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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

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