Inhibition of ARC promoting the apoptosis of rat pulmonary arterial smooth muscle cells after serum deprivation *in vitro*

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Abstract. Apoptosis has important pathophysiological consequences contributing to pulmonary arterial hypertension (PAH). However, the mechanism underlying apoptosis in PAH remains unknown. Apoptosis repressor with caspase recruitment domain (ARC) is an essential factor in cell apoptosis, and regulates intrinsic and extrinsic apoptosis signaling pathways. It is hypothesized that ARC may be involved in the apoptotic responses of pulmonary arterial smooth muscle cells (PASMCs) following mild chronic injury. In the present study, serum deprivation (SD) was used to induce apoptosis of PASMCs. It was demonstrated that the expression of ARC in PASMCs was significantly increased following SD stimulation within 24 h, and ARC downregulation using small interfering RNA significantly enhanced the apoptosis of PASMCs following SD stimulation. In addition, the results demonstrated that ARC downregulation significantly increased the expression of proapoptotic factors and the level of reactive oxygen species (ROS), and decreased the mitochondrial membrane potential following SD exposure, suggesting ARC regulates the apoptosis of PASMCs via modulating mitochondrial function and ROS accumulation. The results of the present study revealed that ARC inhibition promotes the apoptosis of PASMCs following SD stimulation, and that ARC expression increases in the early stages of SD injury.

Introduction

Apoptosis has important pathophysiological consequences contributing to pulmonary arterial remodeling (1,2), that apoptosis eliminates unnecessary cells. For example, cells migrated into the vascular lumen and hypertrophied cells accumulated in the pulmonary vasculature (3). Reversely inhibiting apoptosis of pulmonary arterial smooth muscle cells (PASMCs) can promote thickening of the pulmonary vasculature and raise pulmonary arterial hypertension (PAH) (4). However, compared with PASMCs from normal subjects, PASMCs from PAH patients exhibited a significant resistance to apoptosis (5), whose mechanism is still unknown.

Apoptosis repressor with ARC, an anti-apototic protein, is expressed primarily in cardiac and skeletal myocytes as well as in brain tissue (6-8). ARC comprises the ARC region that is homologous to the CARDs of caspases and caspase adaptor proteins, which can modulate apoptosis signaled by interacting with caspases or adaptor proteins (6). ARC impairs the cellular apoptotic responsiveness to a wide range of stresses and insults, including extrinsic apoptosis initiation via death receptor ligands, dysregulation of cellular Ca^2 homeostasis and endoplasmic reticulum (ER) stress, genotoxic drugs, ionizing radiation, oxidative stress and hypoxia (9). These data give a clue that ARC might be involved in the apoptotic responses of PASMCs.

To test the hypothesis, serum deprivation (SD) is used to induce the apoptosis of PASMCs. Then, it is found that both ARC mRNA and protein levels were gradually increased after SD within 24 h. Later, ARC expression is downregulated using siRNA, which verified that ARC downregulation significantly increased the sensitivity to SD. Thus, the mechanism of ARC in regulation of the apoptosis of PASMCs after SD is revealed. To be specific, it is found that the downregulation of ARC significantly increased the expression of pro-apoptotic factors and the level of reactive oxygen species (ROS), and decreased the mitochondrial membrane potential (MMP) after SD, which suggests that ARC regulates the cell death and the apoptosis of PASMC via controlling mitochondrial function and ROS accumulation. In conclusion, this study revealed that ARC inhibition promotes the apoptosis of PASMCs after SD *in vitro*, and ARC expression in PASMCs would increase after SD injury.

Materials and methods

Cell culture. Experiments were in full compliance with regulations that were enacted by the Animal Ethics Committee of

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Nanjing Medical University (Nanjing, China). Primary cultures of PASMCs were from Sprague-Dawley rats (125-250 g) according to the method reported by Golovina and Blaustein (10). Briefly, pulmonary arteries were isolated from lung tissues, and incubated for 20 min in Hank's Balanced Salt Solution containing 1.5 mg/ml collagenase (Worthington Biochemical Co., Lakewood, NJ, USA). In addition, the endothelium was removed by scratching the luminal surface. The remaining pulmonary artery smooth muscle was then digested with 1.5 mg/ml collagenase and 0.5 mg/ml elastase (Sigma-Aldrich, St. Louis, MO, USA) at 37˚C. Then, the cells were plated on 10-cm dishes, and maintained in DMEM medium supplemented with 10% FBS and 100 IU/ml penicillin (Sigma-Aldrich) at 37˚C with 5% CO_2. By adopting immunofluorescence, we verified the purity and identity of PASMCs with α-smooth muscle actin (α-actin) (Sigma-Aldrich), which is the specific antibody against PASMCs. The cells with typical hill-and-valley morphology at 80% confluence were used for next experiments. SD caused a marked decrease in cell viability (11-13). Then, SD is used to induce PASMC to apoptosis. Briefly, PASMCs were grown to about 80% confluency in complete medium (DMEM with 10% FBS), and then were switched to SD medium for the next 6-72 h to induce cell apoptosis.

Design and transfection of siRNA. ARC-siRNA was designed by GenePharma (Shanghai, China) to inhibit the expression of ARC in PASMCs, and a nonsense sequence was adopted as a negative-siRNA. ARC-siRNA and negative siRNA sequences are listed in Table I. Cells were transfected with these siRNA using Lipofectamine 2000 (Invitrogen Life Technologies, Waltham, MA, USA) by following the manufacturer's instructions. Generally speaking, cells were plated in medium without antibiotics the day before transfection so that 40 to 50% confluence was obtained at the time of transfection. Cells were also transfected with 200 nM ARC-siRNA or negative-siRNA for 12 h, exposed to SD for 48 h and collected for western blot analysis, RT-qPCR, immunofluorescence and flow cytometry assays.

Immunofluorescence. Primary antibodies used were anti-ARC rabbit polyclonal antibody (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-active caspase-3 rabbit monoclonal antibody (1:400; Cell Signaling Technology, Inc., Danvers, MA, USA), and α-actin (1:200; Sigma-Aldrich). Briefly, cells were fixed in 4% paraformaldehyde. Nonspecific binding sites were blocked for 1 h in 0.3% Triton X-100 and 10% (v/v) heat-inactivated normal serum in PBS (PBT1). Samples were then incubated overnight at 4˚C in PBT1 with primary antibodies. After unbound antibodies were removed, samples were incubated with the corresponding secondary antibodies conjugated with tetramethylrhodamine (TRITC), fluorescein isothiocyanate or Cy5 (Abcam, Cambridge, UK). Counterstaining with DAPI (Sigma-Aldrich) caused the visualization of the cell nucleus. Specimens were observed with confocal fluorescence microscopy (Leica SP5; Leica, Heidelberg, Germany). Negative control experiments were performed as above by omitting primary antibodies.

TUNEL kit (Roche, Indianapolis, IN, USA) was adopted to detect apoptotic cells, according to the instructions of the manufacturer. Tetramethylrhodamine ethyl ester (TMRE) and Mitochondrial Membrane Potential Assay kit (Sigma-Aldrich) were used to determine the MMP. Besides, Mito-SOX Red (Life Technologies, Waltham, MA, USA) was used to detect ROS. PASMCs were cultured in SD medium for 48 h, then washed with PBS and incubated with TMRE or MitoSOX Red for 10 min at 37˚C. Cells were washed with prewarmed PBS and observed under confocal microscopy (LSM700; Carl Zeiss, Heidenheim, Germany).

Real-time quantitative PCR. For real-time quantitative PCR, total RNA was extracted with TRIzol reagent (Protein Biotechnology, Beijing, China) and the integrity of all RNA samples was evaluated by OD260/280. In addition, cDNA was obtained by using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Real-time PCR with SYBR-Green (Roche, Basel, Switzerland) was carried out by Biosystems CFX96 Real-Time PCR (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primer sequences are shown in Table II. The specificity of the PCR amplification was confirmed by agarose gel electrophoresis. The thermal cycling condition was 20 sec at 95˚C followed by 40 cycles of 15 sec at 95˚C, 1 min at 62˚C, and 20 sec at 72˚C. Melting curves were directly drawn after amplification. Gene expression was measured by semi-quantitative analysis and GAPDH was used as a normalization control. Besides, it should be noticed that each analysis was performed in triplicate.

Western blot analysis. PASMCs were harvested and lysed with RIPA buffer (Protein Biotechnology) that contains the protease inhibitor cocktail (Sigma, St Louis, MO, USA) for 30 min at 4˚C. The lysates were centrifuged at 12,000 g for 10 min at 4˚C. Protein concentrations were calculated by using the BCA Protein Assay kit (Protein Biotechnology). An equal amount of protein was loaded onto 12% Tris-glycine SDS-PAGE gel and separated at 120 V for 1.5 to 2 h. The protein was then transferred to nitrocellulose membrane and blocked with 5% milk in TBST buffer. Immunoblotting was performed with anti-ARC rabbit polyclonal antibody (1:1,000) and anti-active caspase-3 rabbit monoclonal antibody (1:500). Anti-β-actin Mouse Monoclonal Antibody (1:5,000; Abcam) was used as a control for the equal loading of samples. Peroxidase-conjugated goat anti-rabbit (or anti-mouse) immunoglobulin G (Abcam) was employed as the secondary antibody, while proteins were detected with SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Flow cytometry. Annexin V-FITC and propidium iodide (PI) (BD Biosciences, San Jose, CA, USA) was used for apoptosis analysis according to the manufacturer's instructions. To be specific, the cells were collected, washed twice with cold PBS and then resuspended in 1X binding buffer at a concentration of $1x10^6$ cells/ml. Annexin V-FITC (5 μl) and 5 μl PI were added, gently mixed with 100 μl cells and incubated for 15 min at room temperature in the dark. Then, 400 μl 1X binding buffer was added to tubes. The samples were analyzed by flow cytometry (FACSCanto; BD Biosciences) as soon as possible. The number of acquired events was over 100,000 and the flow cytometry threshold was set to exclude cell debris. All tests were repeated at least three times.

Table I. siRNA sequences used in the experiments.

siRNA	Forward sequence	Reverse sequence
ARC-siRNA 1	CGGAAACGGCUGGUAGAAATT	UUUCUACCAGCCGUUUCCGTT
ARC-siRNA 2	GAGUAUGAAGCCUUGGAUGTT	CAUCCAAGGCUUCAUACUCTT
ARC-siRNA 3	CCCAGCAAACUGUGAGCAUTT	AUGCUCACAGUUUGCUGGGTT
Negative siRNA	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

ARC, apoptosis repressor with caspase recruitment domain.

Table II. PCR sequences used in the experiments.

Gene	Forward sequence	Reverse sequence
Caspase-3	AATCATGCCATTTGCCCAGC	CTCAAGTGTGTAGGGGGAGG
Caspase-9	CTGTCCCGTGAAGCAAGGAT	CAGGGCACACATGACAATGC
Fadd	ACAATGTGGGGAGAGACTGG	CCCTTACCCGATCACTCAGG
Bax	GTCCTCACTGCCTCACTCAC	TTCCCCGTTCCCCATTCATC
Bad	AGGCTTGAGGAAGTCCGATCC	TTGTCGCATCTGTGTTGCAG
Bcl-2	GGTGAACTGGGGGAGGATTG	AGAGCGATGTTGTCCACCAG
Lpo	GTTCCAGCCAACTCACACCA	CTCCCACCAGAACTTGCCTGT
Sod1	GGAGCAAGGTCGCTTACAGA	AGTGACAGCGTCCAAGCAAT
Glrx	AGTCTGGAAAGGTGGTCGTG	CCATTAGCATGGCTGGACGA
ARC	CAGTGTAGGGGAACGCAAAT	CCGGTCAATGGTCTCCGATG
GAPDH	GCAAGAGAGAGGCCCTCAG	TGTGAGGGAGATGCTCAGTG

ARC, apoptosis repressor with caspase recruitment domain.

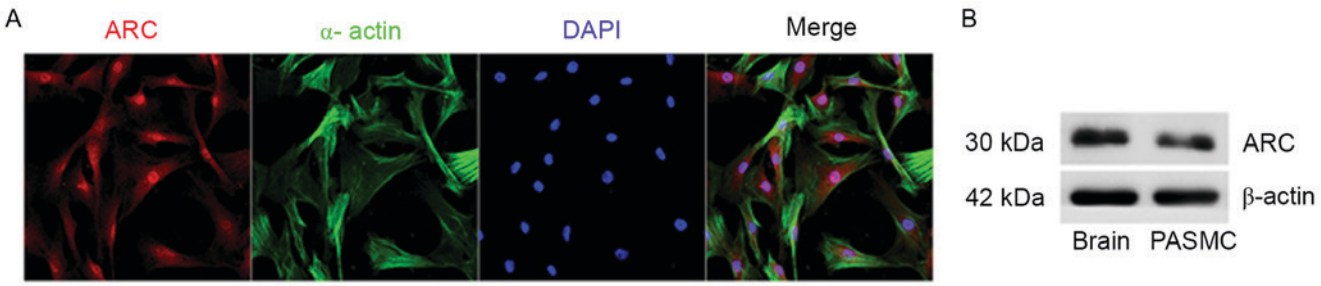

Figure 1. ARC was expressed in the PASMCs. (A) Immunofluoresence staining showed ARC was expressed in the PASMCs. α-actin was used as PASMCs markers. Scale bars, 20 μm. (B) Western blot analysis showed that ARC was expressed in the PASMCs. β-actin served as a loading control in each lane. Brain samples were used as positive controls. ARC, apoptosis repressor with caspase recruitment domain; PASMCs, pulmonary arterial smooth muscle cells; α-actin, α-smooth muscle actin.

Statistical analysis. Results were presented as mean ± standard deviation. A two-tailed unpaired Student's t-test was performed when two groups were compared. In addition, one-way ANOVA was used followed by Dunnett's test when more than two groups were compared. $P<0.05$ was considered statistically significant.

Results

ARC was expressed in PASMCs. The immunoflurescence results demonstrated that ARC was expressed in PASMCs, which was further proved by western blot analysis data (Fig. 1).

The ARC mRNA and protein levels in PASMCs were upregulated in PASMCs after SD. SD was used to induce PASMC to apoptosis. PASMCs were cultured to about 80% confluency in complete medium (DMEM with 10% FBS), and then were switched to SD medium for the next 6-72 h. The RT-qPCR and western blot results demonstrated that both ARC mRNA and protein levels increased after SD when compared with control cells. Thus, both ARC mRNA and protein levels increased to the maximum at 24 h (Fig. 2).

The ARC level downregulation with siRNA caused the decrease of PASMCs viability after SD. To verify the role of

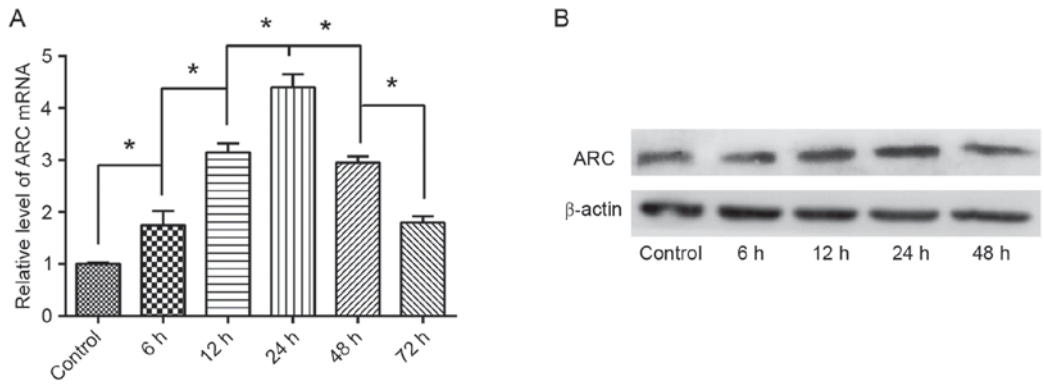

Figure 2. The (A) RT-qPCR and (B) western blot analysis results demonstrated that both ARC mRNA level and protein level increased after SD treatment compared to control cells, and both ARC mRNA level and protein level increased to maximum at 24 h point. The PASMCs were cultured in medium without serum from 6 to 72 h, then harvested for RT-qPCR and western blot analysos. Data is shown as mean ± standard deviation, *P<0.05. ARC, apoptosis repressor with caspase recruitment domain; SD, serum deprivation; PASMCs, pulmonary arterial smooth muscle cells.

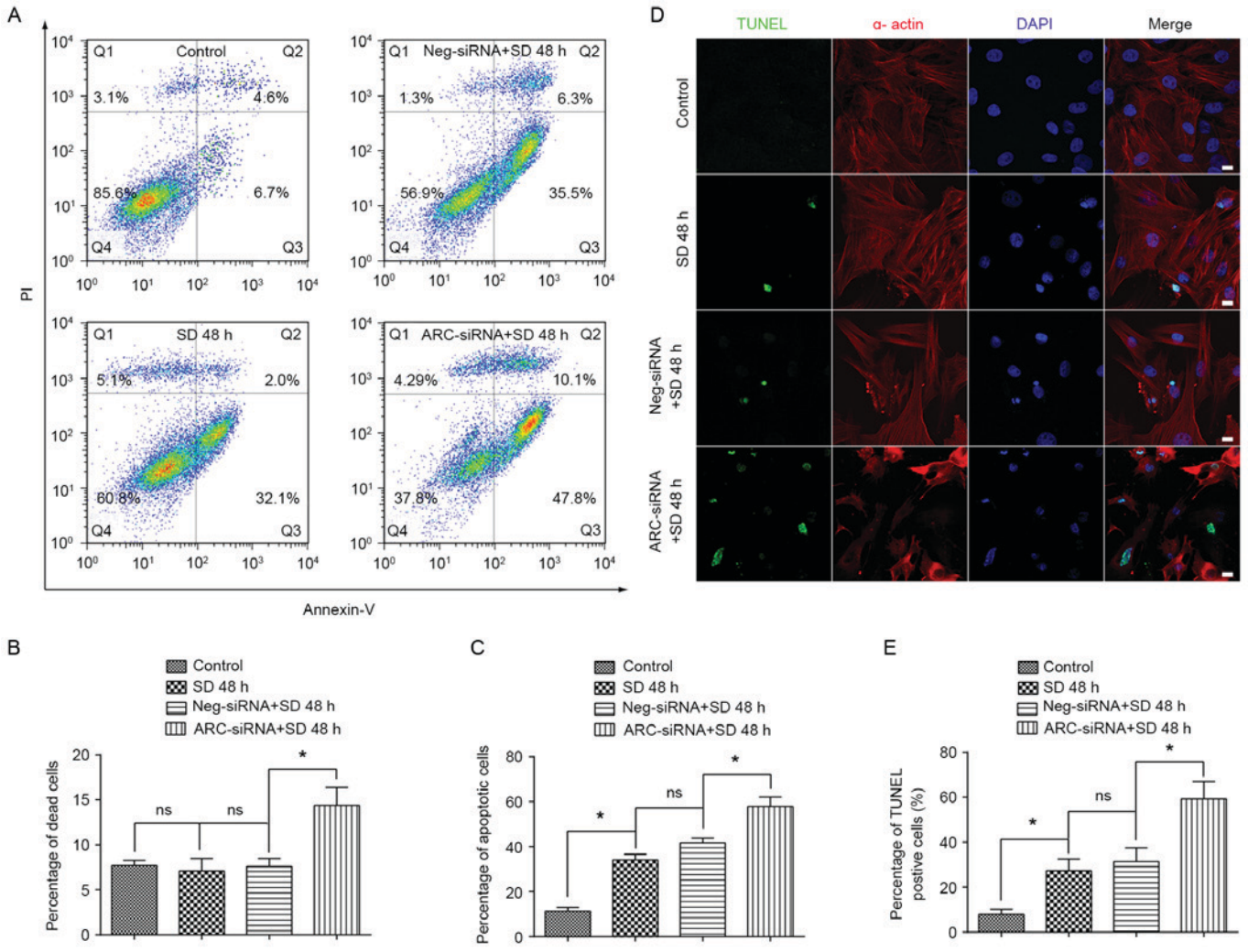

Figure 3. The ratio of both cell death and apoptosis of PASMCs were increased after SD injury when ARC was downregulated by siRNA. (A-C) Flow cytometry analysis revealed the ratio of both death and apoptotic cells were significantly increased when cultured with SD medium for 48 h compared to normal medium control. And the ARC-siRNA transfected groups had significantly higher death and apoptotic cells ratio than the negative-siRNA transfected groups when cultured with SD medium for 48 h. (D and E) The percentage of TUNEL positive apoptotic cells increased in the ARC-siRNA transfected groups compared with the negative siRNA transfected controls. Data is shown as mean ± standard deviation, *P<0.05. Scale bars, 20 μm. PASMCs, pulmonary arterial smooth muscle cells; SD, serum deprivation; ARC, apoptosis repressor with caspase recruitment domain; α-actin, α-smooth muscle actin; ns, not significant.

ARC on PASMCs apoptosis after SD, ARC was downregulated with siRNA, and flow cytometric analysis and TUNEL assay were used to determine cell apoptosis. The results of flow cytometric analysis showed that the apoptosis and death of

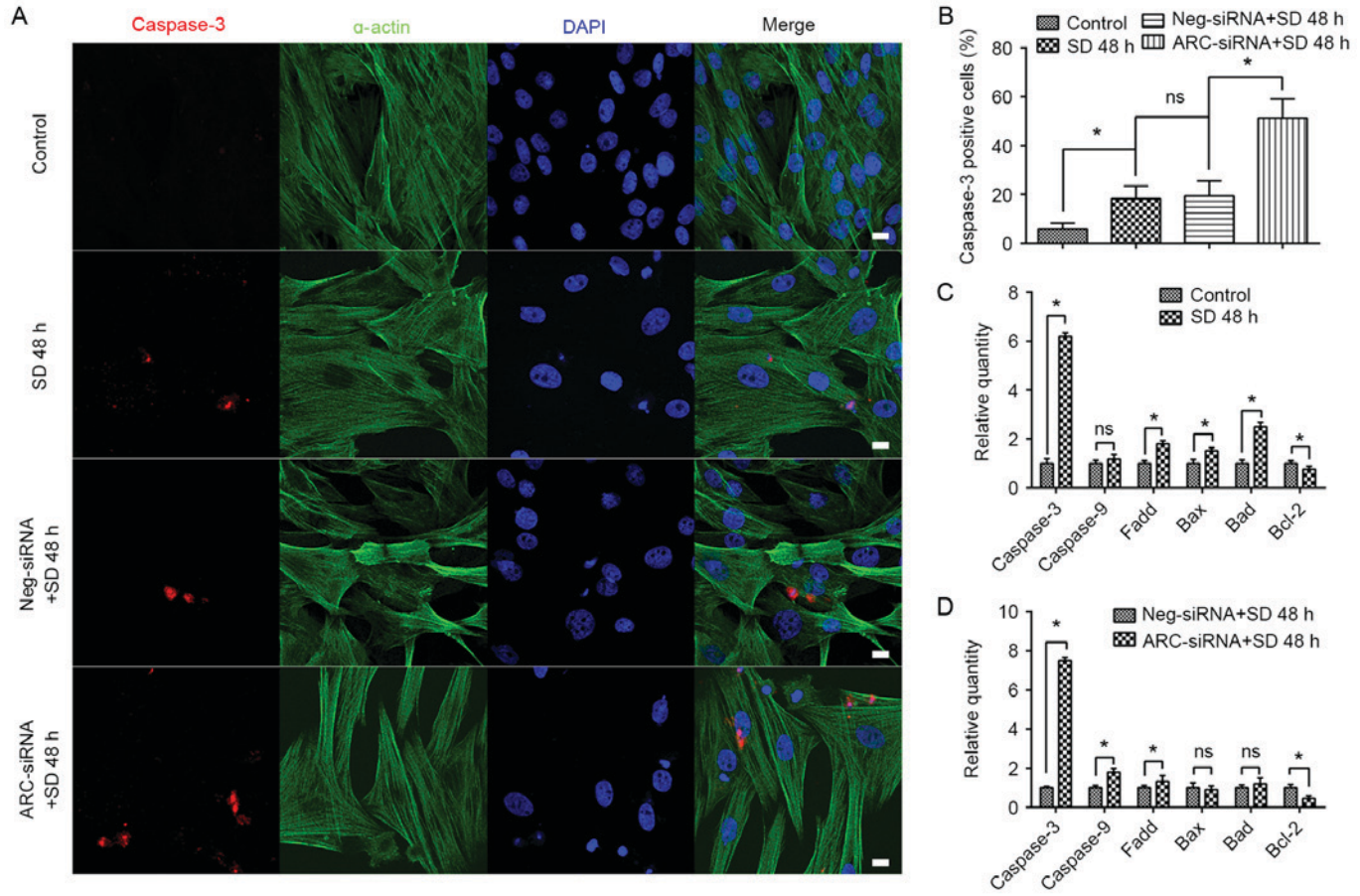

Figure 4. ARC downregulation increased the expression of proapoptotic factors in PASMCs after SD injury. (A and B) The immunofluoscence showed caspase-3 positive cells increased insulted by SD treatment for 48 h compared with control cells. And caspase-3 positive cells increased in the groups transfected with ARC-siRNA compared to the negative siRNA transfected controls. (C) RT-qPCR results showed that the expression of caspase-3, Fadd, Bax and Bad were significantly increased and the anti-apoptotic factor like Bcl-2 was significantly decreased, while the expression of caspase-9 was not significantly changed in SD treated groups compared with control cells. (D) RT-qPCR results showed ARC-siRNA transfected groups had significantly higher expression of caspase-3, caspase-9 and Fadd, while anti-apoptotic factor like Bcl-2 was significantly lower compared to the negative siRNA transfected controls. Data is shown as mean ± standard deviation, *P<0.05. Scale bars, 20 μm. ARC, apoptosis repressor with caspase recruitment domain; PASMCs, pulmonary arterial smooth muscle cells; SD, serum deprivation; α-actin, α-smooth muscle actin; ns, not significant.

PASMCs increased significantly after 48 h SD compared with no damage control (Fig. 3A-C). More notably, PASMCs with ARC-siRNA transfection had a more apoptosis and death ratio than PASMCs with Negative-siRNA transfection (Fig. 3A-C). Besides, the number of TUNEL-positive cells significantly increased in the SD group, and the effect was enhanced by treating with ARC-siRNA transfection (Fig. 3D and E).

The ARC level downregulation with siRNA increases apoptotic factor expression in PASMCs after SD. To investigate the mechanism of ARC on the role of PASMCs apoptosis, the expression of pro-apoptotic factors and anti-apoptic factors in PASMCs were measured by using immunoflurescence and RT-qPCR methods. The results of immunoflurescence and RT-qPCR demonstrated that pro-apoptotic factors like caspase-3 increased significantly after 48 h SD compared with control cells (Fig. 4A-C). Moreover, the ARC-siRNA transfection group had more caspase-3 expression than the Negative-siRNA transfection group (Fig. 4A, B and D). RT-qPCR data also showed that pro-and anti-apoptic factors such as caspase-9, Fadd, Bax and Bad increased after 48 h SD (Fig. 4C). Furthermore, ARC downregulation with ARC-siRNA significantly increased the expression of caspase-9 and Fadd (Fig. 4D). In addition, both SD and ARC downregulation decreased anti-apoptotic factors like Bcl-2 expression (Fig. 4C and D).

ARC downregulation with siRNA reduced MMP of PASMCs after SD. TMRE kit was used to assess the changes in MMP. The quantitative analysis of TMRE intensity showed a significant decrease in SD-treated cells when compared with normal control cells which were cultured in the presence of 10% fetal bovine serum (FBS) (Fig. 5). Moreover, the quantitative analysis of TMRE intensity also showed a significant decrease in ARC-siRNA-transfected cells when compared with negative-siRNA transfected cells (Fig. 5). The result showed that ARC protected against the SD-induced loss of MMP and maintained mitochondrial integrity.

ARC downregulation with siRNA disrupted the balance of oxidative stress and induced the accumulation of ROS in PASMCs after SD. MitoSOX Red, a redox fluorophore detecting selectively mitochondrial superoxide, was used to evaluate mitochondrial ROS generation. The intensity of

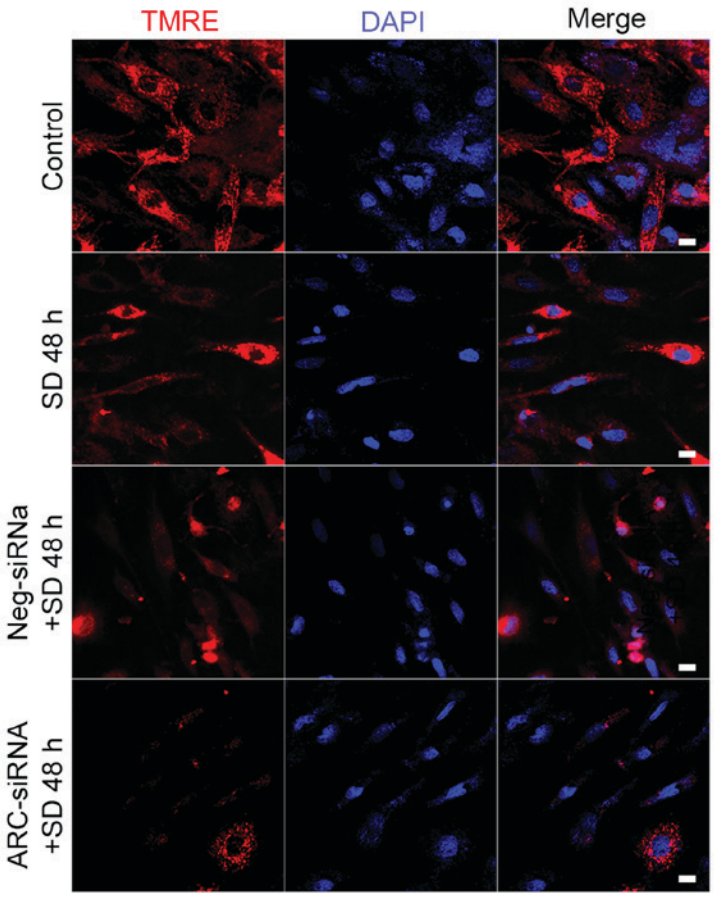

Figure 5. ARC downregulation decreased the MMP of PASMCs after SD injury. The immunofluoscence intensity of TMRE was decreased after SD treatment for 48 h compared with the controls; particularly, TMRE intensity in ARC-siRNA transfected groups was significantly reduced compared with the controls transfected with negative siRNA. Scale bars, 20 μm. ARC, apoptosis repressor with caspase recruitment domain; MMP, mitochondrial membrane potential; PASMCs, pulmonary arterial smooth muscle cells; SD, serum deprivation; TMRE, tetramethylrhodamine ethyl ester.

MitoSOX demonstrated a significant decrease in SD-treated cells when compared with normal control cells, and the intensity of MitoSOX decreased significantly in the ARC-siRNA groups than the negative-siRNA groups (Fig. 6A). The result demonstrated that ARC downregulation could promote ROS accumulation in PASMCs after SD.

Either decreased antioxidant levels or increased prooxidant levels might lead to the increment of ROS levels. Therefore, the antioxidant-prooxidant balance is important to the cell redox homeostasis (8). Here, the mRNA expression of three redox related genes was analyzed by qPCR. The result showed the expression of oxidant factors was disrupted after SD-treatment compared with no damage controls (Fig. 6B); while ARC downregulation with ARC-siRNA transfection significantly decreased the expression of antioxidant factors like Sod1 and Glrx, and increased the expression of prooxidant factors like LPO (Fig. 6C). Meanwhile, the data demonstrated that the downregulation of ARC inhibited the expression of antioxidant genes and increased the intracellular ROS levels in PASMC cells after SD, which would further lead to cell apoptosis.

Discussion

Compared with PASMCs from normal subjects, PASMCs from PAH patients exhibited a significant resistance to apoptosis (4), and the mechanism of it is still unknown. ARC is unique since it suppresses the activation of both the intrinsic apoptosis pathway and the extrinsic apoptosis pathway (6,14). It is assumed that ARC may be implicated in PASMCs' resistance to apoptosis from mild chronic injury. In this study, PASMSCs isolated from rats were used to explore the role of ARC in PASMCs with SD exposure. The result showed that ARC was expressed in PASMCs (Fig. 1), and both ARC mRNA and protein levels increased gradually in PASMCs when being cultured in SD medium within 24 h (Fig. 2). Then, it is found that ARC downregulation dramatically increased the cell death and the apoptosis of PASMCs after SD (Fig. 3). All of the results suggested that ARC inhibition promotes the apoptosis of PASMCs after SD *in vitro*.

Apoptosis is primarily regulated activating caspases through either the intrinsic pathway or the extrinsic pathway (15). Then, the study revealed that ARC is involved in both intrinsic and extrinsic apoptotic pathways. When ARC was inhibited, it is observed that the expression of both intrinsic and extrinsic pro-apoptotic genes including caspase-3, caspase-9 and Fadd was significantly increased and expression of the anti-apoptotic gene Bcl-2 was decreased after SD injury, which suggests that ARC inhibits the apoptosis of PASMCs by both intrinsic and extrinsic apoptotic pathways after SD injury (Fig. 4).

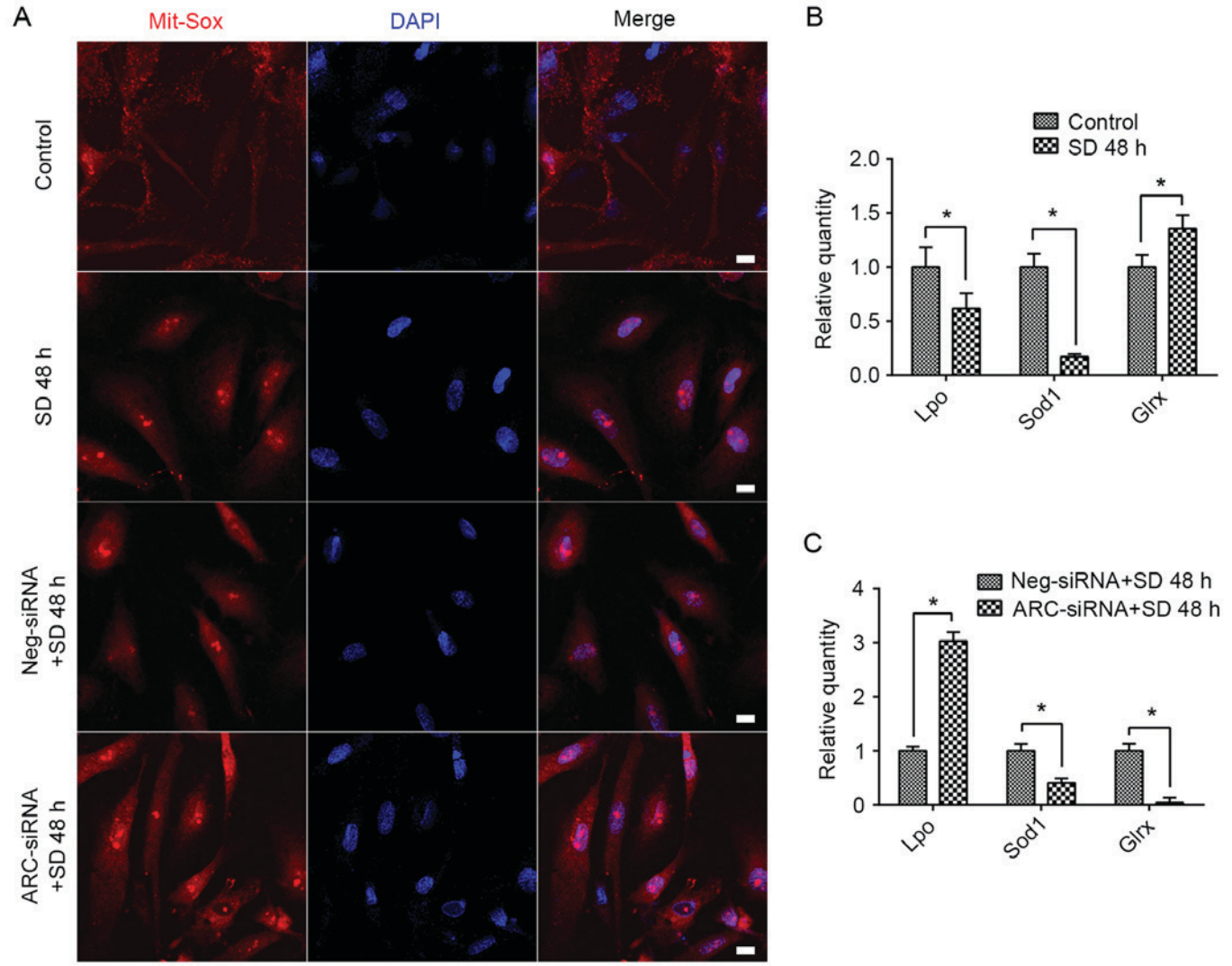

Figure 6. Downregulation of ARC with siRNA elevated the ROS and prooxidant factors level, while decreased antioxidant facts level. (A) The immunofluorescence showed Mito-SOX intensity was increased after SD treatment for 48 h compared with controls; while Mito-SOX intensity in ARC-siRNA transfected groups was more significantly increased compared with the negative siRNA controls. (B) RT-qPCR results showed the expression of prooxidant factors like Lpo and antioxidant factors such as Sod1, Glrx were changed after SD treatment for 48 h compared with controls. (C) RT-qPCR results demonstrated the expression of antioxidant factors such as Sod1, Glrx were significantly decreased; while the expression of prooxidant factor like Lpo was significantly increased in ARC-siRNA transfected groups compared with the negative siRNA controls. Data is shown as mean ± standard deviation, $^{*}P<0.05$. Scale bars, 20 μm. ARC, apoptosis repressor with caspase recruitment domain; ROS, reactive oxygen species; SD, serum deprivation.

Apoptosis is closely related to mitochondrial dysfunction, including increased ROS and decreased MMP (16,17). Previous studies have shown that the accumulation of ROS triggers mitochondrial depolarization, leads to the loss of MMP, and further results in the opening of mitochondrial permeability transition pores that enables the release of pro-apoptotic factors from the mitochondria into the cytosol (18-21). Then, it was demonstrated that ARC inhibition caused the dramatic increase of the mitochondrial ROS level and decrease of MMP after SD (Figs. 5 and 6), suggesting ARC downregulation exacerbated the mitochondrial dysfunction in PASMCs after SD.

Under physiological conditions, the ROS level is kept within a certain range because of the balance between production and scavenging, which is the result of many mutually-coordinated genes. Once this balance is altered, an increase in intracellular ROS will occur (17). In this study, it is found that ARC inhibition resulted in the significant decrease in the expression of several crucial antioxidant genes, including Sod1and Glrx, while it increased the expression of LPO, a prooxidative factor (Fig. 6). In addition, this balance disruption between prooxidant and antioxidant genes might contribute to the increased ROS levels in response to ARC inhibition.

In conclusion, the result showed that ARC mRNA and protein levels increased gradually in PASMCs after SD within 24 h. It also demonstrated that ARC downregulation disrupts the balance between prooxidant and antioxidant genes, and leads to the increased ROS level, which further contributes to mitochondrial dysfunction and the release of apoptotic factors from the mitochondria, at last promotes the apoptosis of PASMCs after SD exposure.

Acknowledgements

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