $[Ca^{2+}]_i$ experiments were performed to distinguish which subtype was in a dominant position in PGE_2-induced cardiac fibroblasts. And our result showed that EP_3 knockdown by siRNA had no obvious effect on $[Ca^{2+}]_i$ and PGE_2-induced cardiac fibroblasts (Fig.S2-3). EP_1 couples to $G\alpha_q$ protein and signals through the phospholipase C (PLC)/inositol 1,4,5-trisphosphate (IP_3) pathway resulting in the formation of the second messengers diacylglycerol (DAG) and IP_3, with the latter rapidly increased $[Ca^{2+}]_i$ [26]. And in our study, EP_1 interference attenuated the calcium increases induced by PGE_2 (fig.5B-D). Moreover, specific antagonist of EP_1 or siRNA- EP_1 could suppress the protein expression of fibrosis markers increased by PGE_2, whereas the specific antagonist of EP_4 (Fig.4D-E) had no obvious effect on cardiac fibrosis induced by PGE_2, even though EP_4 was most abundantly expressed subtype in CFs (Fig.4A). Based on these findings, we speculated that PGE_2 could significantly induce calcium influx and release and promote cardiac fibrosis *via* EP_1 in CFs. However, in C Brilla's research, high concentration (10^{-3}M) of PGE_2 could reduce the collagen synthesis in human cardiac fibroblasts [27]. We postulate that the different species may display different reaction to pathological stimulation because of the expression of dominant receptor(s) and high level PGE_2 may activate the other receptors, such as EP_2 or EP_4, since there are many researches showing that the elevation of cAMP is an antifibrotic factor [28-31]. It has been well established that calcium signals are essential for the development of cardiac fibrosis [32].

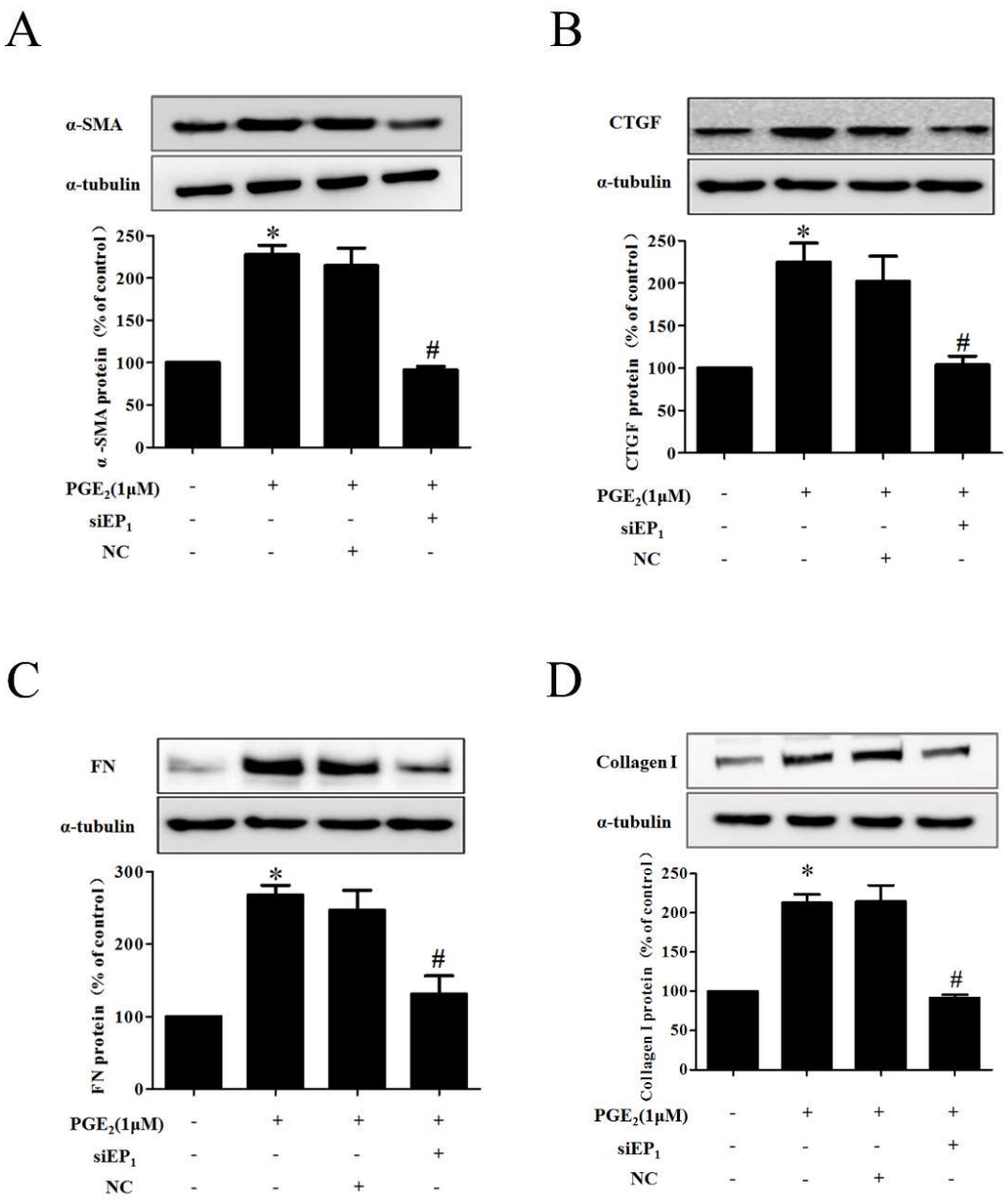

Figure 7. Changes of α-SMA, CTGF, FN, Collagen I expression in siRNA-EP_1 and negative control cells. After transfection with siRNA-EP_1, protein expression of α-SMA (A), CTGF (B), FN (C) and Collagen I (D) was examined by Western blot. The results are expressed as mean±SEM of three independent experiments. $^{*}P<0.05$ vs. control, $^{\#}P<0.05$ vs. PGE_2 treated group. α-SMA: α-smooth muscle actin; CTGF: connective tissue growth factor; FN: fibronectin; PGE_2: Prostaglandin E_2; TG: thapsigargin.

Intervention and modulation of calcium channels, as well as calcium related proteins were connected with anti-fibrosis effects [33, 34]. We speculated that the increase of calcium at least partially contributes to cardiac fibrosis. Moreover, as an important nuclear translocation factor, NFAT is necessary for regulating cardiac hypertrophy, which is also involved in fibrogenesis. Previous research in our laboratory has also demonstrated that NFATc4 translocation triggered by Ang II was restrained by calcium signal inhibition [35]. Our data from immunofluorescent staining indicated that PGE_2 could induce NFATc4 translocation, which was significantly inhibited in siRNA-EP_1 cells (Fig. 6). These results indicated that EP_1 may play an important role in controlling Ca^{2+} signaling

that contributes to the process of fibrogenesis in CFs. In summary, the present study shows that PGE_2 could promote cardiac fibrosis through EP_1 receptor. Inhibition of EP_1 remarkably alleviated fibrotic effect, calcium influx and release, and translocation of NFATc4 induced by PGE_2. These findings provide new insight into the mechanisms by which PGE_2 effectively induces cardiac fibrosis and might provide a novel therapeutic strategy for cardiac fibrosis by selectively inhibiting EP_1.

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Supplementary Figures

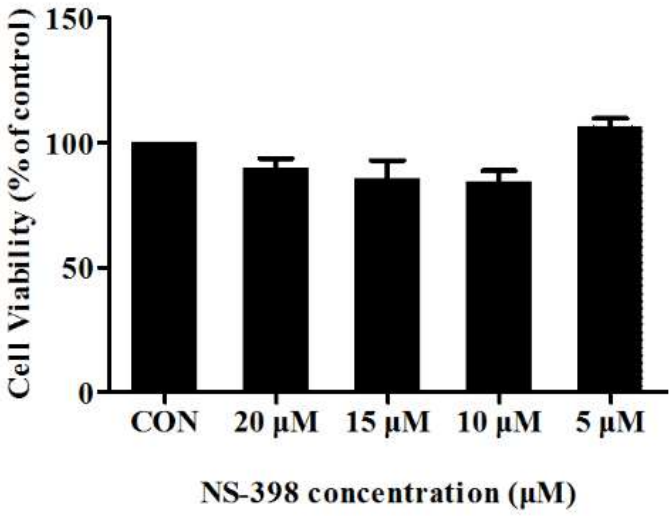

Figure S1. Effect of NS-398 on cell viability of CFs. The cells were incubated with different concentration of NS-398 for 24 h, and the viability ratio was determined by MTT assay. Data are presented as mean±SEM of three independent experiments. P>0.05.

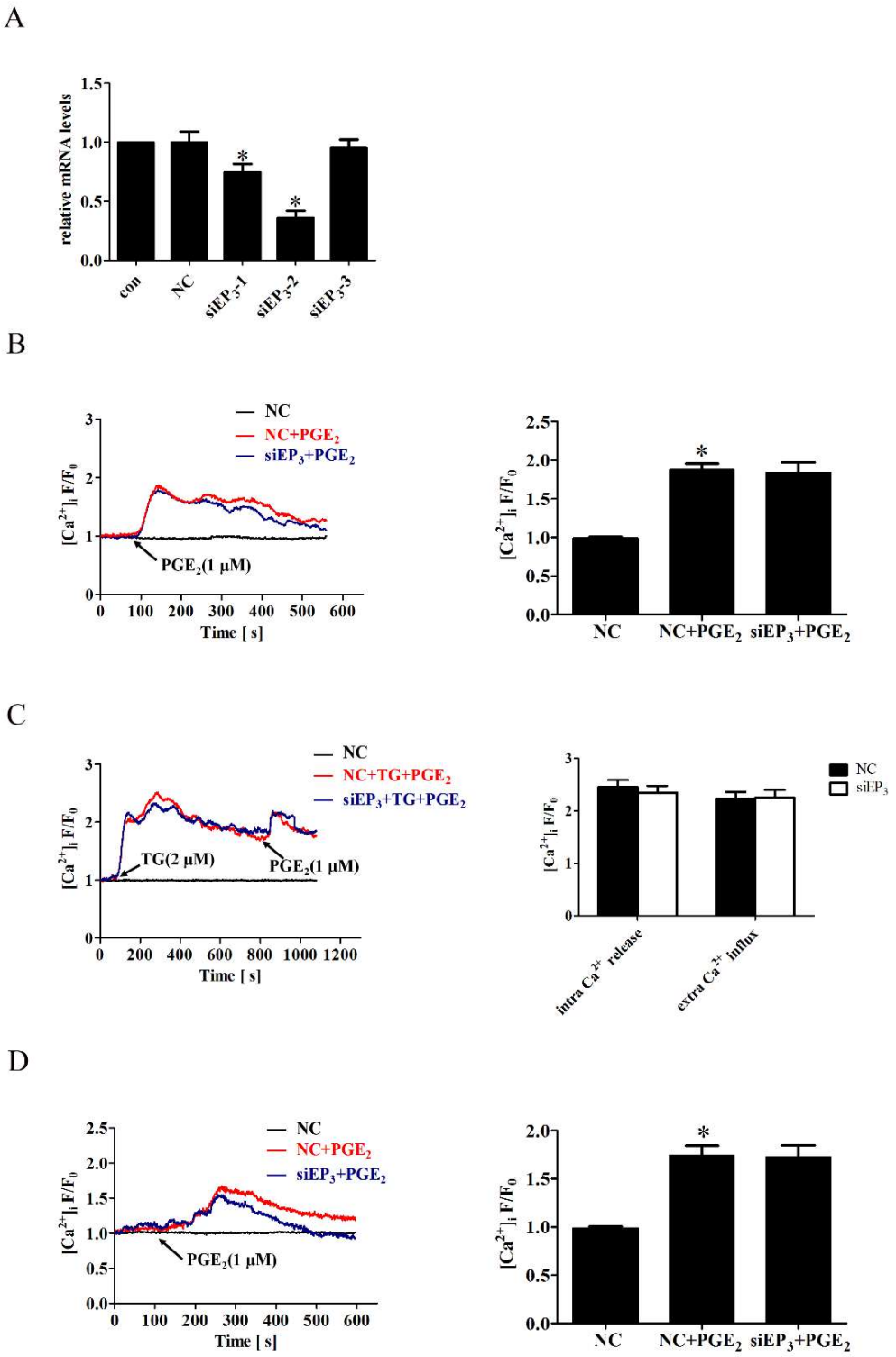

Figure S2. Changes of $[Ca^{2+}]_i$ in siRNA-EP3 and negative control cells. (A) The efficiency for EP3 knockdown was determined by RT-qPCR. Data shown are the fold increase in signal intensity compared with those of control cells and are presented as the means ± SD of values obtained from three independent experiments. *P＜0.05 vs. control. Negative control cells or siRNA-EP3 cells in Tyrode's solution were stimulated with TG (2 μM) first, and then were stimulated with PGE_2 (C) or directly with PGE_2 (1 μM) (B), and then the variation of $[Ca^{2+}]_i$ was detected (n = 10, the trace is representative mean of ten cells). (D) Negative control cells or siRNA-EP3 cells in Ca^{2+}-free Tyrode's solution were stimulated with PGE_2 (1 μM), and then the variation of $[Ca^{2+}]_i$ was detected (n = 10, the trace is representative mean of ten cells). *P＜0.05 vs. blank. PGE_2: Prostaglandin E_2; TG: thapsigargin.

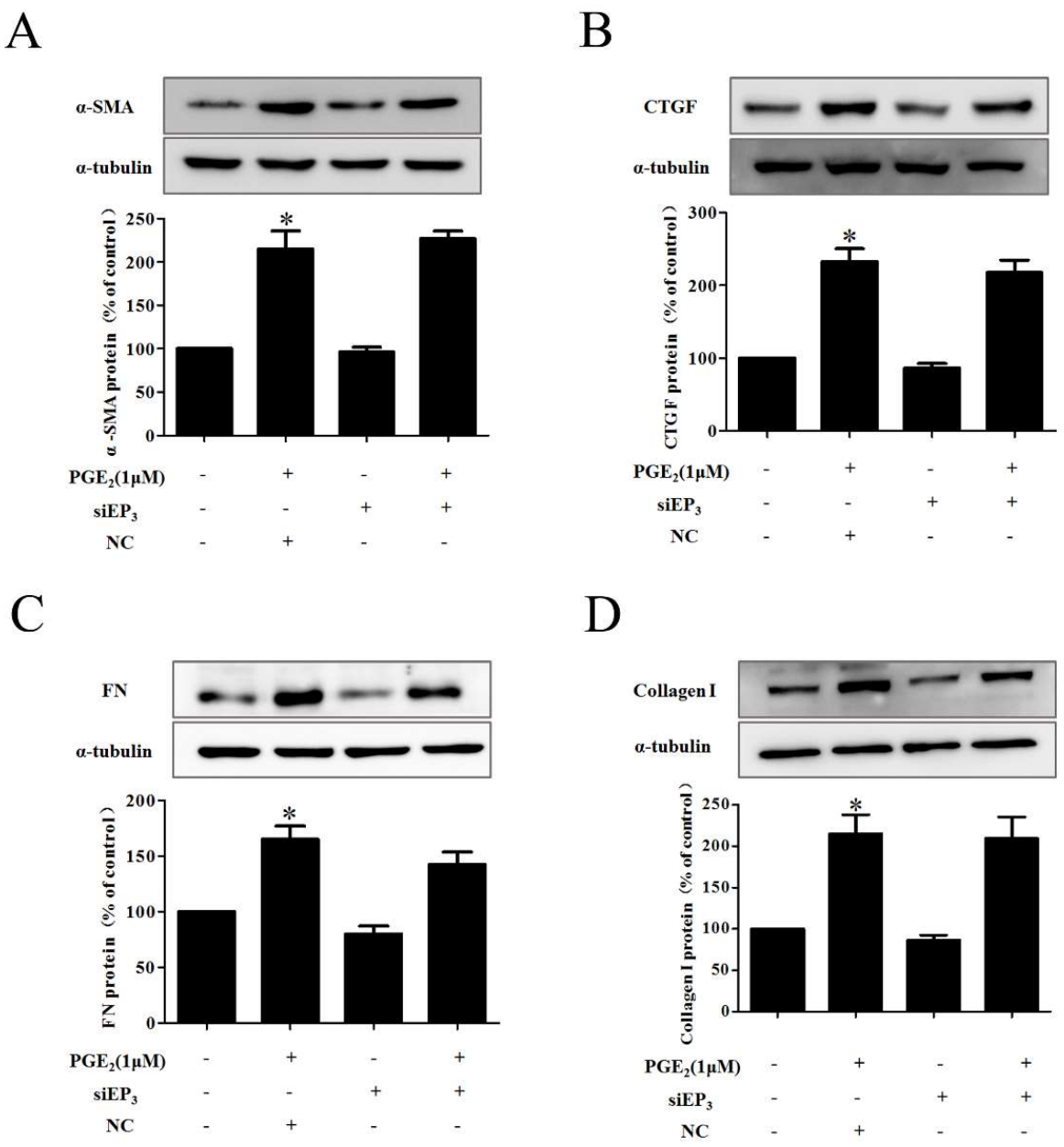

Figure S3. Changes of α-SMA, CTGF, FN, Collagen I expression in siRNA-EP3 and negative control cells. After transfection with siRNA-EP3, protein expression of α-SMA (A), CTGF (B), FN (C) and Collagen I (D) was examined by Western blot. The results are expressed as mean±SEM of three independent experiments. *P＜0.05 vs. control. No statistical differences between PGE_2 treated siRNA-EP3 group vs. PGE_2 treated group (P>0.05). α-SMA: α-smooth muscle actin; CTGF: connective tissue growth factor; FN: fibronectin; PGE_2: Prostaglandin E_2.