Cellular Physiology and Biochemistry

Cell Physiol Biochem 2016;39:1521-1536
DOI: 10.1159/000447855
Published online: September 12, 2016
Accepted: August 04, 2016

Original Paper

Activation of Cannabinoid Receptor Type II by AM1241 Ameliorates Myocardial Fibrosis *via* Nrf2-Mediated Inhibition of TGF-β1/Smad3 Pathway in Myocardial Infarction Mice

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Key Words
Cannabinoid receptor II • Myocardial Infarction • Fibrosis • Nrf2 • TGF-β1 • Smad3

Abstract
Aims: Myocardial interstitial fibrosis is a major histologic landmark resulting in cardiac dysfunction after myocardial infarction (MI). Activation of cannabinoid receptor type II (CB2 receptor) have been demonstrated to reduce fibrosis in hepatic cirrhotic rat. However, the anti-fibrotic effect of CB2 receptor activation in infarcted hearts was still unclear. In this study, we aimed to investigate the effects of a CB2 receptor selective agonist AM1241 on myocardial fibrosis post MI in mice. ***Methods:*** Echocardiograph was conducted to assess cardiac function. Fibrosis markers such as type I and type III collagen, fibronectin, Plasminogen activator inhibitor(PAI)-1 and tissue inhibitor of metalloprotease(TIMP)-1 were examined by Western blot, while collagens were directly observed by Sirius-red staining. Primary cultured cardiac fibroblasts(CFs) were subjected to hypoxia/serum deprivation (H/SD) injury to simulate ischemic conditions *in vivo*. Nrf2 siRNA were applied to explore the role of Nrf2 and TGF-β1/Smad3 pathway in this process. ***Results:*** Echocardiography showed that AM1241 significantly improved cardiac function, suppressed the expression of fibrosis markers such as collagen I and collagen III, fibronectin, PAI-1 and TIMP-1 in mice with MI. In cardiac fibroblasts subjected to H/SD injury, AM1241 reduced the elevated levels of α-SMA, collagen I and collagen III, which were partially abrogated by the Nrf2 siRNA transfection. Furthermore, AM1241 not only activated and accelerated the translocation of Nrf2 to nucleus, but also inhibited TGF-β1/Smad3 pathway in an Nrf2 dependent manner. ***Conclusion:*** CB2 receptor agonist AM1241 alleviated myocardial interstitial fibrosis *via* Nrf2 -mediated down-regulation of TGF-β1/Smad3 pathway, which suggested that CB2 receptor activation might represent a promising target for retarding cardiac fibrosis after MI.

X. Li and D. Han contributed equally to this work and should be considered as co-first authors.

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Introduction

Myocardial infarction (MI) is still a common fatal disease despite advances in drug treatment and percutaneous coronary intervention. According to the report from American Heart Association, there will be approximately one American suffering from MI within every 42 seconds [1]. Cardiac fibrosis is the key part of cardiac remodeling after MI which was characterized by excessive collagen deposition resulting in ventricular stiffening, diastolic and systolic dysfunction and eventually leading to heart failure [2, 3]. It not only involves the infarct zone, but the non-infarct region is also affected. The cardiac fibrosis after MI to date is still a thorny issue for clinical doctors due to few available effective strategies [4].

The cannabinoid receptor-type II (CB2 receptor), a seven-transmembrane and G protein-coupled receptor, is widespread in peripheral tissues including liver, skin, heart and immune cells [5]. In the past several years, the CB2 receptor activation has emerged as a promising therapeutic target to protect against fibrosis in various organs, for instance, CB2 receptor agonist was reported to significantly reduce hepatic collagen content in cirrhotic rats and exert antifibrotic effects in experimental dermal fibrosis mice [6, 7]. Moreover, CB2 receptor activation has exhibited great potential in anti-oxidative stress and anti-inflammation in various disease models [8-10]. In our previous study, we also found that CB2 receptor agonist AM1241 activated PI3K/Akt/Nrf2 signaling to reduce excess oxidative stress and inflammation in ischemic heart to promote endogenous myocardial regeneration [11]. Incidentally, it was well known that excess oxidative stress and inflammation in heart aggravated cardiac fibrosis [12, 13]. Thus, we were curious to know whether CB2 receptor activation could ameliorate cardiac fibrosis post MI.

Transforming growth factor-β1 (TGF-β1) has long been demonstrated to play a crucial role in regulation of extracelluar matrix (ECM) and homeostasis of tissue. TGF-β1 predominantly transmits cell signaling through a downstream mediator protein named Smad3. Numerous evidences have proved that the TGF-β1/Smad3 pathway was a canonical pathway in the development of organ fibrosis [14-16]. On the other hand, nuclear factor-erythroid-2- (NF-E2-) related factor 2 (Nrf2), a major regulator of the antioxidant response elements, has long been known for resisting oxygen-free radicals and oxidative stress [17, 18]. Previously, we and others have reported the involvement of Nrf2-reduced oxidative stress in alleviation of cardiac fibrosis [19-21]. So our current study was designed to investigate whether Nrf2 and TGF-β1/Smad3 signaling were involved in CB2 receptor activation-regulated cardiac fibrosis post MI.

In the present research, both *in vivo and in vitro* models were conducted to imitate cardiac fibrosis after MI. We attempted to evaluate the impact of CB2 receptor agonist AM1241 on cardiac fibrosis post MI and investigate the underlying mechanisms with a focus on Nrf2 and TGF-β1/Smad3 signaling pathways.

Materials and Methods

Animal model and treatment

Eight-week-old C57BL/6 male mice between 20–25 g were purchased and housed in the Animal Center of Fourth Military Medical University. Mice were randomly divided into 4 experiment groups: 1) sham group (n=20), 2) sham+AM1241 group (n=20), 3) MI group (n=20), 4) MI+AM1241 group (n=20). All animal experiment protocols were performed in conformity with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Fourth Military Medical University Ethic Committee on Animal Care.

The MI mice model were conducted as described previously [22], In brief, the left anterior descending (LAD) artery was ligated tightly with a 6-0 suture until the anterior wall of the left ventricle (LV) turned pale. The sham group were performed with similar procedure except LAD ligation. After operation, AM1241 (20mg/kg/d, Selleck, USA) was intraperitoneally injected for 7 consecutive days in 2) and 4) groups, while the (1) and (3) groups were given equal volume DMSO for 7 consecutive days.

Histologic analysis of cardiac fibrosis

To quantify fibrosis area in histological staining, we randomly selected 10 fields in remote region of heart section in "MI" group (n=5) and "MI+AM 1241" group (n=5) under polarized light microscope. The percent of the positive staining areas were quantified with Image J software by 2 independent experienced investigators.

Cardiac fibroblast isolation, culture and purity assessment

Primary cardiac fibroblast cells (CFs) were isolated from the left ventricular of neonatal C57BL/6 mice using the enzymatic digestion and selective plating methods as previously described [23]. Briefly, the hearts were removed from the sterilized mouse with 75% ethanol and then washed with cold PBS twice, the left ventricular were then minced before incubated with 0.1% collagenase at 37°C. The digested cell suspension was mixed with DMEM containing 20% fetal bovine serum to stop digestion and then been centrifuged (800r, 5min) and suspended again. At last, the fibroblasts were separated from myocytes and other cells by selective plating for 1 hour. The isolated CFs were cultured in DMEM containing 10% fetal bovine serum, streptomycin(100U/ml) and penicillin(100U/ml). Cultured CFs of second to third passage were used for further experiments.

The identity and purity of CFs was assessed by morphology examination and immunostaining methods as described previously [24]. CFs should have a thin and triangular profile under light microscope and be positive for fibroblast marker vimentin, and negative for endothelial marker von Willebrand factor(vWF).

H/SD injury to simulate MI

The hypoxia/serum deprivation injury cell model was used to mimic MI as we have previously described [25]. In brief, after indicated treatment, CFs were exposed to hypoxia (94% N_2 , 5% CO_2 and 1% O_2) in an anaerobic system (Thermo Forma, USA) at 37 ℃ for indicated period of time in Hanks buffer.

The small interfering RNA targeting of Nrf2

The small interfering RNA targeted to Nrf2 (Nrf2−siRNA) and control siRNA (Scramble siRNA) were purchased from Gene-Pharma Company (Shanghai, China). The mouse Nrf2 siRNA sequence is 5′-UCCCGUUUGUAGAUGACA-3′(Nrf2, NCBI Reference Sequence: NM_010902.3). Cardiac fibroblasts were transfected with 100nM Nrf2-siRNA or Control siRNA with Lipofectamine 2000 (Invitrogen) for 24 hours according to the manufacturer' protocol. The silencing effects were confirmed by Western blot.

Echocardiography

Transthoracic echocardiography (VEVO2100, VisualSonic, Canada) was performed by 3 independent investigators to evaluate cardiac function post operation as previously described [26]. In brief, mice were anesthetized with inhalation of isoflurane (2%). Images were captured with M-mode echocardiogaphy. Left ventricular end-diastolic volume (LVEDV), Left ventricular end-systolic volume (LVESV), LV ejection fraction (LVEF) and LV fraction shortening (LVFS) were measured by the use of computer algorithm.

Western blot

Subcellular protein fractions of cardiac LV tissues and fibroblasts were extracted with nuclear and cytoplasmic protein extraction kit (Beyotime, Guangzhou, China) following manufacturer's instructions. Protein concentration quantitation was determined by the Bradford method. Proteins were separated *via* SDS-PAGE (12%) (Beyontime, Guangzhou, China) and then transferred onto NC membrane. After incubated with the following primary antibodies: anti-collagen I(ab34710, abcam, 1:5000), anti-collagen III(ab7778, abcam, 1:5000), anti-α-SMA(ab7817, abcam, 1:300), anti-PAI-1(ab66705, abcam, 1:1000), anti-TIMP-1(ab86482, abcam, 1:1000), anti-fibronectin (ab2413, abcam, 1:1000), anti-TGF-β1 (ab92486, abcam, 1:1000), anti-Smad3(ab40854, abcam, 1:1000), anti-P-Smad3(ab52903, Ser423/425, abcam, 1:1000), anti-Nrf2(ab31163, abcam, 1:1000), anti-HO-1(ab13243, abcam, 1:1000), overnight at 4°C, the membrane was then incubated secondary antibodies(PR-0255, ZhongshanJinqiao Biotechnology Co., 1:2000) for 1 hour, protein levels were detected using chemiluminescene system (Amersham Biosciense, Buchinghamshire, UK) and analyzed with Image J software.

Immunofluorescent staining and confocal microscopy

Confocal dishes with CFs were washed with PBS three times and fixed in 4% paraformaldehyde for 20 min. After being permeabilized in 0.03% Triton X-100 for 10 min, the dishes were then blocked with goat serum for 1 h at room temperature. After that, CFs were incubated with primary antibodies at 4°C overnight. The next morning, secondary antibodies were incubated for 1 h and DAPI were counterstained for 10 min according to the manufacture's instructions. All images were captured with a digital confocal microscopy (FV-1000, Olympus, Japan).

Flow cytometry for ROS production

The sensitive ROS fluorescent probe- DCFH-DA (KGT010-1; KeyGEN BioTECH, Nanjing, China) was used to detect the ROS production following the manufacturer's instructions. In brief, cardiac fibroblasts incubated with 5 μM DCFH-DA at 37°C for 30 min. The fluorescence was then detected on flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, NJ).

Measurement of malondialdehyde (MDA), glutathione(GSH) and superoxide dismutase(SOD) activity

The level of MDA, GSH, SOD activity in the supernatant of CFs were determined by commercially available assay kit (Nanjing Jiancheng Bioengineering Institute, China) in accordance with the manufacturer's protocols. All samples were stored at -80°C before assay and the amount of MDA, GSH, SOD activity in the samples were then measured by comparing the OD450 to the standard curves.

Statistical Analysis

All data were analyzed using the GraphPad Prism 6.02 and expressed as mean ± standard deviation (SD). The differences between groups were subjected to one-way ANOVA or Student's two-tailed unpaired *t* test. The statistical significance was accepted at $P < 0.05$.

Results

Cardiac fibrosis was ameliorated by CB2 receptor activation after MI in mice

Western blot analysis of cardiac tissue in remote region revealed that collagen I, the major cardiac extracellular agents in fibrosis formation, was remarkably augmented after MI and approached the peak at 4 weeks. The content of collagen I at 4 weeks was approximately fivefold compared with that in sham-operated tissues ($P< 0.05$, Fig. 1 A, B). Moreover, content of collagen reflected by picrosirius red staining were notably higher in remote region of MI group as compared to sham group, which was attenuated by AM1241 treatment (fibrosis area: 18.37±1.12% in MI group *vs.* 11.40±0.66% in MI+AM1241 group, $P< 0.05$, Fig. 1 C, D). However, collagen content of sham group and sham+AM1241 group, infarct region of MI group and MI+AM1241 group did not differ. These results demonstrated that activation of CB2 receptor by AM1241 mainly ameliorated cardiac fibrosis in remote region after MI in mice.

Expression of Collagen I, Collagen III, fibronectin, PAI-1 and TIMP-1 were decreased in AM1241treated MI mice

In order to confirm the anti-fibrosis effect of AM1241 after MI at protein level, we used Western blot to examine the fibrosis associated proteins collagen I, collagen III, fibronectin, Plasminogen activator inhibitor(PAI)-1 and tissue inhibitor of metalloprotease(TIMP)-1. As shown in Fig. 1F-H, the expressions of cardiac collagen I, collagen III and fibronectin, the main components constituting cardiac extracellular matrix, were not significantly altered by AM1241 treatment in sham operated mice. However, significantly decreased expressions of collagen I, collagen III and fibronectin were observed in MI+AM1241group ($P < 0.05$ *vs.* MI). Meanwhile, PAI-1 and TIMP-1 which were markers of extracellular matrix degradation inhibitors, were also markedly increased in MI group as compared to sham group, whereas, this increase was partially reversed by AM1241 treatment ($P < 0.05$, Fig. 1 I, J). Taken together, these findings demonstrated that AM1241 inhibited synthesis and accelerated the degradation of extracellular matrix in infarcted hearts.

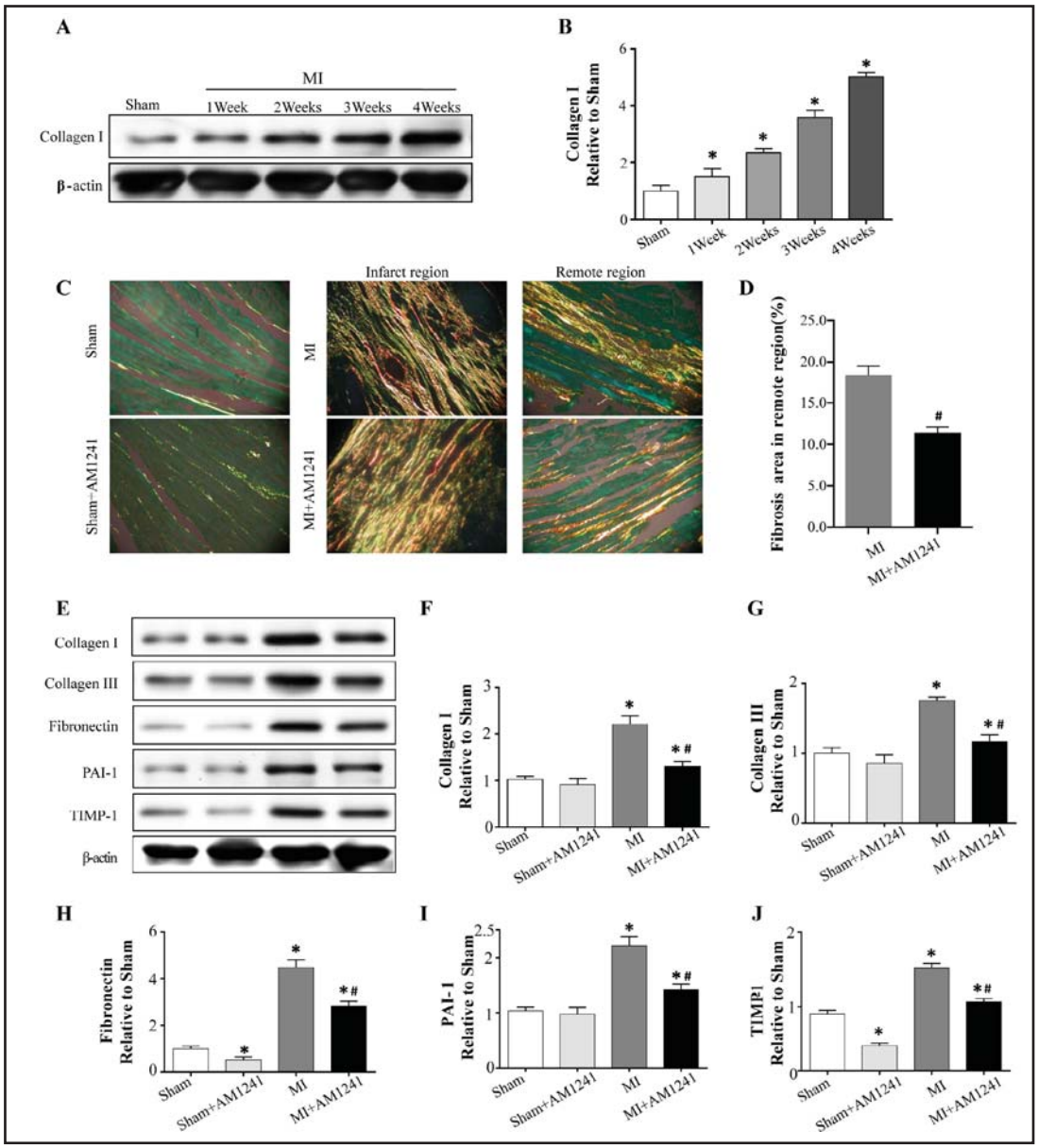

Fig. 1. Activation of CB2 receptor by AM1241 ameliorated cardiac fibrosis post MI observed at tissue and protein level. A: expression level of collagen I post operation at different time points. B: quantification of A. *p<0.05 *vs.* Sham group; C: Picrosirus red staining for collagens at infarct region and remote region in mouse heart, golden color indicated collagen deposition under polarized light. D: quantifications of fibrosis area in remote region of C. E: Western blot analysis of the expression of cardiac collagen I, collagen III, fibronectin, PAI-1, TIMP-1; F, G, H, I and J: quantifications of cardiac collagen I (F), Collagen III (G), Fibronectin (H), PAI-1 (I) and TIMP-1 (J). n=5-10. *p<0.05 *vs.* Sham group;# p<0.05 *vs.* MI group.

Cardiac functional improvement by CB2 activation evaluated by echocardiograph

To evaluate the cardiac functional improvement of CB2 receptor agonist AM1241 in MI mice, we performed echocardiograph 4 weeks after MI. As shown in Fig. 2, AM1241 significantly increased LVEF, LVFS in MI+AM1241 group compared with that of MI group (EF: 60.04±5.93% *vs.* 42.87±3.16%, P < 0.05; FS: 26.17±1.88% *vs.* 21.64±1.03%, P < 0.05). Meanwhile, AM1241 treatment also decreased LVEDV, LVESV as compared to MI group (LVEDV: 0.32±0.01 ml *vs.* 0.38±0.02 ml, P < 0.05; LVESV: 0.12±0.01 ml *vs.* 0.19±0.01 ml, P

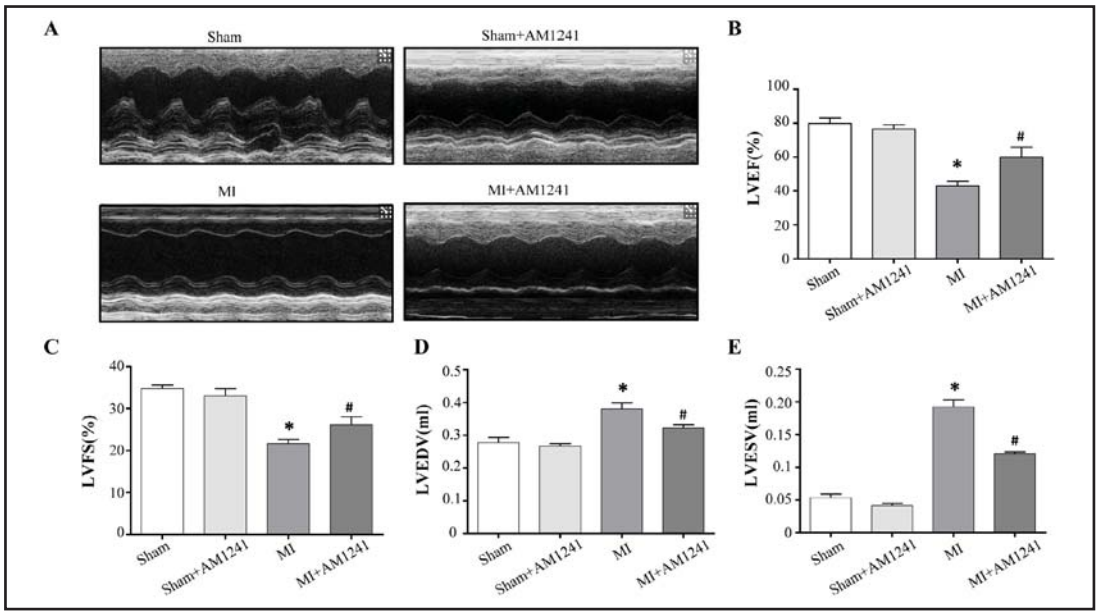

Fig. 2. Cardiac function 4 weeks post operation. A: M-mode echocardiographic images of mice in Sham, Sham+AM1241, MI and MI+AM1241 group at 4 weeks after surgery; B: LV ejection fraction (LVEF); C: LV fraction shortening (LVFS); D: left ventricular end-diastolic volume(LVEDV); E: Left ventricular end-systolic volume (LVESV). n=10-15. *p<0.05 *vs.* Sham group; # p<0.05 *vs.* MI group.

< 0.05). These results indicated that CB2 receptor agonist AM1241 ameliorated cardiac fibrosis, which thereby partly prevented the loss of cardiac systolic and diastolic function.

AM1241 activated CB2 receptor/Akt/Nrf2/HO-1 signaling pathway and accelerated the translocation of Nrf2 into nucleus

To confirm the anti-fibrosis effect of AM1241 *in vitro*, we isolated cardiac fibroblasts(CFs) as described in methods section and used *in vitro* hypoxia/serum deprivation(H/SD) cell model to mimic *in vivo* MI as described in methods section. As shown in Fig. 3A, the majority of the cells of third passage had a thin and triangular profile under light microscope in accordance with description in previous reports. Next, we identified cultured CFs by immunofluorescent staining for CFs marker vimentin, and endothelial marker von willebrand factor (VWF). The results showed that >98% cultured cells were positive for vimentin and negative for VWF (Fig. 3B, C). To identify the proper dosage of AM1241 *in vitro*, α-smooth muscle actin (α-SMA), a fibroblast-myofibroblast transformation marker, was examined by Western blot in CFs subjected to H/SD injury. As shown in Fig. 3D, F, α-SMA expression elevated with the duration of H/SD injury within 12 hours and reached the peak at 12 hours. Next, the expression levels of α-SMA with different concentrations of AM1241 (0, 0.1, 1, 5, 10μM) treatment in CFs subjected to 12 hours' H/SD injury were detected. The results indicated that 0.1, 1, 5, 10μM AM1241 all decreased α-SMA expression level, of which 5μM AM1241 was the most effective one (P < 0.05, Fig. 3. E, G). Thus, the AM1241 dosage of 5 μM (mass concentration, 2.52 mg/L) and H/SD injury for 12 hours were chosen in the following experiments.

Previously, we and others have demonstrated that CB2 receptor activation could activate PI3K/Akt pathway [11, 27], activation of Akt was shown to activate Nrf2, a well-known anti-oxidative protein which is frequently related to anti-fibrotic actions [20, 28]. To achieve a better understanding of the possible signaling pathways and mechanisms implicated in the action of CB2 receptor activation, we detected the expression of CB2 receptor, Akt, Nrf2 and Nrf2 downstream heme oxygenase-1(HO-1) in CFs. Our data showed that H/SD could induce a mild elevation of CB2 receptor, phosphorylation-Akt(p-Akt), nucleus Nrf2, and HO-

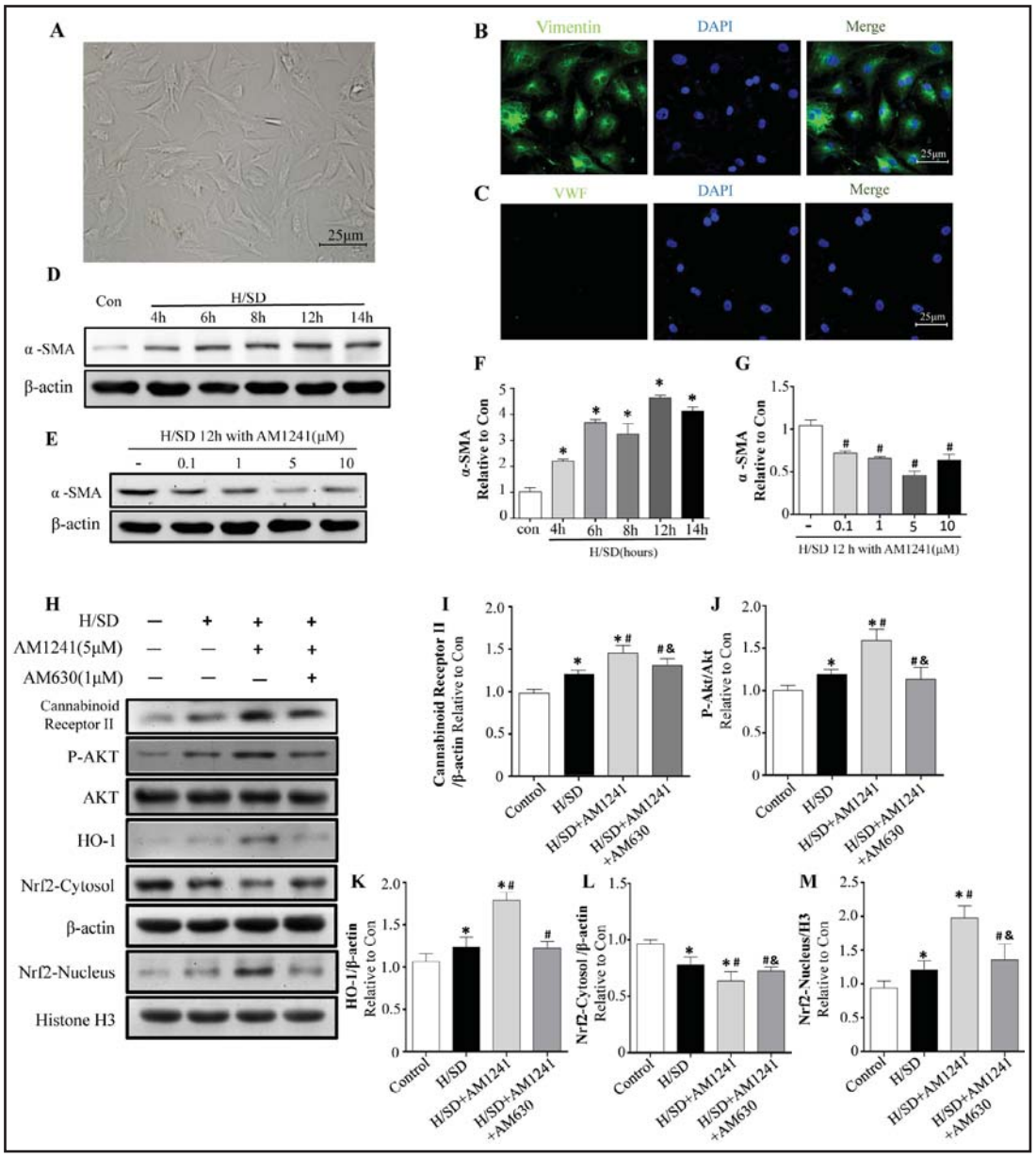

Fig. 3. AM1241 activated CB2 receptor/Akt/Nrf2/HO-1 signaling pathway and accelerated the translocation of Nrf2 into nucleus. A: Morphology of isolated cardiac fibroblasts of third passage under light microscope; B: fluorescence staining of CFs marker vimentin; C: fluorescence staining of endothelial marker Von Willebrand Factor(VWF); D: expression levels of α-smooth muscle actin (α-SMA) in CFs exposed to different period of H/SD injury; E: expression levels of α-SMA in CFs treated with gradient concentrations of AM1241(0-10um/L) under H/SD injury; F: quantification of D. G: quantification of E. H: The expression of cannabinoid receptor type II, Akt, p-Akt, HO-1, Nrf2 in cytosol and Nrf2 in nucleus were detected by Western blot; I, J, K, L and M: quantifications of cannabinoid receptor type II, p-Akt/Akt, HO-1, Nrf2 in cytosol and Nrf2 in nucleus respectively; n=3-5. *p<0.05 *vs.* the control group; # p<0.05 *vs.* H/SD 12 hours with no AM1241 treatment group, & p<0.05 *vs.* H/SD+AM1241 group.

1($P < 0.05$ *vs.* Control), the effect of which was further augmented by AM1241 treatment ($P < 0.05$ *vs.* H/SD, Fig. 3 H-J). However, treatment with a CB2 receptor selective antagonist AM630(1μM, Selleck, USA) reversed these effects of AM1241 ($P < 0.05$ *vs.* H/SD+AM1241, Fig. 3 H-M). These data suggested a pivotal role of CB2 receptor/Akt/Nrf2/HO-1 signaling

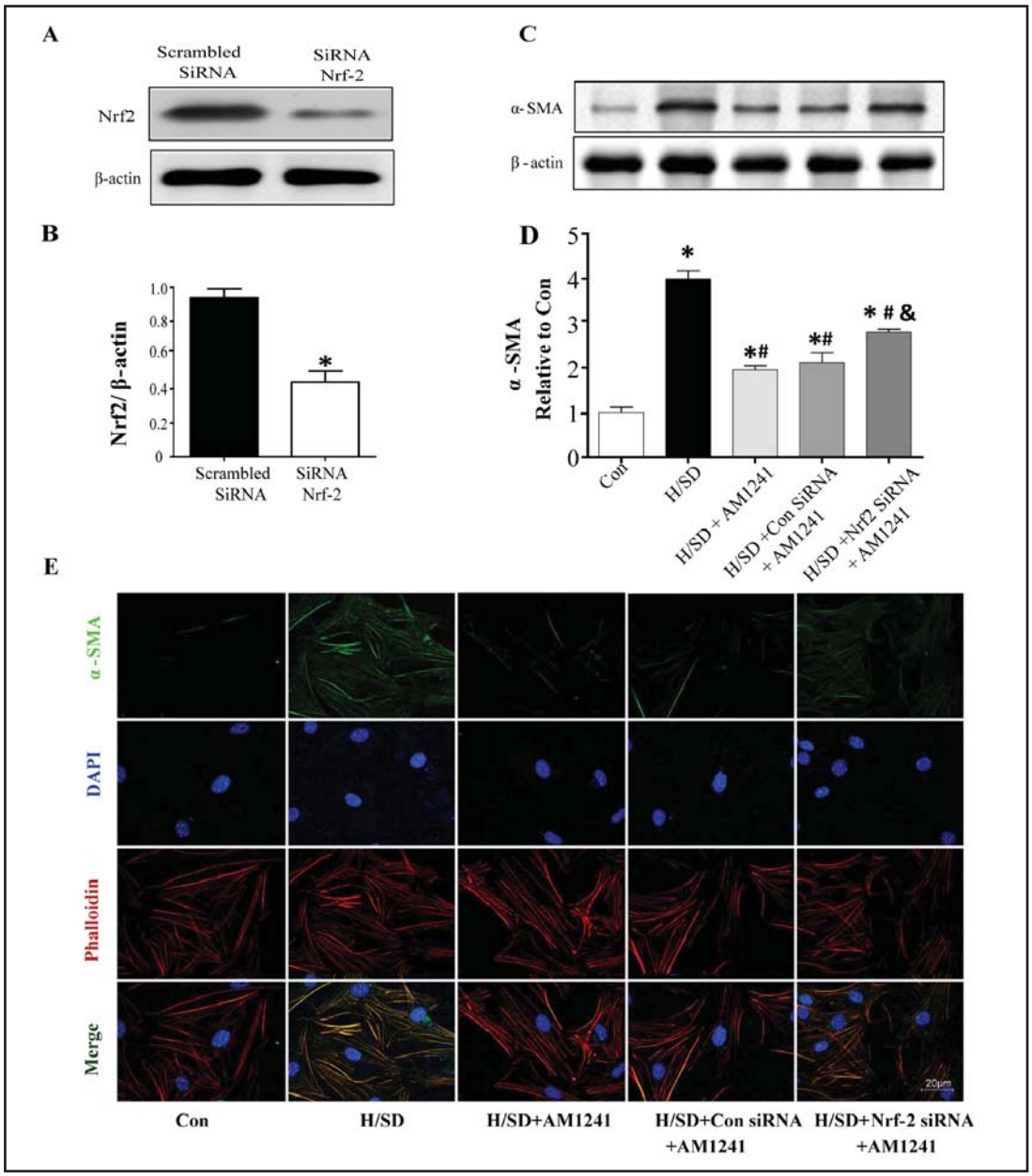

Fig. 4. AM1241 inhibited H/SD-induced fibroblast-to-myoblast transformation of CFs in an Nrf2 dependent manner. A: Total protein of Nrf2 in cardiac fibroblasts under normal condition transfected with Scrambled siRNA and Nrf2 siRNA was determined by Western blot. B: quantification of A. *p<0.05 *vs.* the Scrambled siRNA group; C: Western blot evaluation of the expression of α-SMA in CFs under indicated conditions. D: quantification of C. E: Immunofluorescent staining for α-SMA (green) and nuclear marker DAPI (blue) in CFs after indicated treatment. Phalloidin (red) was used to exhibit actin filament in CFs. n=3-5. *p<0.05 *vs.* Con group; # p<0.05 *vs.* H/SD group; &p<0.05 *vs.* H/SD+AM1241 group.

pathway in the anti-fibrotic effects of AM1241.

AM1241 suppressed the transformation of CFs under H/SD condition via Nrf2 signaling

A Nrf2 targeted small interfering RNA(siRNA) were used to determine the role of Nrf2 in the effects of AM1241 on H/SD induced cardiac fibroblast-to-myoblast transformation. The interfering efficiency of siRNA was determined in Fig. 4A, B, which indicated more than 60% down-regulation of Nrf2 protein level. As expected, AM1241 significantly decreased

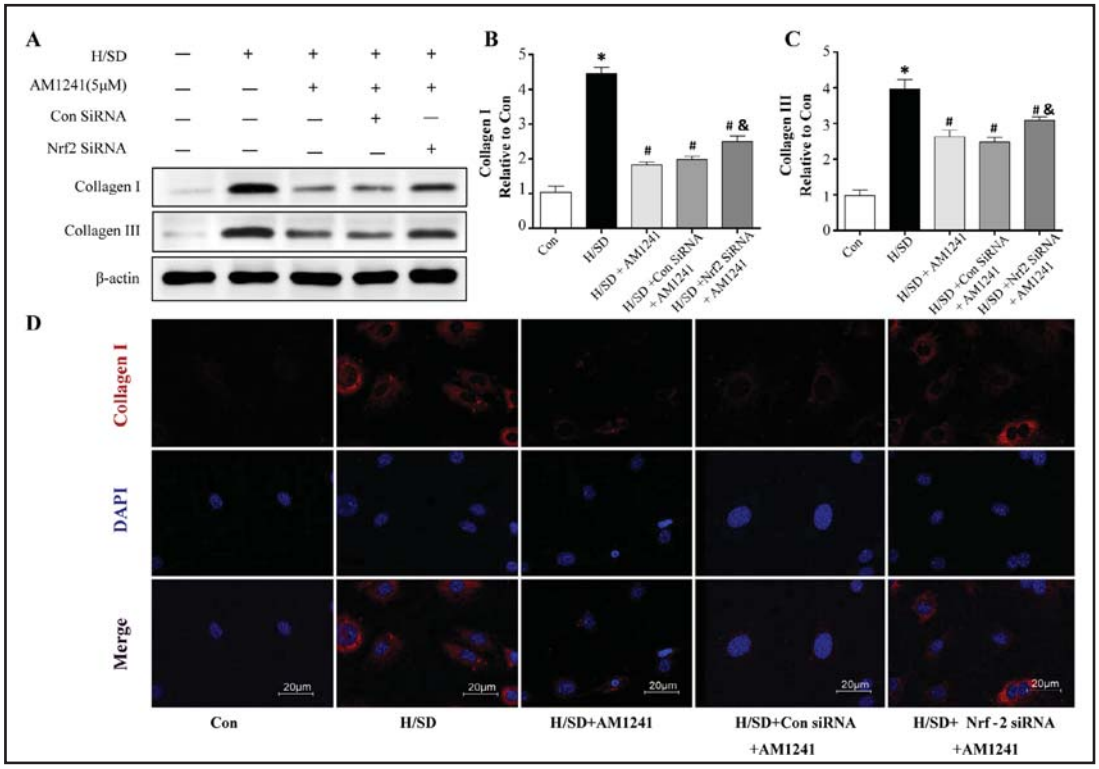

Fig. 5. AM1241 inhibited H/SD-induced collagen production of CFs in an Nrf2 dependent manner. A: Western blot evaluation of the expression of collagen I and collagen III; B, C: quantification of collagen I protein level (B) and collagen III(C) in A; D: The expression of collagen I (red) secreted by CFs after indicated treatment was confirmed by immunofluorescent staining. The nuclei positions were determined by DAPI (blue). n=3-5. *p<0.05 *vs.* Con group; # p<0.05 *vs.* H/SD group; &p<0.05 *vs.* H/SD+AM1241 group.

H/SD induced up-regulation of α-SMA, a common marker for fibroblast-to-myoblast transformation, which was partially reversed by Nrf2 siRNA ($P < 0.05$, Fig. 4 C, D). A similar trend was confirmed in immunofluorescent staining for α-SMA under confocal microscope (Fig. 4 E). Collectively, these data revealed that AM1241 suppressed the transformation of CFs under H/SD condition, in which Nrf-2 was strongly involved.

Collagen production were inhibited by AM1241 after H/SD injury in vitro

The cardiac fibroblasts were attracted to the site of infarct region to secrete collagen which helps scar formation by various chemotactic factors [29]. Therefore, we evaluated the effects of AM1241 on the secretory properties of CFs. The results illustrated that the up-regulated levels of collagen I and collagen III induced by H/SD injury were significantly decreased by AM1241, and Nrf2 siRNA partially abolished this trend ($P < 0.05$, Fig. 5 A-C). Next, to further confirm the result, we used the immunofluorescence staining to detect collagen I secretion in CFs. Unsurprisingly, AM1241 reduced the secretion of collagen I in H/SD+AM1241 group compared with that of the H/SD group, and Nrf2 siRNA partially abrogated this reduction (Fig. 5 D). These data demonstrated that AM1241 inhibited the collagen production properties of CFs under H/SD injury in an Nrf2 dependent manner.

AM1241 decreased oxidative stress levels in CFs subjected to H/SD

Oxidative stress levels has been demonstrated to be closely related to fibrosis [30]. So we next sought to explore the effects of AM1241 on oxidative stress levels in CFs subjected to H/SD injury. Fluorescence intensity of ROS probe DCFH-DA was detected by flow cytometer in response to the levels of ROS. Fluorescence intensity enhanced by H/SD was

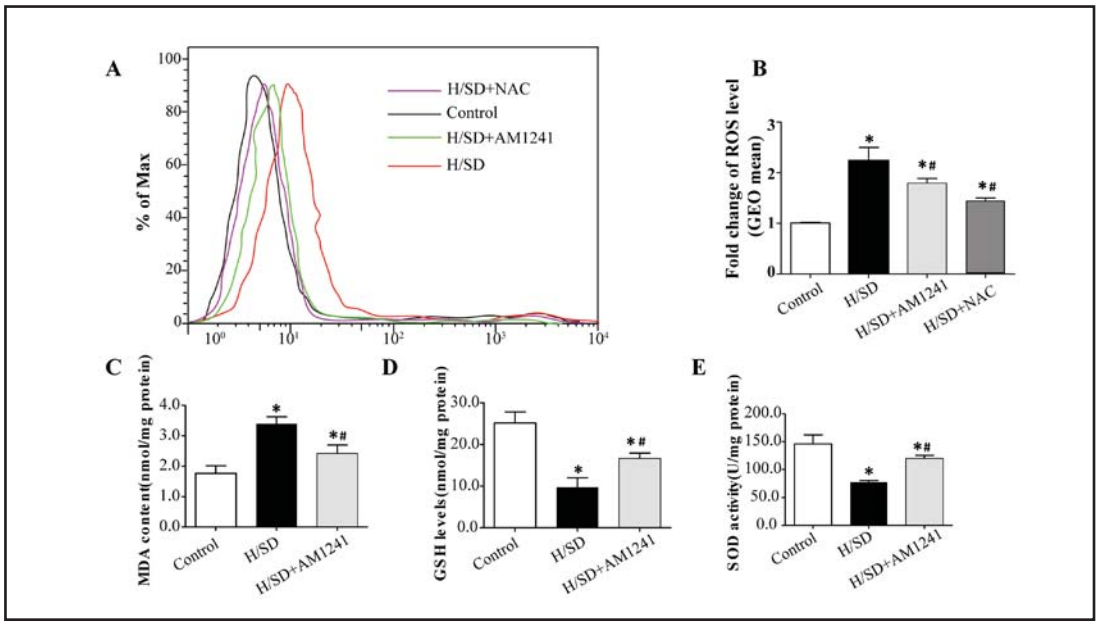

Fig. 6. AM1241 prevented H/SD-induced ROS generation and MDA production, and meanwhile increased H/SD-reduced GSH level and SOD activity in CFs. A: Flow cytometry analysis of ROS production in CFs after indicated treatment; B: quantification of A; C, D and E: The MDA, GSH, and SOD activity determined by respective commercially available assay kit. n=3-5. *p<0.05 *vs.* Con group; # p<0.05 *vs.* H/SD group.

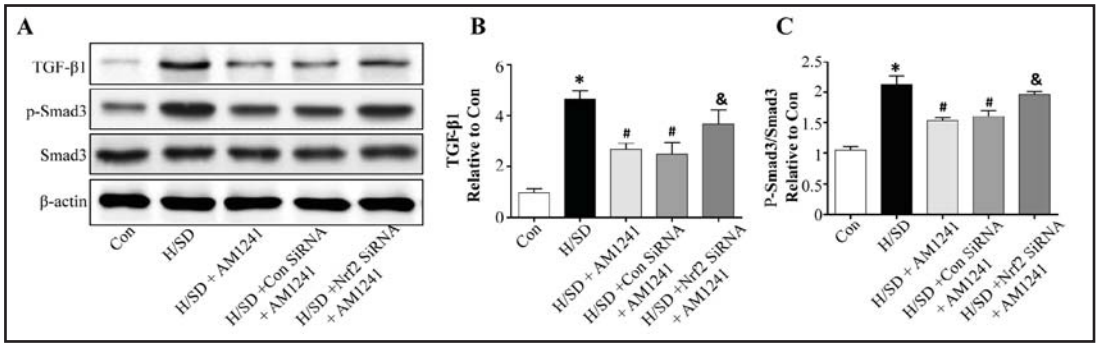

Fig. 7. AM1241 inhibited TGF-β1/Smad3 pathway in CFs in an Nrf2 dependent manner. A: The expression levels of TGF-β1, Smad3 and P-Smad3 in CFs were measured by Western blot; B and C: quantifications of protein levels of TGF-β (B) and P-Smad3/Smad3 (C). n=3-5. *p<0.05 *vs.* Con group;# p<0.05 *vs.* H/SD group; &p<0.05 *vs.* H/SD+AM1241 group.

significantly reduced by AM1241(P < 0.05, Fig. 6 A, B). N-acetylcysteine (NAC) was selected for its common use as a well-known antioxidant of ROS measurement. Our data showed that pre-treatment with NAC (5 mM) for 2 h could remarkably alleviate the ROS triggered by H/SD injury. Furthermore, Malondialdehyde (MDA), recognized as a marker for oxidative stress, is the product of oxidative free radical. Superoxide dismutase (SOD) and Glutathione (GSH) were ROS scavengers which protect cells from ROS damage. As shown in Fig. 6 C-E AM1241 treatment significantly decreased H/SD-induced MDA up-regulation (2.45±0.29 *vs.* 3.42±0.27 nmol/mg protein, P < 0.05) and increased H/SD-reduced GSH level and SOD activity (GSH: 17.96±1.90 *vs.* 9.62±1.84 nmol/mg protein, P < 0.05; SOD: 126.10±4.90 *vs.* 77.85±2.61 U/mg protein, P < 0.05). These findings suggested that the anti-fibrotic effect of AM1241 might be related to reduction in oxidative stress level, and promotion of anti-oxidative GSH and SOD activity.

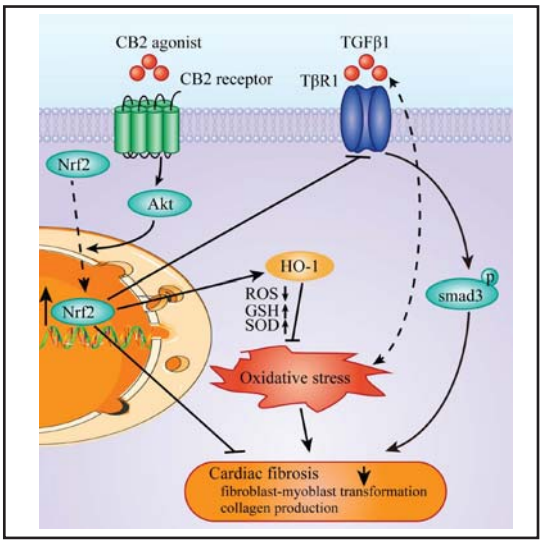

Fig. 8. Schematic diagrams depicting the mechanisms of CB2 receptor activation in alleviating cardiac fibrosis in MI mice. CB2 receptor activation was supposed to alleviate cardiac fibrosis in MI mice through several mechanisms: (a). CB2 receptor activation activated Akt and subsequently accelerated the translocation of Nrf2 to nucleus, Nrf2 activation directly retarded cardiac fibrosis. (b). CB2 receptor activation activated Akt and subsequently accelerated the translocation of Nrf2 to nucleus, thereby reducing oxidative stress and alleviating oxidative stress-aggravated cardiac fibrosis. (c). CB2 receptor activation activated Akt and subsequently accelerated the translocation of Nrf2 to nucleus, thereby inhibiting the pro-fibrotic TGF-β1/Smad3 pathway.

AM1241 suppressed expression of TGF-β1/Smad3 in an Nrf2 dependent pathway

It is well known that the TGF-β1/Smad3 pathway is a classic cell signaling pathway involved in the development of fibrosis. So we next sought to explore the effects of AM1241 on TGF-β1/Smad3 signaling pathway in CFs subjected to H/SD injury. Western blot assay revealed that the levels of TGF-β1, Smad3 phosphorylation significantly increased under H/SD conditions as compared to Control group (Fig. 7 A-C, $P < 0.05$). However, these increases were inhibited by AM1241. In contrast, silencing the Nrf2 by siRNA partially abolished the AM1241's effect. These data indicated that AM1241 inhibited TGF-β1/Smad3 pathway activation in CFs subjected to H/SD injury in an Nrf-2 dependent manner.

Discussion

In the present study, we demonstrated that cannabinoid II (CB2 receptor) receptor agonist AM1241 ameliorated cardiac fibrosis and enhanced cardiac functional recovery in myocardial infarcted mice. In cardiac fibroblasts subjected to H/SD injury, AM1241 inhibited cardiac fibroblast-to-myoblast transformation, collagen production including collagen I and collagen III in an Nrf2 dependent manner. Moreover, AM1241 reduced ROS generation and MDA production, and meanwhile increased anti-oxidative GSH level and SOD activity. Furthermore, AM1241 not only activated and accelerated the translocation of Nrf2 to nucleus, but also inhibited TGF-β1/Smad3 Pathway in an Nrf2 dependent manner.

Cardiac fibrosis and remodeling is one of the major pathological process during the post myocardial infarction phase (post-MI) which is characterized by the imbalance between extracellular matrix(ECM) synthesis and degradation. The overproduction of collagen due to dysregulation of repair inevitably deposited not only in infarct region but also in remote region, resulting in cardiac fibrosis [29]. Cardiac fibrosis curbed cardiac diastolic and systolic function severely which ultimately resulted in heart failure and death [2, 31-33]. In our present study, we demonstrated that CB2 receptor agonist AM1241 ameliorated cardiac fibrosis, which involved in decreased synthesis and increased degradation of extracellular matrix. By retarding cardiac fibrosis, CB2 receptor agonist AM1241 provides incremental benefits for cardiac diastolic and systolic functional recovery. However, relieved cardiac fibrosis may not be the only reason for AM1241's improvement of cardiac function post MI. In our previous study, we proved that CB2 receptor agonist AM1241 could activate cardiac progenitor cells and facilitate endogenous myocardial regeneration [11]. Furthermore, the direct cardio-protection of CB2 receptor agonist in ischemic heart has been well elucidated by a number of studies [34, 35]. Thus, multiple mechanisms may be involved in CB2 receptor

agonist mediated cardiac diastolic and systolic functional recovery in ischemic heart diseases.

Previous evidences have indicated the feasibility of CB2 receptor agonist in the treatment of tissue fibrosis. Munoz-Luque and colleagues reported that CB2 receptor activation reduced liver fibrosis in cirrhotic rats [6]. Moreover, CB2 receptor knockout mice was reported to exhibit increased dermal fibrosis in bleomycin-induced dermal fibrosis [7]. Not surprisingly, CB2 receptor-deficiency in mice reportedly leaded to interstitial fibrosis after repetitive cardiac ischemia/reperfusion injury [35]. In present study, CB2 receptor agonist AM1241 is proved to possess a significant anti-fibrotic effect in MI mice, which may represent a novel strategy to retard cardiac fibrosis in ischemic heart disease and other fibrosis associated heart disorders in a much broader context.

Cardiac fibroblasts (CFs) were thought to play a pivotal role in the pathogenesis of cardiac fibrosis. Under normal circumstances, cardiac fibroblasts (CFs) were protected by the ECM framework and produced minimal amounts of ECM. Once injury happened, fibroblasts were activated and differentiated into contractile myofibroblast characteristicly expressing α-smooth muscle actin (α-SMA) and exhibiting secretory properties [36, 37]. Previous reports have proved that hypoxic stimuli increased the fibroblast-to-myofibroblast transformation and collagen synthesis of CFs [38, 39]. In addition, another previous study has shown that exposure the primary fibroblasts from wild-type and CB2 receptor knockout mice to H_2O_2 for 15 min, the expression of α-SMA in CB2 receptor knockout fibroblasts were significantly higher than that of wildtype fibroblasts. However, JWH133, a CB2 receptor agonist, could inhibit α-SMA up-regulation [40]. Consistent with previous findings that certain environmental stimuli could promote the differentiation of CFs into myofibroblasts that drive aggressive remodeling of the ECM, we observed that simulated MI *in vitro*—H/SD stimuli remarkably increased the expression of α-SMA and enhanced the secretion of collagens that aggravate ECM remodeling. Moreover, we also found that CB2 receptor agonist AM1241 retarded cardiac fibrosis in MI mice by reversing the fibroblast-to-myofibroblast transformation and collagen synthesis of CFs. After MI, CFs quickly differentiated to myofibroblasts which exhibited increased fibrillar collagen synthesis, αSMA expression and capacity to aggravate cardiac fibrosis [37]. By reversing this process, CB2 receptor agonist provided incremental benefits to infarcted hearts—alleviating cardiac fibrosis as well as facilitating cardiac functional recovery .

Nrf2, a well-known anti-oxidative gene, has been reported to be closely related to cardiac fibrosis. Nrf2 reportedly inhibit cardiac fibrosis directly. Nrf2 knockout mice exhibited myocardial fibrosis significantly after transverse aortic constriction surgery, while the overexpression of Nrf2 dramatically inhibited myocardial fibrosis [41]. Moreover, as a crucial regulator of oxidative stress and reactive oxygen species(ROS) level, Nrf2 could reduce cardiac fibrosis through regulating ROS levels. Accumulating evidences already demonstrated that ROS generation promoted the development of cardiac interstitial fibrosis and reducing the overproduction of ROS contributed to ameliorated cardiac fibrosis [42-44]. Nrf2 was dissociated with Keap1 following ROS generation and translocated into nucleus to bind the antioxidant response elements (AREs), then, initiated the transcription of antioxidant genes such as Heme oxygenase-1 (HO-1), superoxide dismutase (SOD), glutathione (GSH) to defense the ROS productions [17, 45, 46]. In line with these reports, we observed the down-regulated levels of ROS and MDA and up-regulated levels of GSH and SOD activity en route to the beneficial actions of CB2 receptor agonist AM1241 against cardiac fibrosis. Furthermore, by using an Nrf2 siRNA, we confirmed the anti-fibrotic effects of AM1241 on CFs *in vitro* were Nrf2 dependent. Thus, CB2 receptor activated Nrf2 signaling might reduce cardiac fibrosis through direct anti-fibrotic effects of Nrf2 and indirect down-regulation of ROS levels. In addition, we also observed that CB2 receptor, its downstream molecular Akt, nucleus Nrf2 and HO-1 slightly increased under H/SD condition. AM1241 treatment further enhanced the CB2 receptor/Akt/Nrf2 activation, the effect of which was inhibited by CB2 receptor antagonist AM630. These data suggested a pivotal role of CB2 receptor/Akt/Nrf2/HO-1 signaling pathway in the anti-fibrotic effects of AM1241.

TGF-β1/Smad3 Pathway, a canonical signaling pathway, has been proved by numerous reports to be involved in maintaining normal cardiac structure extracellular matrix (ECM) homeostasis and regulating cardiac fibrosis [14, 39, 47]. Once TGF-β1 was activated, the signals transmitted through phosphorylating a cytoplasmic protein called Smad3 into nucleus, thus regulating the fibrosis related gene expression, such as *collagen I*, *collagen III*, *α-SMA*, *PAI-1* and *TIMP-1* [12, 14, 15, 39, 48, 49]. Interestingly, Nrf2 has been reported to attenuate dystrophic muscle fibrosis through inhibition of TGF-β1/Smad3 Pathway [50]. In our present study, we used siRNA to inhibit Nrf2 expression, and demonstrated that Nrf2 silencing significantly increased the expression of TGF-β1 and phosphorylation of Smad3. ROS have been shown to promote the development of fibrosis through activating latent TGF-β. ROS upregulated TGF-β expression in various ways, such as oxidizing latency-associated protein(LAP), activating MMPs and up-regulating Nox/Duox family, which in turn cleaved LAP to release more TGF-β, thus forming a positive feedback cycle [51, 52]. On the other hand, TGF-β was reported to directly promote reactive oxygen species (ROS) generation, while deletion of Smad3 abolished this effect of TGF-β, suggesting a pivotal role of TGF-β-Smad3 signaling in ROS generation [53]. Thus, there may be a crosstalk between TGF-β signaling and ROS generation is cardiac fibrogenesis. In our research, we also observed the co-activation of TGF-β signaling and ROS generation in CFs subjected to H/SD injury, while AM1241 treatment leaded to a concurrent decrease of both TGF-β signaling and ROS generation.

Despite the clinical relevance of our present study, there are still some limitations in our study. Firstly, the detailed mechanisms for how Nrf2 interacted with and inhibited TGF-β1/Smad3 pathway was not yet elucidated in the present study, which need further investigations. Secondly, in our current research, we observed that 10 μM AM1241 was not as effective as 5 μM AM1241. Yao B and colleagues have also reported that AM1241 exhibited partial agonist activity in the activation of p42/p44 ERK [54]. While some reports have denoted that activation of ERK inhibited progression of fibrosis, other reports indicated that ERK pathway was implicated in pro-fibrotic actions [55] and diabetic mouse heart [56]. In addition, TGF-β1 reportedly activated Erk in lung fibroblasts [57]. Thus, the overall effects of AM1241 on cardiac fibrosis might be ascribed to the complex interplay among ERK, Nrf2 and TGF-β1 signals. Whether higher concentrations of AM1241 in CFs might make a reversion of the anti-fibrotic actions and the underlying mechanisms remained to be investigated.

In conclusion, our work demonstrated a beneficial role of CB2 receptor activation in ameliorating cardiac fibrosis after myocardial infarction possibly through Nrf2 mediated inhibition of TGF-β1/Smad3 pathway. It is suggested that therapeutic strategy targeting CB2 receptor might be a potential approach to attenuate myocardial fibrosis induced by MI and other fibrosis associated heart disorders in a clinical perspective application.

Acknowledgements

This work was supported by the National Key Research Program of China (2016YFA0100903), National Funds for Distinguished Young Scientists of China (81325009) and National Nature Science Foundation of China (No. No.81270168, 81530058,81570272), (FCao BWS12J037), Beijing Nature Science Foundation (No.7152131), Innovation Team Grant of Shaanxi Province (No.2014KCT-20).

Disclosure Statement

None.

References

1 Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Isasi CR, Jimenez MC, Judd SE, Kissela BM, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Magid DJ, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Rosamond W, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Woo D, Yeh RW, Turner MB, American Heart Association Statistics C, Stroke Statistics S: Heart disease and stroke statistics-2016 update: A report from the american heart association. Circulation 2016;133:e38-e360.

2 Jugdutt BI: Limiting fibrosis after myocardial infarction. N Engl J Med 2009;360:1567-1569.

3 de Haas HJ, Arbustini E, Fuster V, Kramer CM, Narula J: Molecular imaging of the cardiac extracellular matrix. Circ Res 2014;114:903-915.

4 Tamargo J, López-Sendón J: Novel therapeutic targets for the treatment of heart failure. Nat Rev Drug Discov 2011;10:536-555.

5 Di Marzo V, Bifulco M, De Petrocellis L: The endocannabinoid system and its therapeutic exploitation. Nat Rev Drug Discov 2004;3:771-784.

6 Munoz-Luque J, Ros J, Fernandez-Varo G, Tugues S, Morales-Ruiz M, Alvarez CE, Friedman SL, Arroyo V, Jimenez W: Regression of fibrosis after chronic stimulation of cannabinoid cb2 receptor in cirrhotic rats. J Pharmacol Exp Ther 2008;324:475-483.

7 Akhmetshina A, Dees C, Busch N, Beer J, Sarter K, Zwerina J, Zimmer A, Distler O, Schett G, Distler JH: The cannabinoid receptor cb2 exerts antifibrotic effects in experimental dermal fibrosis. Arthritis Rheum 2009;60:1129-1136.

8 Hochhauser E, Lahat E, Sultan M, Pappo O, Waldman M, Sarne Y, Shainberg A, Gutman M, Safran M, Ari ZB: Ultra low dose delta 9-tetrahydrocannabinol protects mouse liver from ischemia reperfusion injury. Cell Physiol Biochem 2015;36:1971-1981.

9 Mukhopadhyay P, Rajesh M, Pan H, Patel V, Mukhopadhyay B, Bátkai S, Gao B, Haskó G, Pacher P: Cannabinoid-2 receptor limits inflammation, oxidative/nitrosative stress, and cell death in nephropathy. Free Radic Biol Med 2010;48:457-467.

10 Rajesh M, Pan H, Mukhopadhyay P, Bátkai S, Osei-Hyiaman D, Haskó G, Liaudet L, Gao B, Pacher P: Pivotal advance: Cannabinoid-2 receptor agonist hu-308 protects against hepatic ischemia/reperfusion injury by attenuating oxidative stress, inflammatory response, and apoptosis. J Leukoc Biol 2007;82:1382-1389.

11 Wang Y, Ma S, Wang Q, Hu W, Wang D, Li X, Su T, Qin X, Zhang X, Ma K, Chen J, Xiong L, Cao F: Effects of cannabinoid receptor type 2 on endogenous myocardial regeneration by activating cardiac progenitor cells in mouse infarcted heart. Sci China Life sci 2014;57:201-208.

12 Zheng D, Dong S, Li T, Yang F, Yu X, Wu J, Zhong X, Zhao Y, Wang L, Xu C, Lu F, Zhang W: Exogenous hydrogen sulfide attenuates cardiac fibrosis through reactive oxygen species signal pathways in experimental diabetes mellitus models. Cell Physiol Biochem 2015;36:917-929.

13 Bache RJ, Chen Y: Nox2-induced myocardial fibrosis and diastolic dysfunction: Role of the endothelium. J Am Coll Cardiol 2014;63:2742-2744.

14 Ruiz-Ortega M, Rodriguez-Vita J, Sanchez-Lopez E, Carvajal G, Egido J: Tgf-beta signaling in vascular fibrosis. Cardiovasc Res 2007;74:196-206.

15 Kim MJ, Park SA, Kim CH, Park SY, Kim JS, Kim DK, Nam JS, Sheen YY: Tgf-beta type i receptor kinase inhibitor ew-7197 suppresses cholestatic liver fibrosis by inhibiting hif1alpha-induced epithelial mesenchymal transition. Cell Physiol Biochem 2016;38:571-588.

16 He T, Bai X, Yang L, Fan L, Li Y, Su L, Gao J, Han S, Hu D: Loureirin b inhibits hypertrophic scar formation via inhibition of the tgf-beta1-erk/jnk pathway. Cell Physiol Biochem 2015;37:666-676.

17 Howden R: Nrf2 and cardiovascular defense. Oxid Med Cell Longev 2013;2013:104308.

18 Liu W, Wang B, Wang T, Liu X, He X, Liu Y, Li Z, Zeng H: Ursodeoxycholic acid attenuates acute aortic dissection formation in angiotensin ii-infused apolipoprotein e-deficient mice associated with reduced ros and increased nrf2 levels. Cell Physiol Biochem 2016;38:1391-1405.

19 Wang Y, Li C, Cheng K, Zhang R, Narsinh K, Li S, Li X, Qin X, Zhang R, Li C, Su T, Chen J, Cao F: Activation of liver x receptor improves viability of adipose-derived mesenchymal stem cells to attenuate myocardial ischemia injury through tlr4/nf-kappab and keap-1/nrf-2 signaling pathways. Antioxid Redox Signal 2014;21:2543-2557.

20 Zhou S, Sun W, Zhang Z, Zheng Y: The role of nrf2-mediated pathway in cardiac remodeling and heart failure. Oxid Med Cell Longev 2014;2014:260429.
21 Li X-H, Li C-Y, Xiang Z-G, Hu J-J, Lu J-M, Tian R-B, Jia W: Allicin ameliorates cardiac hypertrophy and fibrosis through enhancing of nrf2 antioxidant signaling pathways. Cardiovasc Drugs Ther 2012;26:457-465.
22 Gao E, Lei YH, Shang X, Huang ZM, Zuo L, Boucher M, Fan Q, Chuprun JK, Ma XL, Koch WJ: A novel and efficient model of coronary artery ligation and myocardial infarction in the mouse. Circ Res 2010;107:1445-1453.
23 Dubey RK, Gillespie DG, Mi Z, Jackson EK: Exogenous and endogenous adenosine inhibits fetal calf serum-induced growth of rat cardiac fibroblasts: Role of a2b receptors. Circulation 1997;96:2656-2666.
24 Chen X, Liu G, Zhang W, Zhang J, Yan Y, Dong W, Liang E, Zhang Y, Zhang M: Inhibition of mef2a prevents hyperglycemia-induced extracellular matrix accumulation by blocking akt and tgf-β1/smad activation in cardiac fibroblasts. Int J Biochem Cell Biol 2015;69:52-61.
25 Han D, Huang W, Li X, Gao L, Su T, Li X, Ma S, Liu T, Li C, Chen J, Gao E, Cao F: Melatonin facilitates adipose-derived mesenchymal stem cells to repair the murine infarcted heart via the sirt1 signaling pathway. J Pineal Res 2016;60:178-192.
26 Pan Q, Qin X, Ma S, Wang H, Cheng K, Song X, Gao H, Wang Q, Tao R, Wang Y, Li X, Xiong L, Cao F: Myocardial protective effect of extracellular superoxide dismutase gene modified bone marrow mesenchymal stromal cells on infarcted mice hearts. Theranostics 2014;4:475-486.
27 Palazuelos J, Ortega Z, Diaz-Alonso J, Guzman M, Galve-Roperh I: Cb2 cannabinoid receptors promote neural progenitor cell proliferation via mtorc1 signaling. J Biol Chem 2012;287:1198-1209.
28 Yeh YH, Kuo CT, Chang GJ, Chen YH, Lai YJ, Cheng ML, Chen WJ: Rosuvastatin suppresses atrial tachycardia-induced cellular remodeling via akt/nrf2/heme oxygenase-1 pathway. J Mol Cell Cardiol 2015;82:84-92.
29 van Nieuwenhoven FA, Turner NA: The role of cardiac fibroblasts in the transition from inflammation to fibrosis following myocardial infarction. Vascul Pharmacol 2013;58:182-188.
30 Walters DM, Cho H-Y, Kleeberger SR: Oxidative stress and antioxidants in the pathogenesis of pulmonary fibrosis: A potential role for nrf2. Antioxid Redox Signal 2008;10:321-332.
31 Opie LH, Commerford PJ, Gersh BJ, Pfeffer MA: Controversies in ventricular remodelling. Lancet 2006;367:356-367.
32 Cohn JN, Ferrari R, Sharpe N: Cardiac remodeling--concepts and clinical implications: A consensus paper from an international forum on cardiac remodeling. Behalf of an international forum on cardiac remodeling. J Am Coll Cardiol 2000;35:569-582.
33 Li W, Yan S, Zhao J, Ding X, Zhang S, Wang D, Liu L, Peng W, Li H, Wang D, Liu Z, Li Y: Metoprolol inhibits cardiac apoptosis and fibrosis in a canine model of chronic obstructive sleep apnea. Cell Physiol Biochem 2015;36:1131-1141.
34 Li Q, Wang F, Zhang Y-M, Zhou J-J, Zhang Y: Activation of cannabinoid type 2 receptor by jwh133 protects heart against ischemia/reperfusion-induced apoptosis. Cell Physiol Biochem 2013;31:693-702.
35 Duerr GD, Heinemann JC, Suchan G, Kolobara E, Wenzel D, Geisen C, Matthey M, Passe-Tietjen K, Mahmud W, Ghanem A: The endocannabinoid-cb2 receptor axis protects the ischemic heart at the early stage of cardiomyopathy. Basic Res Cardiol 2014;109:1-15.
36 van den Borne SW, Diez J, Blankesteijn WM, Verjans J, Hofstra L, Narula J: Myocardial remodeling after infarction: The role of myofibroblasts. Nat Rev Cardiol 2010;7:30-37.
37 van Nieuwenhoven FA, Turner NA: The role of cardiac fibroblasts in the transition from inflammation to fibrosis following myocardial infarction. Vascul Pharmacol 2013;58:182-188.
38 Wang K, Liu CY, Zhou LY, Wang JX, Wang M, Zhao B, Zhao WK, Xu SJ, Fan LH, Zhang XJ, Feng C, Wang CQ, Zhao YF, Li PF: Apf lncrna regulates autophagy and myocardial infarction by targeting mir-188-3p. Nat Commun 2015;6:6779.
39 Zhao X, Wang K, Liao Y, Zeng Q, Li Y, Hu F, Liu Y, Meng K, Qian C, Zhang Q, Guan H, Feng K, Zhou Y, Du Y, Chen Z: Microrna-101a inhibits cardiac fibrosis induced by hypoxia via targeting tgfbetari on cardiac fibroblasts. Cell Physiol Biochem 2015;35:213-226.
40 Defer N, Wan J, Souktani R, Escoubet B, Perier M, Caramelle P, Manin S, Deveaux V, Bourin MC, Zimmer A, Lotersztajn S, Pecker F, Pavoine C: The cannabinoid receptor type 2 promotes cardiac myocyte and fibroblast survival and protects against ischemia/reperfusion-induced cardiomyopathy. FASEB J 2009;23:2120-2130.

41 Li J, Ichikawa T, Villacorta L, Janicki JS, Brower GL, Yamamoto M, Cui T: Nrf2 protects against maladaptive cardiac responses to hemodynamic stress. Arterioscler Thromb Vasc Biol 2009;29:1843-1850.
42 Hafstad AD, Nabeebaccus AA, Shah AM: Novel aspects of ros signalling in heart failure. Basic Res Cardiol 2013;108:359.
43 Huynh K, Bernardo BC, McMullen JR, Ritchie RH: Diabetic cardiomyopathy: Mechanisms and new treatment strategies targeting antioxidant signaling pathways. Pharmacol Ther 2014;142:375-415.
44 Zhao W, Zhao T, Chen Y, Ahokas RA, Sun Y: Oxidative stress mediates cardiac fibrosis by enhancing transforming growth factor-beta1 in hypertensive rats. Mol Cell Biochem 2008;317:43-50.
45 Yamamoto T, Suzuki T, Kobayashi A, Wakabayashi J, Maher J, Motohashi H, Yamamoto M: Physiological significance of reactive cysteine residues of keap1 in determining nrf2 activity. Mol Cell Biol 2008;28:2758-2770.
46 Xu M, Li XX, Wang L, Wang M, Zhang Y, Li PL: Contribution of nrf2 to atherogenic phenotype switching of coronary arterial smooth muscle cells lacking cd38 gene. Cell Physiol Biochem 2015;37:432-444.
47 Li S, Fan Q, He S, Tang T, Liao Y, Xie J: Microrna-21 negatively regulates treg cells through a tgf-beta1/smad-independent pathway in patients with coronary heart disease. Cell Physiol Biochem 2015;37:866-878.
48 Todorovic V, Jurukovski V, Chen Y, Fontana L, Dabovic B, Rifkin DB: Latent tgf-beta binding proteins. Int J Biochem Cell Biol 2005;37:38-41.
49 Qi HP, Wang Y, Zhang QH, Guo J, Li L, Cao YG, Li SZ, Li XL, Shi MM, Xu W, Li BY, Sun HL: Activation of peroxisome proliferator-activated receptor gamma (ppargamma) through nf-kappab/brg1 and tgf-beta1 pathways attenuates cardiac remodeling in pressure-overloaded rat hearts. Cell Physiol Biochem 2015;35:899-912.
50 Sun C, Li S, Li D: Sulforaphane mitigates muscle fibrosis in mdx mice via nrf2-mediated inhibition of tgf-beta/smad signaling. J Appl Physiol (1985) 2016;120:377-390.
51 Hecker L, Vittal R, Jones T, Jagirdar R, Luckhardt TR, Horowitz JC, Pennathur S, Martinez FJ, Thannickal VJ: Nadph oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. Nat Med 2009;15:1077-1081.
52 Liu RM, Gaston Pravia KA: Oxidative stress and glutathione in tgf-beta-mediated fibrogenesis. Free Radic Biol Med 2010;48:1-15.
53 Black D, Lyman S, Qian T, Lemasters JJ, Rippe RA, Nitta T, Kim J-S, Behrns KE: Transforming growth factor beta mediates hepatocyte apoptosis through smad3 generation of reactive oxygen species. Biochimie 2007;89:1464-1473.
54 Yao BB, Mukherjee S, Fan Y, Garrison TR, Daza AV, Grayson GK, Hooker BA, Dart MJ, Sullivan JP, Meyer MD: In vitro pharmacological characterization of am1241: A protean agonist at the cannabinoid cb2 receptor? Br J Pharmacol 2006;149:145-154.
55 Stratton R, Rajkumar V, Ponticos M, Nichols B, Shiwen X, Black CM, Abraham DJ, Leask A: Prostacyclin derivatives prevent the fibrotic response to tgf-beta by inhibiting the ras/mek/erk pathway. FASEB J 2002;16:1949-1951.
56 Peake BF, Nicholson CK, Lambert JP, Hood RL, Amin H, Amin S, Calvert JW: Hydrogen sulfide preconditions the db/db diabetic mouse heart against ischemia-reperfusion injury by activating nrf2 signaling in an erk-dependent manner. Am J Physiol Heart Circ Physiol 2013;304:H1215-H1224.
57 Caraci F, Gili E, Calafiore M, Failla M, La Rosa C, Crimi N, Sortino MA, Nicoletti F, Copani A, Vancheri C: Tgf-beta 1 targets the gsk-3 beta /beta-catenin pathway via erk activation in the transition of human lung fibroblasts into myofibroblasts. Pharmacol Res 2008;57:274-282.