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Original Article

Long noncoding RNA SRA1 attenuates hypoxia-induced injury in H9c2 cardiomyocytes through regulating PPARγ/NF-κB signaling pathway

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Abstract: This study aimed to investigate the effects and mechanisms of long noncoding RNA SRA1 on regulating hypoxia-induced injury in H9c2 cardiomyocytes. The H9c2 cardiomyocytes were cultured under hypoxic (3% O_2) conditions and whether hypoxia induced injury was assessed by detecting cell viability, apoptosis and autophagy. Then, SRA1 was overexpressed and suppressed in H9c2 cardiomyocytes by transfection with pc-SRA1 and sh-SRA1, and the effects of SRA1 dysregulation on cell viability, apoptosis, and autophagy of H9c2 cardiomyocytes under hypoxia condition were detected. Furthermore, the regulatory relationship between SRA1 and PPARγ was explored, as well as the association between SRA1 and NF-κB signaling. Hypoxia induced injury to H9c2 cardiomyocytes, such as inhibiting cell viability, and promoting cell apoptosis and autophagy. Moreover, hypoxia resulted in a decreased expression of SRA1 in H9c2 cardiomyocytes, and overexpression of SRA1 alleviated hypoxia-induced injury, while suppression of SRA1 indicated the contrary results. Further studies showed that SRA1 positively regulated PPARγ. Overexpression of SRA1 alleviated hypoxia injury by activating PPARγ. Besides, suppression of SRA1 activated NF-κB pathway in hypoxia-treated H9c2 cardiomyocytes, which were significantly reversed after suppression of SRA1 and overexpression of PPARγ at the same time. Our findings indicated that suppression of SRA1 may aggravate hypoxia-induced injury to H9c2 cardiomyocytes by positive regulation of PPARγ and activation of NF-κB pathway. SRA1 may serve as a promising perspective for the therapy of heart failure induced by hypoxia.

Keywords: Heart failure, hypoxia-induced injury, long non-coding RNA SRA1, PPARγ, NF-κB pathway

Introduction

Heart failure is a lethal disorder featuring progressive deterioration of left ventricular function [1, 2]. In heart failure patients, cardiovascular morbidity and mortality comprise a major health and economic burden [3]. It is estimated that the five year survival of patients is approximately 50%-no better than that for many cancers [4]. Therefore, to improve clinical outcome, it is imperative to perform more studies to elucidate the key mechanism underlying heart failure.

Long noncoding RNAs (lncRNAs) are confirmed as important regulators involving in the pathological development of cardiovascular diseases [5-7]. In heart failure, the crucial role of lncRNA dysregulation is also revealed [8-10]. Identification of key lncRNAs will help to design an effective therapeutic strategy for heart failure. SRA1, a long non-coding RNA (lncRNA), is identified as a critical regulator in several cancers [11, 12]. A recent study reveals that lncRNA SRA1 can bind to and coactivate peroxisome proliferator–activated receptor γ (PPARγ) in mice [13]. Moreover, the PPARγ agonist pioglitazone is found to have a ability of relieving severe pulmonary hypertension and vascular remodeling and preventing right heart failure in rats [14]. However, whether SRA1 plays a key role in heart failure is largely known.

In the present study, H9c2 cardiomyocytes were cultured under hypoxia condition to induce cell injury. SRA1 was overexpressed and sup-

pressed in H9c2 cardiomyocytes, and the effects of SRA1 dysregulation on cell viability, apoptosis and autophagy of H9c2 cardiomyocytes under hypoxia condition were detected. Furthermore, the regulatory relationship between SRA1 and PPARγ was explored, as well as the association between SRA1 and NF-κB signaling. The objective of our study was to elucidate the roles and regulatory mechanism of SRA1 in preventing hypoxia-induced myocardial injury, thus to provide a novel perspective for preventing heart failure induced by hypoxia.

Materials and methods

Cell culture and treatment

The cardiomyocyte cell line H9c2 was obtained from Sigma-Aldrich (St. Louis, MO). H9c2 cardiomyocytes were then cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% FBS, 1% GlutaMAX and 1% Penicillin/Streptomycin (100 U/ml: 100 mg/ml) (Invitrogen) and maintained at 37°C under 5% CO_2. To induce hypoxic injury, H9c2 cardiomyocytes were exposed to a hypoxic condition (3% O_2 concentration). The normoxia culture condition (21% O_2 concentration) was used as control.

Cell transfection

SRA1 or PPARγ overexpression was realized by constructing the full-length sequences of SRA1 or PPARγ into H9c2 cardiomyocytes by pcDNA3.1 plasmids (GenePharma, Shanghai, China). Short-hairpin RNA targeting SRA1 (shSRA1) and its negative control (shNC) were synthesized by GenePharma Co. (Shanghai, China). For cell transfection, pc-SRA1, pc-PPARγ, pcDNA3.1, sh-SRA1 and shNC were transfected into H9c2 cardiomyocytes using Lipofectamine 3000 reagent (Invitrogen). After 48 h of transfection, H9c2 cardiomyocytes were collected for subsequent experiments.

Quantitative PCR (qPCR)

Following the manufacturer's instructions of Trizol reagent (Invitrogen), total RNA was extracted from H9c2 cardiomyocytes in different groups. After determining the quality and concentration of total RNA by SMA 400 UV-VIS (Merinton, Shanghai, China), the Real-Time PCR analysis was performed to test the mRNA or RNA expression levels using the One Step SYBR® PrimeScript®PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, China). The expressions of target genes were normalized to GAPDH and the fold changes were calculated using relative quantification ($2^{-\Delta\Delta Ct}$) method.

Western blot

Total protein was extracted from H9c2 cardiomyocytes in different groups using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing protease inhibitors (Roche, Guangzhou, China). After being quantified by the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA), a Bio-Rad Bis-Tris Gel system was established for western blot following the manufacturer's instructions. Primary antibodies to β-actin, Bcl-2, Bax, pro- or cleaved-caspase-3, pro- or cleaved-caspase-9, LC3-I, LC3-II, Beclin-1, p62, PPARγ, p65, p-p65, IκBα and p-IκBα (1:1,000, Abcam, USA) were added to incubate the Polyvinylidene Difluoride (PVDF) membranes at 4°C overnight. After rinsing, the membranes were probed with recommended secondary antibody marked by horseradish peroxidase (1:5000, Abcam) for 1 h at room temperature. The membranes were then transferred into the Bio-Rad ChemiDoc™ XRS system, and incubated with 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA). The protein signals in membranes were then visualized and quantified by Image Lab™ Software (Bio-Rad, Shanghai, China).

Cell viability assay

For assessment of cell viability, 1×10^5 H9c2 cardiomyocytes were seeded in duplicate in 60-mm dishes. After different treatments, H9c2 cardiomyocytes were washed and live cells were counted by trypan blue exclusion.

Apoptosis assay

For detection of cell apoptosis, H9c2 cardiomyocytes in different groups were harvested and fixed in 70% ethanol. Subsequently, H9c2 cardiomyocytes were stained in propidium iodide (PI) and fluorescein isothiocynate (FITC)-conjugated Annexin V in the presence of 50 μg/ml RNase A (Sigma-Aldrich). Followed by incubation for 1 h at room temperature in the dark, cell apoptosis was analyzed by flow cytometry

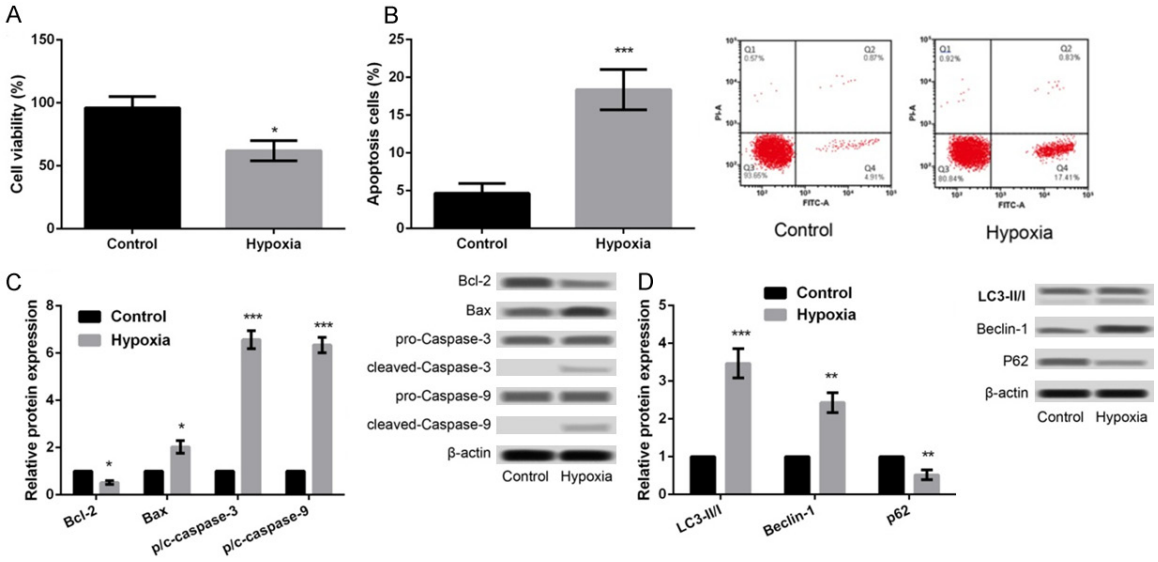

Figure 1. Hypoxia induced hypoxic injury in H9c2 cardiomyocytes. A: Cell viability; B: Apoptosis; C: The expression of apoptosis-related proteins; D: The expression of autophagy-related proteins. All experiments were repeated three times. Data are expressed as mean ± SD. *, P < 0.05, **, P < 0.01, and ***, P < 0.001.

using a FACS can (Beckman Coulter, Fullerton, CA, USA). Apoptotic cells were defined as annexin V positive and PI negative. The percentage of apoptotic cells were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

All experiments were repeated three times. Statistical analyses for data obtained from multiple experiments were performed using Graphpad 6.0 statistical software (GraphPad, San Diego, CA, USA). Data are presented as the mean ± standard deviation (SD) and the *P*-values between any two groups were calculated using a one-way ANOVA. P < 0.05 was considered significant.

Results

Hypoxia caused injury to H9c2 cardiomyocytes

To test hypoxia-induced injury to H9c2 cardiomyocytes, cell viability, apoptosis and autophagy was detected after hypoxic treatment. In comparison with control, cell viability of H9c2 cardiomyocytes was significantly decreased after hypoxic treatment (P < 0.05, **Figure 1A**). Moreover, the percentage of apoptosis cells was remarkably increased after hypoxic treatment (P < 0.001, **Figure 1B**); meanwhile, after hypoxic treatment, the expression of Bcl-2 was obviously down-regulated in H9c2 cardiomyocytes, while the expression of Bax, p/c-caspase-3 and p/c-caspase-9 were all up-regulated (P < 0.05, **Figure 1C**). These data indicated that hypoxia could induce apoptosis of H9c2 cardiomyocytes. Furthermore, increased expression of LC3-II/I and Beclin-1 and decreased p62 expression were revealed in H9c2 cardiomyocytes after hypoxic treatment (P < 0.01, **Figure 1D**), suggesting that hypoxia could induce autophagy of H9c2 cardiomyocytes. These findings indicated that hypoxia caused injury to H9c2 cardiomyocytes.

Hypoxia resulted in a decreased expression of SRA1 in H9c2 cardiomyocytes

The expression of SRA1 was further investigated under hypoxia condition. The results showed that relative to control, hypoxia resulted in a decreased expression of SRA1 in H9c2 cardiomyocytes significantly (P < 0.01, **Figure 2A**).

Overexpression of SRA1 relieved hypoxia-induced injury to H9c2 cardiomyocytes, while suppression of SRA1 aggravated this injury

To further investigate the role of SRA1 in regulating hypoxia-induced injury to H9c2 cardiomyocytes, SRA1 was overexpressed and suppressed in H9c2 cardiomyocytes by transfection with pc-SRA1 and sh-SRA1, and the high

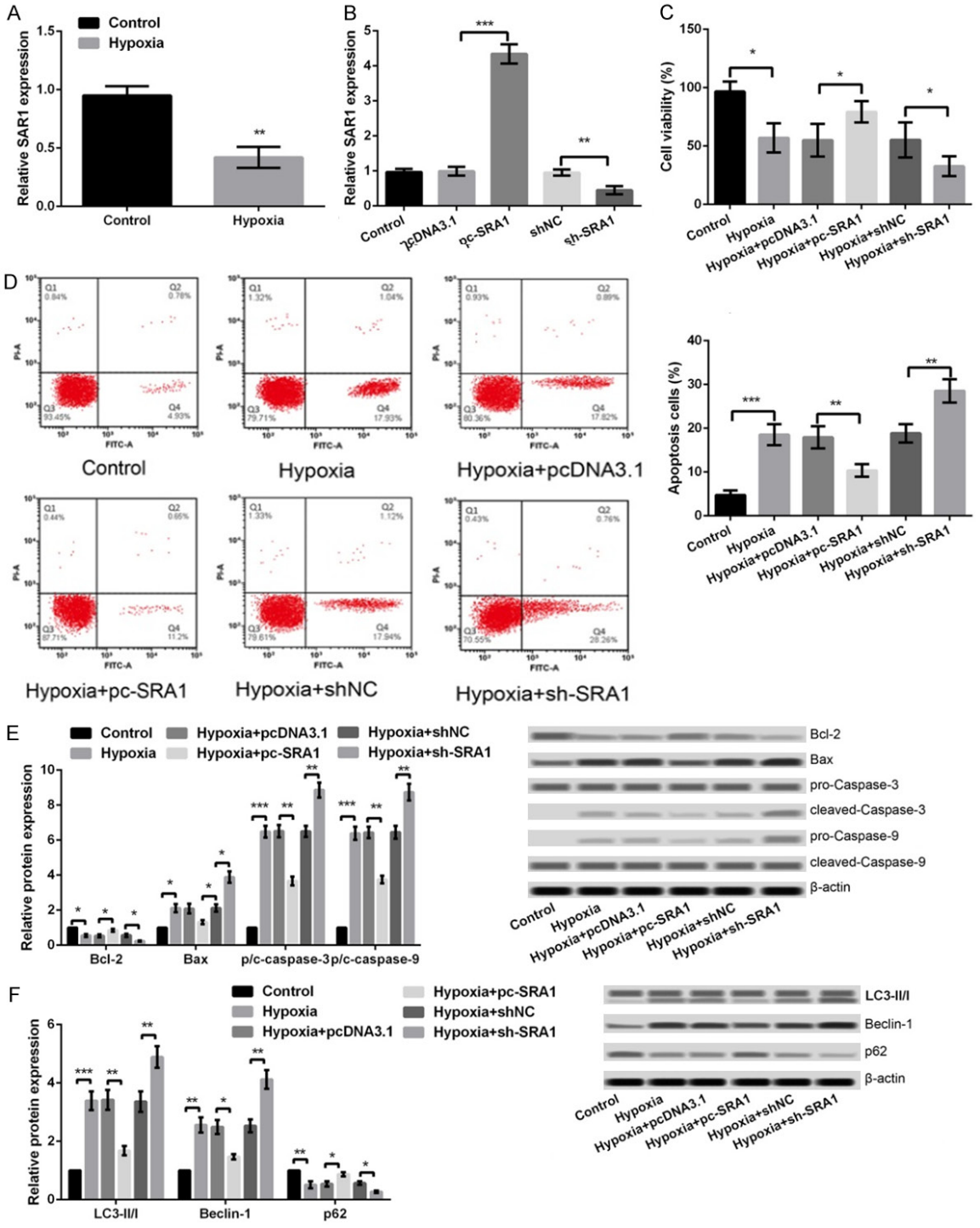

Figure 2. Hypoxia treatment resulted in a decreased expression of SAR1 (A) and overexpression of SRA1 relieved hypoxia-induced injury to H9c2 cardiomyocytes, while suppression of SRA1 aggravated this injury. The experiment was repeated three times. Data are expressed as mean ± SD. **, $P < 0.01$. B: The expression of SRA1 after transfection; C: Cell viability; D: Apoptosis; E: The expression of apoptosis-related proteins; F: The expression of autophagy-related proteins. All experiments were repeated three times. Data are expressed as mean ± SD. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$.

transfection efficiency was confirmed by qPCR ($P < 0.01$, **Figure 2B**). The effects of SRA1 dysregulation on cell viability, apoptosis, and autophagy of H9c2 cardiomyocytes under hypoxic conditions were detected. In comparison with Hypoxia+pcDNA3.1 group, the viability of

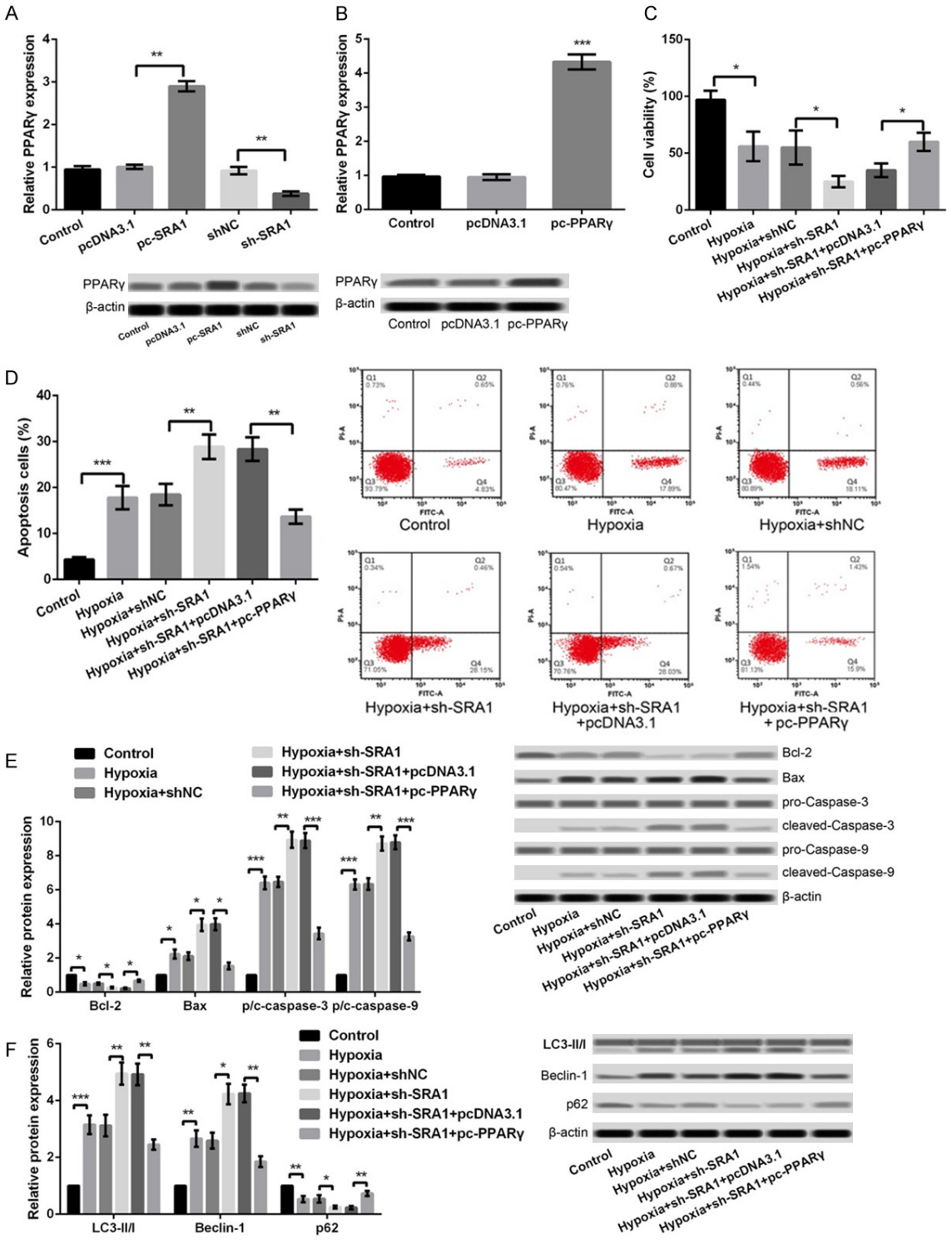

Figure 3. SRA1 activated PPARγ and the effects of SRA1 on hypoxia-induced injury were regulated by PPARγ. A: The mRNA and protein expression of PPARγ after transfection with pc-SRA1, sh-SRA1 and their controls; B: The mRNA and protein expression of PPARγ after transfection with pc-PPARγ and pcDNA3.1; C: Cell viability; D: Apoptosis; E: The expression of apoptosis-related proteins. F: The expression of autophagy-related proteins. All experiments were repeated three times. Data are expressed as mean ± SD. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$.

H9c2 cardiomyocytes in Hypoxia+pc-SRA1 group was significantly increased ($P < 0.05$, **Figure 2C**). However, the viability of H9c2 cardiomyocytes in Hypoxia+sh-SRA1 group was

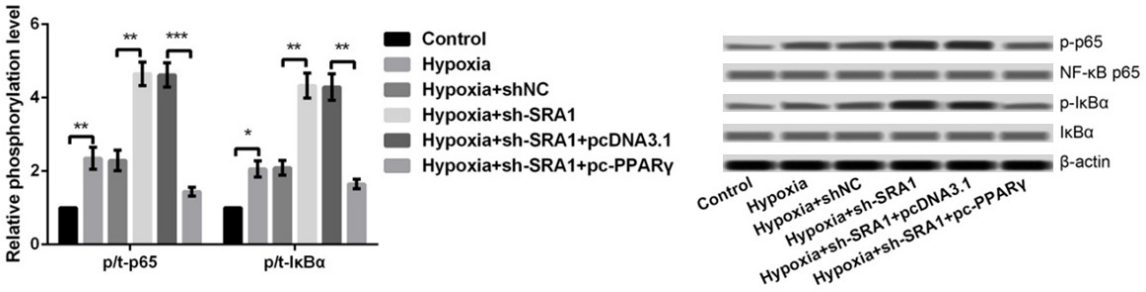

Figure 4. The expression levels of NF-κB pathway-related proteins after different treatments. The experiments were repeated three times. Data are expressed as mean ± SD. *, P < 0.05, **, P < 0.01, and ***, P < 0.001.

markedly lower than that of Hypoxia+shNC group (P < 0.05, **Figure 2C**). Moreover, overexpression of SRA1 in Hypoxia+pc-SRA1 group significantly decreased the percentage of apoptotic cells (P < 0.01, **Figure 2D**), and resulted in consistent expression changes of apoptosis-related proteins (P < 0.05, **Figure 2E**). Suppression of SRA1 had the opposite effect on cell apoptosis (**Figure 2D**, **2E**). Also, overexpression of SRA1 in the Hypoxia+pc-SRA1 group significantly inhibited autophagy of H9c2 cardiomyocytes by increasing p62 expression and decreasing the expression of LC3-II/I and Beclin-1 (P < 0.05, **Figure 2F**), whereas suppression of SRA1 had opposite effect. Taken together, overexpression of SRA1 relieved hypoxia-induced injury to H9c2 cardiomyocytes, while suppression of SRA1 aggravated this injury.

SRA1 activated PPARγ and the effects of SRA1 on hypoxia-induced injury were regulated by PPARγ

A previous study revealed that lncRNA SRA1 can bind to and coactivate PPARγ in mice [13]. The PPARγ agonist pioglitazoneis found to have the ability to relieve severe pulmonary hypertension and vascular remodeling and preventing right heart failure in rats [14]. We thus hypothesized that SRA1 might play a key role in heart failure by activation of PPARγ. As a result, overexpression of SRA1 promoted the expression of PPARγ, while suppression of SRA1 inhibited its expression (P < 0.01, **Figure 3A**). Subsequently, PPARγ was successfully overexpressed in H9c2 cardiomyocytes by transfection with pc-PPARγ (P < 0.001, **Figure 3B**). To further verify whether the effects of SRA1 on hypoxia-induced injury were regulated by PPARγ1, we detected the combined effects of suppression of SRA1 and overexpression of PPARγ on viability, apoptosis, and autophagy of H9c2 cardiomyocytes under hypoxic conditions. In comparison with Hypoxia+sh-SRA1+pcDNA3.1 group, suppression of SRA1 and overexpression of PPARγ at the same time in Hypoxia+sh-SRA1+pc-PPARγ group significantly reversed the effects of suppression of SRA1 alone on hypoxia-induced injury by increasing cell viability (P < 0.05, **Figure 3C**), decreasing apoptosis by regulating the expression changes of Bcl-2, Bax, p/c-caspase-3 and p/c-caspase-3 (P < 0.05, **Figure 3D**, **3E**), and inhibiting autophagy by regulating the expression changes of LC3-II/I, Beclin-1 and p62 (P < 0.01, **Figure 3F**). These data indicated that SRA1 regulated hypoxia-induced injury to H9c2 cardiomyocytes by activating PPARγ.

Protective effects of SRA1 on hypoxia-induced injury in H9c2 cardiomyocytes occurred by regulating the NF-κB signaling

NF-κB signaling is widely involved in the development of heart failure [15, 16], and PPARγ is shown to have an ability of inhibiting NF-κB pathway [17, 18]. We thus investigated whether SRA1 regulate hypoxia-induced injury in H9c2 cells by modulating NF-κB signaling. As shown in **Figure 4**, the expression levels of p/t-p65 and p/t-IκBα in H9c2 cardiomyocytes were significantly increased after hypoxia treatment. Relative to Hypoxia+shNC group, the expression levels of p/t-p65 and p/t-IκBα in hypoxia-treated H9c2 cardiomyocytes were further increased after suppression of SRA1, suggesting that suppression of SRA1 could activate NF-κB signaling in hypoxia-treated H9c2 cardiomyocytes; however, the effects of suppression of SRA1 alone on the expression levels of p/t-p65 and p/t-IκBα in hypoxia-treated H9c2 car-

diomyocytes were significantly reversed after suppression of SRA1 and overexpression of PPARγ at the same time.

Discussion

In this study, we studied the effects and mechanisms of SRA1 on regulating hypoxia-induced injury in H9c2 cardiomyocytes. The results showed that hypoxia induced injury to H9c2 cardiomyocytes, such as inhibiting cell viability, and promoting cell apoptosis and autophagy. Moreover, hypoxia resulted in a decreased expression of SRA1 in H9c2 cardiomyocytes, and overexpression of SRA1 alleviated hypoxia-induced injury, while suppression of SRA1 indicated the contrary results. Further studies showed that SRA1 positively regulated PPARγ. Overexpression of SRA1 alleviated hypoxia injury by activating PPARγ. Besides, suppression of SRA1 activated NF-κB pathway in hypoxia-treated H9c2 cardiomyocytes. These findings imply that SRA1 may be a key player in heart failure and merit further discussion.

Accumulating studies have reported the key role of lncRNAs in heart failure. Yu et al. found that plasma lncRNA UCA 1 could be used as a promising biomarker to diagnose and predict poor outcomes of chronic heart failure [19]. Kumarswamy et al. demonstrated that lncRNA LIPCAR was associated with the developing cardiac remodeling and could serve as an indicator for predicting survival in patients with heart failure [20]. Furthermore, lncRNA CHRF is also shown to be involved in doxorubicin-induced heart failure [21]. In this study, hypoxia resulted in a decreased expression of SRA1 in H9c2 cardiomyocytes. Overexpression of SRA1 alleviated hypoxia-induced injury, while suppression of SRA1 indicated the contrary results. Although the role of SRA1 in heart failure induced by hypoxia has not been fully investigated, our study prompts us to speculate that suppression of SRA1 may contribute to the development of heart failure induced by hypoxia.

In addition, an important finding of our study was that SRA1 positively regulated PPARγ. PPARγ is a ligand-activated transcription factor that affects glucose and mitochondrial fatty acid metabolism in many tissues and organs. A previous study has shown that in nonischemic left ventricular failure, deletion of the PPARγ cofactor and PPARγ coactivator 1 resulted in decreased mitochondrial fatty acid oxidation and lipid homeostasis as well as worsened cardiac function [22, 23]. Hansmann et al. implied the potential beneficial role of PPARγ agonists in pressure-overload heart failure [24]. Furthermore, Yixinshu, a traditional Chinese herbal medication, has been found to protect against heart failure and ameliorate hippocampal abnormality induced by heart failure by regulating the PPARγ pathway, implying the key role of PPARγ in regulating heart failure [25]. In this study, we found that overexpression of SRA1 alleviated hypoxia injury by activating PPARγ. Given the key role of PPARγ in heart failure, our results suggest that overexpression of SRA1 may prevent the development of heart failure induced by hypoxia by positive regulation of PPARγ.

Furthermore, the NF-κB signaling pathway is an important regulator of inflammation, and also is involved in many other biologic processes, such as cell growth and survival. Growing evidence has confirmed that NF-κB activation is implicated in ischemia/reperfusion damage, myocarditis, myocardial infarction, and heart failure [26-28]. Volz et al. revealed that the extracellular heterodimeric protein S100A8/A9 could aggravate post-ischemic heart failure by activation of NF-κB signaling [29]. Xing et al. reported that the YiQiFuMai injection (YQFM), a traditional Chinese medicine, could ameliorate chronic heart failure through regulating NF-κB inactivation [30]. These findings suggest that target NF-κB inactivation may be a potential strategy for the treatment of heart failure. In our study, the results showed that suppression of SRA1 activated NF-κB pathway in hypoxia-treated H9c2 cardiomyocytes, which were significantly reversed after suppression of SRA1 and overexpression of PPARγ at the same time. Therefore, we speculate that suppression of SRA1 may contribute to the development of heart failure induced by activation of the NF-κB pathway. Exploration of the regulatory relationship between SRA1 and NF-κB pathway will provide new insight for the therapy of heart failure induced by hypoxia.

In sum, our findings indicated that suppression of SRA1 may aggravate hypoxia-induced injury to H9c2 cardiomyocytes by positive regulation of PPARγ and activation of NF-κB pathway.

SRA1 may serve as a promising perspective for the therapy of heart failure induced by hypoxia.

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Disclosure of conflict of interest

None.

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