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Original article

STARS knockout attenuates hypoxia-induced pulmonary arterial hypertension by suppressing pulmonary arterial smooth muscle cell proliferation

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ABSTRACT

STARS (STriated muscle Activator of Rho Signaling) is a sarcomeric protein, which expressed early in cardiac development and involved in pathological remodeling. Abundant evidence indicated that STARS could regulate cell proliferation, but it's exact function remains unclear. In this study, we aimed to investigate the role of STARS in the proliferation of pulmonary arterial smooth muscle cells (PASMC) and the potential effect on the progression of pulmonary arterial hypertension (PAH). In this study, we established a PAH mouse model through chronic hypoxia exposure as reflected by the increased RVSP and RVHI. Western blot and RT-qPCR detected the increased STARS protein and mRNA levels in PAH mice. Next, we cultured the primary PASMC from PAH mice. After STARS overexpression in PASMC, STARS, SRF and Egr-1 were up-regulated significantly. The MTT assay revealed an increase in cell proliferation. Flow cytometry showed a marked inhibition of cell apoptosis. However, STARS silence in PASMC exerted opposite effects with STARS overexpression. SRF siRNA transfection blocked the effects of STARS overexpression in PASMC. In order to further confirm the role of STARS in PAH mice *in vivo*, we exposed STARS knockout mice to hypoxia and found lower RVSP and RVHI in knockout mice as compared with controls. Our results not only suggest that STARS plays a crucial role in the development of PAH by increasing the proliferation of PASMC through activation of the SRF/Egr-1 pathway, but also provides a new mechanism for hypoxia-induced PAH. In addition, STARS may represent a potential treatment target.

1. Introduction

Pulmonary arterial hypertension (PAH) is a progressive disease that contributes significantly to morbidity and mortality in children [1]. If not identified and treated early, it will eventually result in right heart failure and death. PAH is characterized by hyperplasia and anti-apoptotic diathesis in pulmonary arterial smooth muscle cells (PASMC) and pulmonary artery endothelial cells (PAECs) [2]. Further, remodeled extracellular matrix and elevated pulmonary vascular resistance were emerged as well. Persistent PAH of the newborn often accompanied by severe hypoxemia [3]. In newborn piglets, chronic hypoxia induces symptoms similar to those caused by vascular abnormalities in infants [4]. For the past few years, researches about the pathophysiological mechanisms of PAH had widely reported. The immediate early gene early growth response-1 (Egr-1) is typically activated in the development of PAH, but the mechanism underlying the development of PASMC abnormalities remains unclear [5].

Egr-1 is a zinc-finger transcription factor that can be stimulated by hypoxia and cytokines. It not only expressed highly in human and animal models of atherosclerosis, but also plays important roles in PAH as a mediator of PASMC growth and proliferation [6,7]. The transcription of Egr-1 is dependent on the multiple serum response elements that locate in Egr-1 promoter region, such as serum response factor (SRF) [8]. SRF belongs to the MADS-box gene family, which could regulate cellular responses to mitogenic stimuli by regulating expression of numerous genes [9]. Previous studies also found that SRF involves the development and function of muscle [10]. Furthermore, upregulation of SRF caused by hypoxia resulted in abnormally enhanced proliferation and migration of PASMC and vascular remodeling. Notably, SRF-specific shRNA was able to attenuate the proliferation and migration of

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PASMC exposed to hypoxia *in vitro* as well as vascular remodeling *in vivo* [11]. Therefore, the SRF/Egr-1 pathway is key to the pathogenesis of PAH.

STARS (STriated muscle Activator of Rho Signaling) is an evolutionarily-conserved sarcomeric protein and expressed highly in skeletal, cardiac and smooth muscle [12]. A previous research found that STARS promoter contains binding sites of several muscle-enriched transcription factors, thus it acts as an acute stress sensor for pathological remodeling [13]. For example, STARS levels are sharply elevated in response to stress conditions, especially left ventricle (LV) pressure overload [14]. Since STARS contains a unique domain that stimulates serum response factor (SRF)-dependent transcription through the activation of Rho GTPase, STARS could control the intracellular signaling cascade by regulating the SRF transcriptional pathway [15]. In several instances, STARS was reported involved in the development and regression of human skeletal muscle hypertrophy. Furthermore, the ablation of STARS impaired arteriogenesis in mice [16]. Although STARS is closely related to the cell proliferation and growth, the role and mechanism of STARS on PAH still remains unclear.

In the present study, we investigated the role of STARS in the pathogenesis of PAH as well as the underlying molecular mechanism. STARS expression was initially measured in an animal model induced by hypoxia. Next, we explored the effect of STARS on the proliferation of PASMC. Our results demonstrated that STARS lead to excessive proliferation of PASMC depend on the SRF/Egr-1 pathway. Understanding the activation, transcriptional control and biological roles of STARS in PASMC will elucidate the progression of PAH.

2. Materials and methods

2.1. Animals and hypoxia-induced PAH model

Twelve pregnant C57BL mice were maintained at constant temperature 22 °C and humidity (60%) for one week before giving birth with access to food and water libitum. The STARS knockout mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All procedures were conducted with the National Institutes of Health Guide for Care and approved by the Committee of Xijing Hospital. Thirty-six male offspring (three male pups from each litter) that had been delivered naturally at full term and fed a standard diet were included in the study. After weaning, pups were exposed to 10% O_2 in a hypoxic chamber for 1 and 2 weeks. The hypoxic chambers were opened briefly (less than 10–15 min) for food, water, and cleaning. Pups were divided in three treatment groups (n = 12 per group): control, hypoxia for 1 week and hypoxia for 2 weeks. For STARS knockout section, 12 male offspring (STARS knockout mice and wildtype mice, n = 12, respectively) were bred on a C57Bl/6 background and used for experiment.

2.2. Right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (RVHI) measurements

Mice exposed to hypoxia for 1 or 2 weeks were anesthetized with 50 mg/kg sodium pentobarbital. The pulmonary artery pressures was recorded as previously described with a 1.4 F polyethylene micro-catheter which inserted into the right ventricle through the right jugular vein [17]. The RVSP was measured and digitally processed by the hemodynamic analyzing system. The hearts of mice were harvested after euthanasia with an overdose of sodium pentobarbital. The right ventricular (RV) free wall was dissected and weighed with an electronic scale. The left ventricle (LV), including the septum (S), was weighed as well. The RVHI is expressed as the tissue weight ratios [18]: RVHI (%) = (RV/[LV + S]) × 100.

2.3. Cell culture

The PASMC was isolated and obtained from offspring mice according to the previously described methods with slight modifications [19]. In this study, the whole lung tissues were obtained and used for cell culture, rather than the left upper lung. In addition, the Hanks balanced salt solution (HBSS) in previous were replaced with Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 1 g/l glucose (Mediatech, Herndon, VA). Next, PASMC were seeded on a 24-well plate in complete DMEM media supplemented with 1% sodium pyruvate, 1% non-essential amino acids, 1% penicillin–amphotericin–streptomycin and 10% FBS. Cells were maintained in a standard incubator flushed with 5% CO_2 at 37 °C and passaged after 80% confluence. During this period, DMEM was changed every 3 days.

2.4. STAR plasmid construction

Full-length mouse STARS cDNA was amplified by reverse transcription PCR(RT-PCR). A STARS expression vector (pcDNA3.1-STARS) was generated by inserting the amplified STARS full-length cDNA into pcDNA3.1(−) attached C-terminal His tag (Invitrogen, Carlsbad, CA). The restriction sites included *Xho I and Apa l* two cloning sites. All procedures of plasmids construction were accorded to the standard methodologies [20]. The correct plasmids were introduced and sequenced during plasmids synthesis and inserted with correct orientation into reading frame. Finally, the plasmids were amplified with the MiniBEST Plasmid Purification Kit (TaKaRa).

2.5. Transfection of the recombinant plasmids and siRNA

Cells were seeded in the 24-well plate and maintained in DMEM with 5% CO_2 at 37 °C for 24 h. For transient transfection assays, 0.5 μg of the pcDNA3.1-STARS, an empty construct pcDNA3.1, STARS small-interfering RNA (siRNA), SRF siRNA or non-specific siRNA was diluted in 200 μL FBS-free DMEM with 6 μL TurboFect™ siRNA Transfection Reagent (#R1401) per well and transfected into PASMC by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. After transfection followed by 24-h incubation, cells were at 80% confluence and collected for further testing.

2.6. Cell proliferation and apoptosis assays

Cell proliferation was detected by MTT (Sigma-Aldrich, St. Louis, MO, USA) assay according to the manufacturer's instructions. Cells were stained with MTT for 2 h after incubation for 24, 48, 72, or 96 h. Next, the absorbance at 490 nm was measured after incubation with DMSO (Sigma) for 5 min. Evaluation of cell apoptosis was performed by using flow cytometry (BD, Franklin Lakes, NJ, USA). The cells were incubated in PBS followed by suspending in 500 μL of binding buffer. Thereafter, 5 μL of Annexin V-FITC and PI was added to label the cells.

2.7. Western blot analysis

Cell lysates from the whole lung tissues homogenates and PASMC were separated by 12% SDS-PAGE, followed by transfer to a PVDF membranes (ImmobilonTM-P; Millipore, Bedford, MA). The blot was blocked with 5% non-fat dry milk for 2 h at room temperature and incubated with the interest primary