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Apocynin attenuates isoproterenol-induced myocardial injury and fibrogenesis

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ABSTRACT

Oxidative stress is mechanistically implicated in the pathogenesis of myocardial injury and the subsequent fibrogenic tissue remodeling. Therapies targeting oxidative stress in the process of myocardial fibrogenesis are still lacking and thus remain as an active research area in myocardial injury management. The current study evaluated the effects of a NADPH oxidase inhibitor, apocynin, on the production of reactive oxygen species and the development of myocardial fibrogenesis in isoproterenol (ISO)-induced myocardial injury mouse model. The results revealed a remarkable effect of apocynin on attenuating the development of myocardial necrotic lesions, inflammation and fibrogenesis. Additionally, the protective effects of apocynin against myocardial injuries were associated with suppressed expression of an array of genes implicated in inflammatory and fibrogenic responses. Our study thus provided for the first time the histopathological and molecular evidence supporting the therapeutic value of apocynin against the development of myocardial injuries, in particular, myocardial fibrogenesis, which will benefit the mechanism-based drug development targeting oxidative stress in preventing and/or treating related myocardial disorders.

1. Introduction

Myocardial fibrosis is one of the most common manifestations of the failing heart [1]. Various pathophysiological mechanisms, for instance, myocardial infarction can lead to myocardial fibrosis. Myocardial fibrosis is one of the major determinants of ventricular remodeling after myocardial infarction and is associated with impeded ventricular systolic function, abnormal cardiac remodeling, increased ventricular stiffness and development of arrhythmias, etc. Mechanism-based therapeutic development for myocardial infarction and post ischemic myocardial fibrosis remains as the area of intensive research.

NADPH oxidase is the primary enzymatic source of oxidant generation in mammalian cells [2]. NADPH oxidase, a multisubunit complex consisting of membrane-associated $gp91^{phox}$ and $p22^{phox}$ and cytosolic subunits including $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$, etc., mediates the production of superoxide anion, a precursor of reactive oxygen species (ROS). Tightly regulated ROS are important intracellular messengers required for various physiological functions including cell growth, differentiation and metabolism, etc. However, overproduction of ROS is implicated in the pathogenesis of various diseases including myocardial infarction [3]. The potential role of NADPH oxidase-mediated ROS generation in myocardial infarction has been suggested by studies performed in genetically modified mouse model [4]. However, the therapeutic value of pharmacological intervention targeting NADPH oxidase-mediated ROS generation in myocardial ischemic injury and the subsequent myocardial fibrosis remains to be investigated. Apocynin (4-hydroxy-3-methoxyacetophenone, APO), a naturally occurring methoxy-substituted catechol, disrupts the formation of the active NADPH oxidase complex by blocking migration of $p47^{phox}$ to the plasma membrane, which is critically involved in initiating assembly of the functional NADPH oxidase complex [5]. Therefore, APO has been extensively adopted as an inhibitor of NADPH oxidase in various experimental models. However, the effects and underlying mechanisms of APO on myocardial injuries remain to be evaluated.

Our current study therefore addressed the therapeutic value of APO in myocardial ischemic injures and fibrogenesis by examining

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the histological and molecular alterations of the hearts in the presence and absence of APO treatment in isoproterenol (ISO)-induced myocardial injury mouse model.

2. Materials and methods

2.1. Animal

Male and female 6- to 8-week-old C57BL/6J mice weighing 20–25 g were obtained from Chinese Academy of Science. The mice were maintained on regular rodent chow and allowed free access to food and water. All the procedures were reviewed and approved by Institutional Animal Care and Use Committee of Shanghai University of TCM. Myocardial ischemic injury was induced by intraperitoneal injection of isoproterenol (ISO) (Sigma, USA) at the dose of 5 mg/kg body weight (bw) daily for 5 days. PBS was used as a vehicle control for ISO. Apocynin (APO) (Sigma, USA) was dissolved in DMSO (Sigma, USA) and was delivered 30 min prior to each ISO administration through intraperitoneal injection. All the treatment was controlled in the volume of 50 μL.

2.2. Histological examination

At the end of the indicated treatment, mice were anesthetized by intraperitoneal injection of a cocktail containing ketamine (6 mg/ml) and xylazine (0.44 mg/ml) in 10 mM sodium phosphate, pH 7.2 and 100 mM NaCl at the dose of 20 μl/g bw. Hearts were dissected and fixed in 4% paraformaldehyde prior to further processing. For histological examination of mouse hearts, paraffin sections in the thickness of 5 μm were prepared and subject to hematoxylin and eosin (H&E) or Masson's trichrome staining.

2.3. Microarray analysis

Total RNA was isolated and purified from the mouse hearts using mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, US) following the manufacturer's instructions. Total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Agilent technologies, Santa Clara, CA, US) according to the manufacturer's instructions. Labeled cRNA were subject to Agilent mouse genome 4×44 K microarray hybridization (Agilent technologies, Santa Clara, CA, US). Differentially expressed genes were defined as the genes with fold changes above 2 and $p < 0.05$ after t-test analysis.

2.4. Real-time PCR analysis

Total RNA was isolated and purified using mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, US) and then reverse-transcribed using RevertAid First Strand cDNA Synthesis kit (Thermo, USA) to generate cDNA for real-time PCR analyses of mRNA expression. Primers are indicated in Supplemental Table. Real-time PCR was carried out using ABI Power SYBR Green PCR Master Mix (ABI, USA) for mRNA expression on 7900 HT Sequence Detection System (ABI, USA) and Light Cycler 480 SYBR I Master (Roche, USA) for miRNA expression on Light Cycler 480 (Roche, USA), respectively.

2.5. Statistical analysis

Results were averaged from at least three independent experiments and data were expressed as mean ± S.E.M. The statistical analyses were carried out using the student's t test. p value less than 0.05 was considered as statistically significant.

3. Results

3.1. Apocynin protects mouse hearts from developing ISO-induced injury through inhibition of ROS production

ISO, a sympathomimetic β-adrenergic receptor agonist, induces infarct like cell death of cardiac muscle. The animal model of ISO-induced myocardial injury recapitulates major metabolic and morphological changes occurring during human myocardial infarction, providing a standardized model for evaluating the cardiac protective effects of pharmacological agents against myocardial ischemic injury [6–10]. The effect of APO on ISO-induced myocardial injury was assessed by administering APO at various doses to mice 30 min prior to ISO challenging at the dose of 5 mg/kg bw daily for 5 consecutive days. As shown in Fig. 1, compared to the intact histological features displayed by the hearts from vehicle controls (Fig. 1A), ISO induced extensive myocardial necrosis, inflammatory infiltration and granulation (Fig. 1B). APO treatment, however, displayed a dose-dependent effect on attenuating ISO-induced myocardial ischemic injury when it was delivered at 12.5 mg/kg bw (Fig. 1C), 25 mg/kg bw (Fig. 1D), 50 mg/kg bw (Fig. 1E) and 100 mg/kg bw (Fig. 1F), respectively.

To further evaluate whether the effects of APO were associated with ROS generation, in situ superoxide production was assessed via DHE, a ROS probe with substrate specificity to superoxide, in the hearts from vehicle controls, ISO-challenged mice and APO-treated mice, respectively. As shown in Supplemental Fig. 1A, compared to the signals recorded in the left ventricular sections in the vehicle controls (Supplemental Fig. 1A.a), mice receiving ISO alone displayed remarkably increased superoxide generation in the left ventricle (Supplemental Fig. 1A.b), whereas this elevation in superoxide signals was not encountered in APO-treated mouse hearts (Supplemental Fig. 1A.c). Myocardial NADPH oxidase activity was further assessed by evaluating NADPH mediated superoxide production via lucigenin chemiluminescence assay. As shown in Supplemental Fig. 1B.a, compared to their normal counterparts, significantly increased myocardial NADPH-dependent ROS generation was observed in the hearts of mice that received ISO. In distinct contrast, APO treatment resulted in significantly reduced ROS signal in the hearts compared to that from ISO controls. Moreover, as shown in Supplemental Fig. 1B.b, the lucigenin signal was significantly inhibited by DPI or APO, supporting the notion that ROS generation was likely NADPH oxidase-dependent. In addition, ROS signal was nearly abolished by Tiron co-incubation, confirming that superoxide was the major source of detected ROS, which was consistent with the results from in situ ROS detection as shown in Supplemental Fig. 1A.a. These results indicated that APO treatment was able to suppress ISO-induced, NADPH oxidase-mediated overproduction of ROS, which may contribute to its protective effects against the development of myocardial injuries.

3.2. APO inhibits ISO-induced myocardial fibrogenesis

The effect of APO treatment on myocardial fibrosis was further evaluated by Masson's trichrome staining, a widely used method for the detection of collagen fibers. As shown in Fig. 2A, small amount of collagen fiber was observed in the interstitial space in the hearts from the vehicle controls, whereas significantly increased amount of Masson's trichrome stained collagen fibers were present at the site of microscopic injury in ISO-treated mouse hearts (29.5% ± 1.55 in ISO vs. 1.27% ± 0.13 in VC). In distinct contrast, APO treatment resulted in significantly diminished Masson's trichrome stained area in a dose-dependent manner (11.92% ± 0.85 in APO 12.5 mg/kg bw group, 7.6% ± 0.34 in APO 25 mg/kg bw

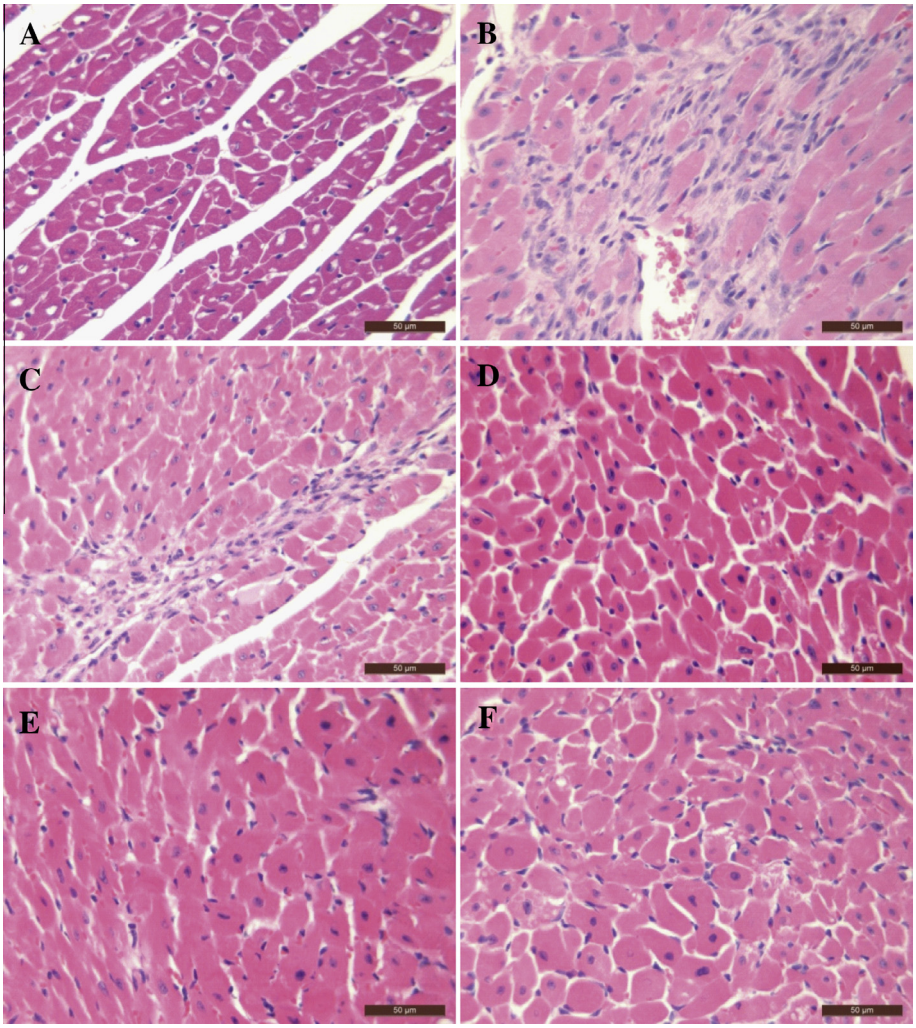

Fig. 1. Dose-dependent effect of APO treatment on ISO-induced myocardial injury. Mice received APO 30 min prior to ISO administration for 5 consecutive days. APO was given via i.p injection at various doses, including 12.5 mg/kg bw, 25 mg/kg bw, 50 mg/kg bw and 100 mg/kg bw, respectively. At the end of 5-day treatment, hearts were collected and sections were stained with H&E staining. (A) Well organized and aligned cardiac muscle fibers were observed in the vehicle controls. (B) Extensive myocardial necrosis and granulation was prominent in mice challenged by ISO in the absence of APO treatment. (C) Minor myocardial necrotic injury was observed in mice receiving APO at the dose of 12.5 mg/kg bw. (D–F) No overt myocardial necrotic changes were encountered when APO was delivered at the doses of 25 mg/kg bw, 50 mg/kg bw and 100 mg/kg bw, respectively.

group, 2.35% ± 0.34 in APO 50 mg/kg bw group and 3.77% ± 0.45 in APO 100 mg/kg bw group, respectively). The representative photomicrographs were presented in Fig. 2B. These data suggest that APO treatment protects against the development of myocardial fibrosis.

The effect of APO treatment on myocardial fibrogenesis was also evaluated by immunohistochemical examination of molecular markers signifying tissue fibrogenesis. The presence and the distribution of myofibroblasts were examined by α-smooth muscle actin (α-SMA) [11] expression in the heart sections. In ISO-challenged mouse hearts, α-SMA expressing myofibroblasts were readily detected at the sites of microscopic injuries (Supplemental Fig. 2a), which were not encountered in the hearts from APO-treated mice (Supplemental Fig. 2b). Moreover, the activity of TGFβ signaling, a major pathway involved in myocardial fibrosis [12] was assessed by first examining p-SMAD2 immunoreactivity in the mouse hearts. As shown in Supplemental Fig. 2c, the immunoreactivity of p-SMAD2 was remarkably increased in ISO-treated mouse heart at the sites of myocardial injuries, whereas, it was rarely observed in APO-treated heart sections (Supplemental Fig. 2d). Furthermore, the expression of Timp1 and Timp2, two factors induced by TGFβ and function to inhibit collagen degradation, was examined. Timp1 and Timp2 expression was readily detected at the sites of myocardial injury in ISO-treated hearts (Supplemental Fig. 2e and g), which was not observed in APO-treated mouse hearts (Supplemental Fig. 2f and h).

3.3. Altered gene expression in response to apocynin treatment

To further explore the molecular mechanisms underlying the protective effects of APO against ISO-induced myocardial injuries, genome-wide gene expression profiling in the absence and presence of APO intervention was examined and compared. As shown in Fig. 3A, compared to the gene expression profile from vehicle controls, 942 genes showed dysregulated expression in ISO-challenged mouse hearts, and 438 of which were significantly modulated by APO treatment. Gene annotation further revealed that myocardial injury responsive genes, chemokine ligands and inflammatory cell markers, as well as collagen and non-collagen ECM genes were induced by ISO and this induction was remarkably suppressed by APO treatment. Examples were shown in Fig. 3B, mainly including injury response gene, Adam8; chemokine ligands

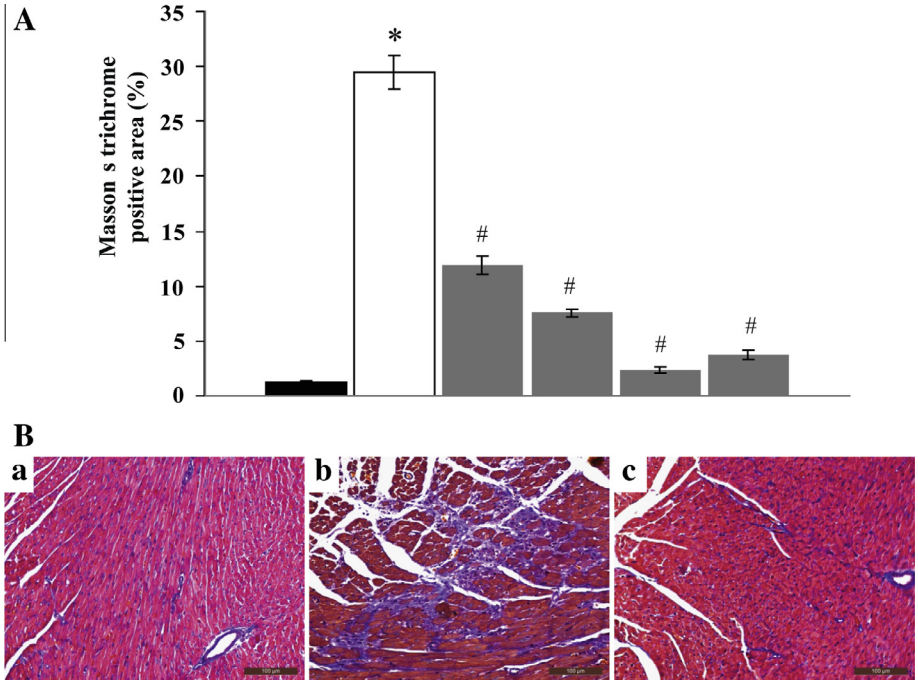

Fig. 2. APO treatment attenuated ISO-induced myocardial fibrogenesis. Masson's trichrome staining was performed in the heart sections harvested after 5-day treatment of ISO in the absence or presence of APO treatment. (A) Percentage of Masson's trichrome positive area was measured and showed that ISO alone significantly increased Masson's trichrome stained area compared to that from the vehicle controls, which was significantly reduced by APO treatment in a dose-dependent manner. (*compared to the VC, $p < 0.01$; #compared to the ISO, $p < 0.01$). (B) Representative images of Masson's trichrome stained left ventricular sections from VC, ISO alone, and APO given at 50 mg/kg bw prior to ISO administration.

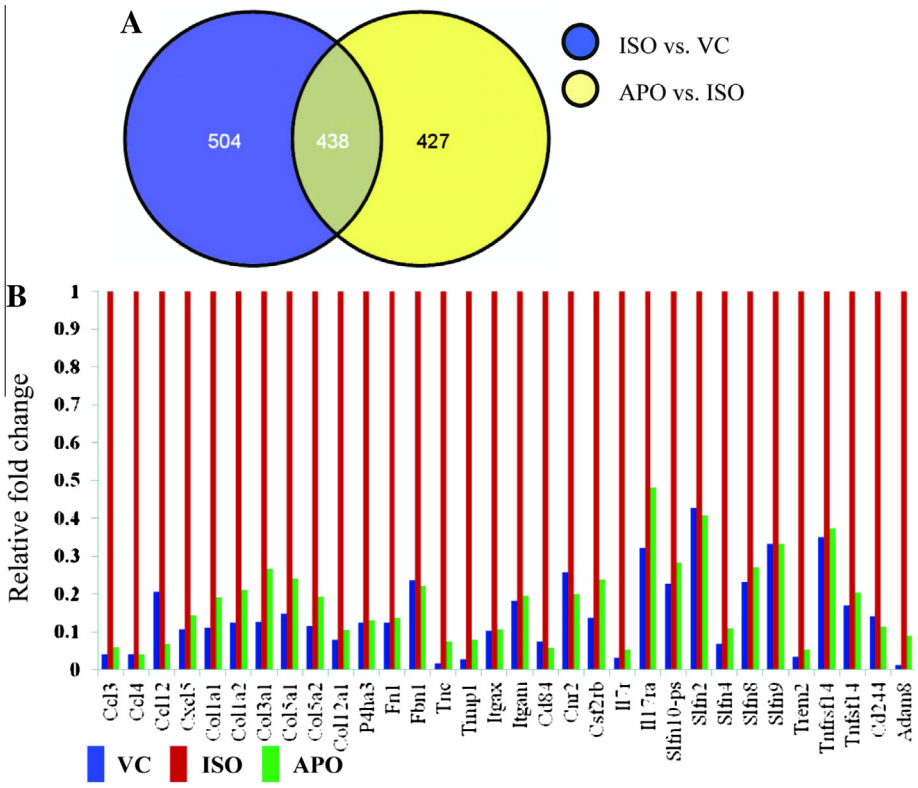

Fig. 3. APO treatment modulated the expression of genes associated with ISO-induced myocardial injury. Genome-wide gene expression profiling analysis was performed after 5-day ISO administration in absence or presence of APO treatment. (A) Differentially expressed genes in ISO-challenged hearts ($n = 3$) vs. that from the VC ($n = 4$) and APO-treated hearts ($n = 5$) vs. ISO-challenged hearts. (Differentially expressed genes were defined as $p < 0.05$ and fold change > 2). (B) Examples of genes implicated in myocardial injury response, inflammation and ECM were differentially regulated by ISO or APO.

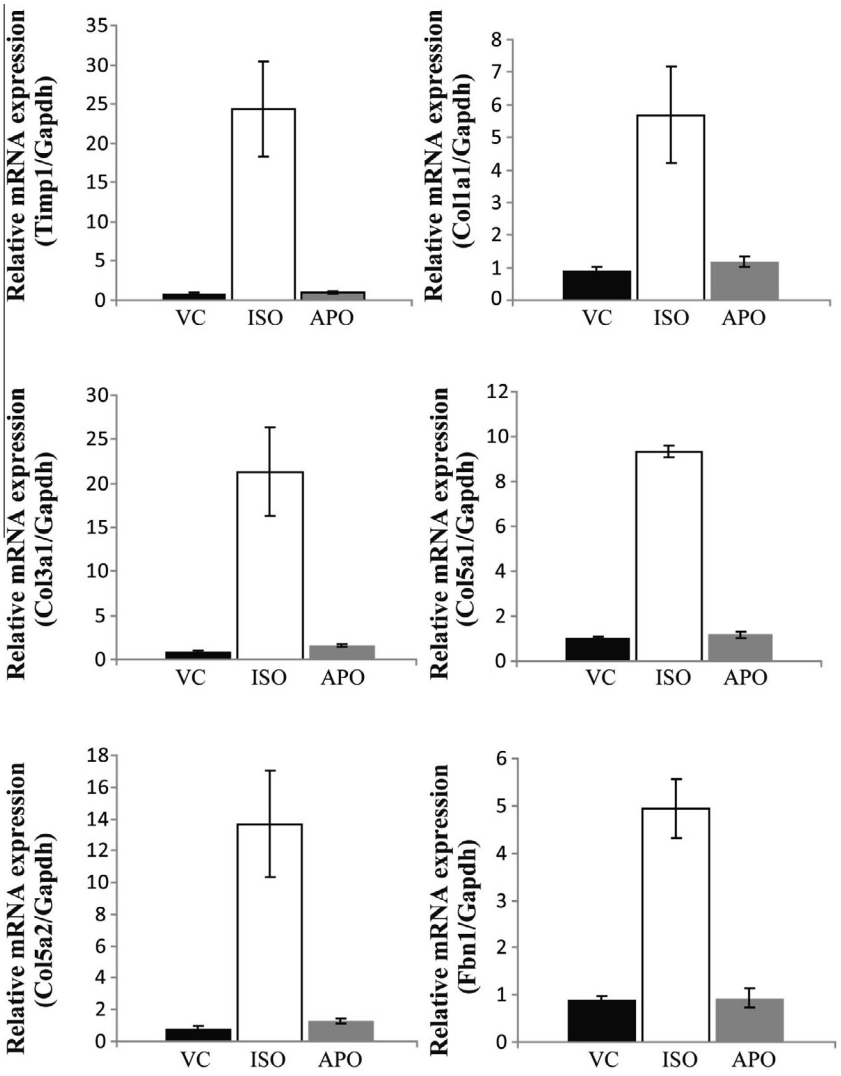

Fig. 4. Real-time PCR validation of the expression of genes implicated in fibrogenesis. Differentially expressed ECM genes associated with fibrogenesis were further validated by real-time PCR and confirmed the findings revealed by gene profiling analysis. The relative expression of mRNA of indicated genes from ISO ($n = 5$) and APO ($n = 5$) was presented after calculating the fold change against that from VC ($n = 5$), which was set at 1.

such as Ccl3 and Ccl4; collagen (Col) genes such as Col1, Col3 and Col5; non-collagen extracellular matrix (ECM) genes including fibronectin, fibrillin and tenascin; fibrogenesis regulator Timp1; immune cell and inflammatory response including monocyte/macrophage surface markers and activation markers such as integrin α-m 1(Itgam, Mac-1, CD11b), integrin α-x (Itgax, CD11c), Schlafen (Slfn) 1, 2, 4, 8 and 9, etc. Real-time PCR was carried out and further validated the changes in the genes implicated in fibrogenesis (Fig. 4).

4. Conclusions

Oxidative stress is mechanistically implicated in the pathogenesis of cardiac injury and progression of cardiac dysfunction under various pathological conditions, regardless of the etiological or anatomical basis. NADPH oxidase is the primary enzymatic source of superoxide generation in mammalian cells [2]. However, the pharmacological value of targeting NADPH oxidase remains to be evaluated in myocardial injury. Our data demonstrate that a NADPH oxidase inhibitor, APO, significantly attenuated the histopathological and molecular changes associated with myocardial injury and ensuing fibrogenesis.

Myocardial fibrosis is a result of injuries of different causes to cardiac tissue, which ultimately leads to destruction of physiological tissue architecture and progressive organ dysfunction. Fibrogenesis is initiated by a variety of cytokines and growth factors produced by activated macrophages and inflammatory cells during the initial inflammatory phase. Following the inflammatory phase, fibrogenesis occurs prominently in the granulation tissue around the necrotic myocardium. Fibroblasts that proliferate and infiltrate into the infarct zone are transformed into myofibroblasts, which express and synthesize a variety of collagenic and non-collagenic ECM components that reconstruct the ECM in the infarcted myocardium and directly contributes to myocardial fibrosis [11]. TGF-β1 is one of the main regulators of ECM homeostasis by inducing the expression of various ECM proteins in cardiac myofibroblasts, playing critical roles in promoting fibrotic formation [12]. TIMPs are also under strict regulation by TGF-β1 and are implicated in the pathogenesis of cardiac fibrogenesis by their inhibitory effects on collagen degradation. Pharmacological intervention modulating these aspects of fibrogenesis may have therapeutic value in limiting fibrogenic processes and ventricular remodeling. Our results indicate that upregulated TGFβ signaling is involved in ISO-induced myocardial fibrogenesis and, APO treatment atten-

uated the development of this pathology in part through suppressing the activity of TGFβ signaling.

At molecular level, ISO-induced myocardial injury was marked by altered expression of an array of genes implicated in the inflammatory and fibrogenic responses. For instance, Adam8 has been suggested as a potential surrogate of inflammation [13]. Adam8 is expressed mainly in macrophages, B cells and cells of the nervous system and contributes to the regulation of leukocyte adhesion and infiltration. The expression of Adam8 was increased by about 74-fold in ISO-challenged mouse hearts compared to that from the normal controls, APO treatment significantly downregulated Adam8 expression to about 0.09% of that in ISO-treated hearts. ISO-induced myocardial injury was also marked by increased expression of a panel of CC chemokine ligands, including Ccl3, 4, 9, 12 and 17. These Ccls mainly function to induce the migration of monocytes to enter surrounding tissue to become tissue macrophages thereby promoting inflammation in injured tissue [14]. Therefore, the upregulated expression of these CC chemokine ligands could contribute to the elevated inflammatory response and the pathogenesis of ISO-induced myocardial injury. Notably, the expression of Ccl11 was significantly decreased in ISO-treated mouse hearts. Ccl11 has been shown to be constitutively expressed in the heart and other tissues such as lung, gut and placenta. The function of Ccl11 has been suggested to negatively regulate neutrophil recruitment and might be involved in the amelioration of acute inflammatory response within these tissues [15]. APO treatment resulted in significant reduction in the expression of Ccl3, Ccl4, Ccl9, Ccl12 and Ccl17, and upregulation of Ccl11 expression in the hearts of ISO-challenged mice, indicating that APO treatment exert cardiac protective effect in part through modulating the expression of chemokine ligands that are involved in the inflammatory response during myocardial injury. Additionally, APO treatment downregulated the constitutive expression of Ccl6 and Ccl8 although their expression was not altered by ISO, which may further enhance the interventional effect of APO on inflammatory response during myocardial injury.

The effect of APO on inflammation and immune response was further supported by its impact on the expression of a panel of molecular markers of monocyte/macrophage, leukocyte and lymphocyte that were upregulated in ISO-challenged mouse hearts, including Csf2rβ2, Itgax, Itgam, CD84, CD244, Cnr2, Trem2, interleukin receptors (ILR) such as IL7R, IL17Rα, IL1R1, IL1R2, IL1Rl2, IL2Rα, several Slfn including Slfn1, Slfn2, Slfn4, Slfn8, Slfn9, Tnfsf14, Tnfrsf14, Tnfaip2, etc. It is worth noting that APO treatment significantly downregulated the constitutive expression of IL1β, IL18, IL3Rα, IL21R and Nfil3, which could further enhance its effects on modulating immune and inflammatory response during myocardial injury. Slfn family genes are predominantly expressed in the cells of immune system and play roles in cell proliferation, survival, development and differentiation. Slfn2 is required for the maturation and/or survival of monocyte [16]. Significantly increased expression of Slfn1, 2, 4, 8 and 9 was observed in the hearts from ISO-challenged mice with Slfn4 being most upregulated, indicating the macrophage activation is an active process in ISO-induced myocardial injury. APO treatment exhibited a remarkable effect on downregulating the expression of these Slfns, suggesting that inhibition of macrophage activation could in part contribute to its effects on protecting against ISO-induced myocardial injury.

In addition to altered expression of genes contributory to inflammatory response, aberrant expression of ECM genes was evident in ISO-induced myocardial injury. The expression of a large number of ECM genes including collagens, collagen synthesis-related genes implicated in fibrotic formation was significantly upregulated. For instance, Col1a1 and Col3a, the predominant components of cardiac collagens [17], were increased by 11-fold and 8.7-fold, respectively, which corroborates the histopathological manifestations of ISO-induced myocardial fibrogenesis. Notably, APO treatment significantly downregulated the expression of nearly 50% of collagen and collagen synthesis related genes, which were upregulated by ISO. In particular, the expression of Col1a1 and Col3a1was brought down by APO treatment to 17% and 27% of that in ISO-challenged hearts, respectively. Moreover, APO treatment exhibited a striking effect on the expression of non-collagen ECM genes, including Fibronectin [18], Fibrillin 1 [19] and Tenascin C [20]. Fibronectin gene encodes the most ubiquitous noncollagen ECM protein associated with cardiac fibrosis. Fibrillin 1 is a member of the fibrillin family and encodes a large, extracellular matrix glycoprotein that serves as a structural component of calcium-binding microfibrils. Fibrillin 1 is abundantly expressed throughout the myocardium and also highly inducible in response to fibrotic stimuli in cardiac fibroblasts, indicating that it is closely associated with reactive and reparative cardiac fibrosis. For instance, fibrillin 1 expression was significantly increased at both mRNA and protein level in ANG II or DOCA-salt-induced cardiac fibrosis rat model. Besides, APO treatment also significantly reduced the mRNA expression of Timp1, which was dramatically upregulated in ISO-treated mouse heart. This was consistent with increased Timp1 expression revealed by immunohistochemical assessment.

Our results further supported the notion that oxidative stress contributes to the inflammatory and fibrogenic responses that are mechanistically important for the development of myocardial injury. Most importantly, our study demonstrated for the first time a remarkable therapeutic effect of APO on ameliorating myocardial ischemic injury, which is in part mediated through suppressing inflammatory and fibrogenic responses. These data therefore justify further evaluation of the clinical value of APO in managing related myocardial disorders.

Authors' contributions

L. Liu and J.C. performed the experiments and analyzed the data. Q.Y., C.J., M.X., B.N., X.D., P.W., X.Y., L. Li performed the experiments and W.W. helped analyze the data. Y.C. and T.Z. designed the experiments and wrote the manuscript.

Competing interest

The authors declare no competing interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.157.

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