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ORIGINAL PAPER

A soluble receptor for advanced glycation end-products inhibits myocardial apoptosis induced by ischemia/reperfusion via the JAK2/STAT3 pathway

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Abstract sRAGE can protect cardiomyocytes from apoptosis induced by ischemia/reperfusion (I/R). However, the signaling mechanisms in cardioprotection by sRAGE are currently unknown. We investigated the cardioprotective effect and potential molecular mechanisms of sRAGE inhibition on apoptosis in the mouse myocardial I/R as an in vivo model and neonatal rat cardiomyocyte subjected to ischemic buffer as an in vitro model. Cardiac function and myocardial infarct size following by I/R were evaluated with echocardiography and Evans blue/2,3,5-triphenyltetrazolium chloride. Apoptosis was detected by TUNEL staining and caspase-3 activity. Expression of the apoptosis-related proteins p53, Bax, Bcl-2, JAK2/p-JAK2, STAT3/p-STAT3, AKT/p-AKT, ERK/p-ERK, STAT5A/p-STAT5A and STAT6/p-STAT6 were detected by western blot analysis in the presence and absence of the JAK2 inhibitor AG 490. sRAGE (100 μg/day) improved the heart function in mice with I/R: the left ventricular ejection fraction and fractional shortening were increased by 42 and 57 %, respectively; the infarct size was decreased by 52 %, the TUNEL-positive myocytes by 66 %, and activity of caspase-3 by 24 %, the protein expression of p53 and ratio of Bax to Bcl-2 by 29 and 88 %, respectively; protein expression of the p-JAK2, p-STAT3 and p-AKT were increased by 92, 280 and 31 %, respectively. sRAGE have no effect on protein expression of p-ERK1/2, p-STAT5A and p-STAT6 following by I/R. sRAGE (900 nmol/L) exhibited anti-apoptotic effects in cardiomyocytes by decreasing TUNEL-positive myocytes by 67 % and caspase-3 activity by 20 %, p53 protein level and the Bax/Bcl-2 ratio by 58 and 86 %, respectively; increasing protein expression of the p-JAK2 and p-STAT3 by 26 and 156 %, respectively, p-AKT protein level by 33 %. The anti-apoptotic effects of sRAGE following I/R were blocked by JAK2 inhibitor AG 490. The effect of sRAGE reduction on TUNEL-positive myocytes and caspase-3 activity were abolished by PI3K inhibitor LY294002, but not ERK 1/2 inhibitor PD98059. These results suggest that sRAGE protects cardiomyocytes from apoptosis induced by I/R in vitro and in vivo by activating the JAK2/STAT3 signaling pathway.

Keywords sRAGE · Myocardium · Cardiac function · Ischemia/reperfusion · Apoptosis · JAK2/STAT3

Abbreviations

sRAGE	Soluble receptor for advanced glycation end-products
I/R	Ischemia/reperfusion
JAK/STAT	Janus kinase/signal transducer and activator of transcription
JAK2	Janus activated kinase 2
STAT3	Signal transducer and activator of transcription 3
RAGE	Receptor for advanced glycation end-products

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EF	Ejection fraction
FS	Fractional shortening
LV	Left ventricular
ERK ½	Extracellular signal-regulated kinase 1/2
PI3K	Phosphatidylinositol 3 kinase
AKT	Protein kinase B
TTC	2,3,5-Triphenyltetrazolium chloride
TUNEL	Terminal deoxynucleotidyl transferase-mediated nick end label
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
FBS	Fatal bovine serum
PBS	Phosphate buffer saline

Introduction

Ischemic heart disease is a major cause of morbidity and mortality in hospitalized patients, such as myocardial ischemia/reperfusion (I/R), heart failure, angina, and myocardial infarction. I/R is a pathological process that results in extensive cell death. Early and successful myocardial reperfusion is the best curative effect method to decrease the infarct size and restore cardiac function after acute ischemic events. However, ischemic tissue of the myocardium is susceptible to be further injured deriving from reperfusion, which abolishes the beneficial effects of reperfusion itself. Possible mechanisms of myocardial I/R injury are calcium overload and mitochondrial dysfunction [1], an increase in reactive oxygen species [2, 3], activation and adhesion of neutrophils [4], and cellular apoptosis [5]. Although the mechanism of I/R injury involves multiple cellular processes, cellular apoptosis plays a critical role in the pathogenesis of myocardial injury. Therefore, attention has been focused on prevention and treatment of cellular apoptosis to decrease the degree of injury and improve function in the damaged myocardium. Current prevention and treatment strategies of myocardial I/R injury, including ischemic preconditioning [6], ischemic postconditioning, remote ischemic conditioning [7, 8], and endogenous protective material release, such as the soluble receptor for advanced glycation end products (sRAGE).

Previous studies have shown that sRAGE has a protective effect on coronary artery disease [9]. sRAGE, the N-terminal extracellular domain of the RAGE, can be derived either from enzymatic cleavage of full-length cell-surface receptor or endogenous secretion of splice variants of RAGE. [10, 11]. RAGE, expressed in many tissues, is a transmembrane protein member of the immunoglobulin superfamily. The cellular signaling pathways of RAGE includes the family of mitogen-activated protein (MAP) kinases (ERK 1/2, p38, SAPK/JNK), members of the JAK/STAT signalling family, phosphoinositide 3-kinase, Rho GTPases (Rac1, Cdc42), and the pro-apoptotic pathway p53-Bcl-2-associated X protein. The RAGE signaling pathways lead to activation of various transcription factors, including NF-κB and SP-1. Activation of RAGE induces a number of cell processes, including inflammation, apoptosis, proliferation and autophagy. Therefore, RAGE is involved in the development of many diseases, such as diabetes, cardiovascular diseases, osteoarthritis and cancer [12]. sRAGE may counteract RAGE-mediated pathogenesis by acting as a decoy [13].

Many studies have investigated human circulating sRAGE levels and their association with a variety of pathophysiological conditions, including diabetes [13], immune/inflammatory diseases [14], coronary artery disease [15], I/R injury [16], and hypertension [17]. However, the therapeutic effects of sRAGE in patients with heart injury after I/R remain unclear. Previous studies have reported that sRAGE can protect cardiomyocytes from apoptosis induced by I/R in in vivo and in vitro models. In vivo, sRAGE alleviates RAGE-mediated I/R injury by limiting infarct size and improving left ventricular function post-I/R [16]. In addition, Aleshin et al. showed that the increased phosphorylation of STAT3 by sRAGE attenuated RAGE mediates ischemic injury in vivo [18], but the exact effect of sRAGE on STAT3 in vitro is unknown. In vitro, exogenous administration of sRAGE during I/R is involved in cardioprotection by inhibiting apoptosis via the mitochondrial pathway [19]. However, the underlying molecular mechanisms of sRAGE beneficial effects in the setting of I/R need to be clarified.

Two major cell survival signaling cascades are known to be protect against I/R injury: the reperfusion injury salvage kinase (RISK) pathway and the survivor activating factor enhancement pathway, which involves STAT3. JAK2/STAT3 is a powerful survival signaling pathway in many systems. Activated p-JAK and p-STAT3 are sufficient to protect cardiomyocytes against apoptosis in vitro and in vivo. The JAK2 inhibitor AG 490 abolishes ischemic preconditioning-induced cardioprotection after myocardial I/R [20]. In addition, Aleshin et al. confirmed that STAT signal transduction participates in RAGE-mediated I/R injury [18]. It is of importance to explore the action mechanism of sRAGE as a novel therapeutics for ischemic diseases. In the current study, we investigated the therapeutic effects and potential mechanism of sRAGE on I/R-induced myocardial apoptosis in mouse myocardial and neonatal rat cardiomyocytes I/R injury model. In particular, we focused on the effect of sRAGE on the JAK2/STAT3 pathway.

We hypothesized that sRAGE inhibits apoptosis induced by I/R in cardiomyocytes in vivo and in vitro through activating the JAK2/STAT3 signaling pathway.

Materials and methods

Materials

Male C57BL/6 mice of 6–8 weeks (weight 20–22 g) and 1- to 2-day-old Sprague–Dawley rats were obtained from the Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Mice were housed in a pathogen-free environment and kept under a 12/12-h light–dark cycle at a temperature of 23–25 °C. Standard laboratory animal chow and water were offered, and kept in the Laboratory of Animal Experiments at Capital Medical University. A 2 weeks adaptation time was permitted before any experimental procedures. All animal experimental procedures were authorized by the Animal Subjects Committee of Capital Medical University. Synthetic rat sRAGE was from Aidi Bo biological Ltd. (Beijing, China). JAK2 inhibitor AG 490, PI3K inhibitor LY294002 and ERK 1/2 inhibitor PD98059 were from Sigma Co. (St. Louis, MO, USA). Evans blue/2,3,5-triphenyltetrazolium chloride was provided by Sigma Co. (St. Louis, MO, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) Apoptosis Detection Kit was purchased from Promega and caspase-3 activity was provided by the BIOMOL. Primary antibodies against cell apoptosis-related proteins were purchased from cell signaling technology, Inc. (JAK2, p-JAK2, STAT3, p-STAT3, STAT5A, p-STAT5A, STAT6, p-STAT6, AKT, p-AKT, ERK 1/2, p-ERK 1/2 and Bcl-2) and Abcam Inc. (p53 and Bax). Other chemicals and reagents were of analytical grade.

Myocardial I/R model in mice

C57BL/6 mice myocardial I/R was completed by ligating left anterior descending coronary artery (LAD) with a slipknot as described previously [21]. The left-sided thoracotomy of C57BL/6 male mice was performed to expose the heart after anesthetized and maintained with 2 % isoflurane, and the origin of the LAD was ligated by a 7-0 silk suture, and then the sham-operated animals underwent the same procedure except fastening the suture which was around the LAD. After 30 min of myocardial ischemia, the ligation of LAD was released for 24 h reperfusion. sRAGE at 100 μg/day or equal volumes of vehicle, was administered by intraperitoneal injections twice, which were performed at 12 h prior to LAD ligation and 12 h of reperfusion [22].

Isolation and culture of primary neonatal rat cardiomyocytes

Neonatal rat cardiac myocytes were isolated by enzymatic disassociation from 1- to 2-day-old Sprague–Dawley rats [23]. Briefly, the excised hearts were placed and cleaned in iced-cold Hanks balanced salt solution (HBSS; Ca^{2+}–Mg^{2+} free) and the myocardial tissues were cut up in HBSS containing tyrosine (0.05 %) and collagenase (0.055 %), and digested at 37 °C. Cells were extracted by repeated 2-min rounds of tissue digestion. After each incubation, the supernatant was transferred to an equal volume of DMEM containing 15 % fetal bovine serum. The whole cell suspensions were centrifuged at 1000 rpm for 10 min, then the supernatants were removed and the cell pellets were resuspended by DMEM which contained 15 % fetal bovine serum. The cells were plated onto 150-mm culture dishes for 90 min in order to the non-myocytes attached to the dish, and then the cardiomyocytes stayed in the suspension. The cardiomyocytes were harvested and seeded on to 6-well culture dishes, while 5-Bromo-2′-deoxyuridine (100 μmol/L) was added into the medium during the first 48 h for inhibiting proliferation of non-myocytes. Finally, cells were incubated in a carbon dioxide incubator (MCO-15AC, Sanyo, Japan) in an atmosphere of 5 % CO_2 and 95 % O_2 at 37 °C.

I/R model in vitro

Cardiomyocytes were simulated ischemia by replacing normal DMEM medium with "ischemia buffer" (pH 6.3), which including the following (mmol/L): 118 NaCl, 24 $NaHCO_3$, 1.0 NaH_2PO_4, 2.5 $CaCl_2$–$2H_2O$, 1.2 $MgCl_2$, 20 sodium lactate, 16 KCl and 10 2-deoxyglucose as reported previously [24]. Subsequently, cells were incubated in an atmosphere of 1 % O_2 and 5 % CO_2 at 37 °C for 2 h. The cells were then cultured with serum-free DMEM medium for a further 24 h of reperfusion. In control groups, cells were incubated with DMEM in an atmosphere of 5 % CO_2 at 37 °C all the time.

Treatment of cardiomyocytes with sRAGE, AG 490, LY294002 and PD98059

In the I/R and sRAGE (900 nmol/L) groups, sRAGE was added to the cells 10 min before the simulated I/R and then the cells were incubated with sRAGE at a concentration of 900 nmol/L for ischemia and reperfusion. Cells were pretreated with three different inhibitors for 30 min before the simulated I/R process, which are JAK2 inhibitor AG 490 (5 μmol/L) [25], PI3K inhibitor LY294002 (10 μmol/L) [23], ERK 1/2 inhibitor PD98059 (100 nmol/L) [26], respectively. All of the inhibitors were dissolved in DMSO.

For DMSO group, cells were incubated with DMSO alone for the remainder of the experiment.

Evaluation of cardiac function, myocardial infarct size

At the end of 24 h reperfusion, echocardiography was performed with the Vevo770 imaging system (VisualSonics Inc., Toronto, Ontario, Canada) after the mice were anesthetized with 2 % isoflurane, and LV fractional shortening and LV ejection fraction were calculated at M mode as described previously [27]. After functional evaluated, myocardial infarct size was determined by the Evans blue/2,3,5-triphenyltetrazolium chloride (TTC, Sigma) double staining method [28]. Briefly, at the end of reperfusion, the LAD was retied at the home position. At room temperature, after injecting Evan's blue into the left ventricle, the heart was taken out and perfused with 1× PBS and then directly frozen blocin liquid nitrogen for storage at −80 °C. At the time of analysis, the frozen heart was warmed at −20 °C and sectioned transversely (1 mm), then the tissue was stained by 1 % TTC at 37 °C for 20 min. The tissue was steeped in 10 % neutralized formalin for 12 h in order to stop the staining and then visualized with a digital camera to calculated the fraction of each section with blue, red, or white staining. The area at risk (AAR) was determined as the ratio of red plus white and the total area (red plus white plus blue). Infarct size was represented by the percentage of white in relation to the total area of white plus red. The different region of nonischemic, ischemic (AAR), and infarct were quantified by computer using NIH Image (v1.62). Calculations were averaged over all sections of each heart.

Determination of myocardial apoptosis

Apoptosis of cardiomyocytes and heart tissue was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (Promega, America) and caspase-3 activity (BIOMOL, PA) [29, 30]. Briefly, the heart tissue was embedded by paraffin and cut into Sections 4–5 μm thick. And the sections were incubated with 50 μL of the TUNEL mixture (47.5 μL of TUNEL label containing fluorescein isothiocyanate-conjugated dUTP and 2.5 μL of TUNEL enzyme) in a humidified chamber at 37 °C for 60 min. Control sections were incubated in 50 μL of TUNEL label solution except TUNEL enzyme. All of the sections were taken photograph at 20× objective with an Olympus BX51 Fluorescence Microscope (Olympus America Inc., Center Valley, PA). Total nuclei and the TUNEL-positive nuclei were counted and the percentage of TUNEL-positive myocytes was calculated automatically as the ratio of TUNEL positive nuclei/total number of nuclei by NIH Image software. Results from 8 to 10 random fields per section under a microscope taken from the same animal were averaged and counted as one sample. The caspase-3 activity of cardiomyocytes and heart tissue was performed by cleavage of a fluorogenic substrate. According to the supplier's instructions, all samples were lysed with lysis buffer, and then centrifuged at 14,000 rpm for 10 min at 4 °C, supernatant will be used only. Fluorescence was measured 0 min as background value, and after incubated for 1.5 h at 37 °C and avoid light as 90 min value. Activity of caspase-3 was determined using a spectrophotometer at 405 nm (Molecular Devices, Sunnyvale, CA). The results were expressed as—fold of the sham or control group.

Western blot

Western blotting was performed as described previously [31]. In brief, cardiac tissues and cells were lysed with lysis buffer containing 20 mM Tris (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1 mM EGTA, 1 % Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 4 μg/mL aprotinin, 4 μg/mL leupeptin, 4 μg/mL pepstatin, and 1 mM PMSF. 50–60 μg of total proteins were separated by electrophoresis on SDS-PAGE and then transferred to PVDF membranes. After blocking with 5 % skim milk at room temperature for 60 min, membranes were incubated with primary antibody against JAK2 (1:1000; Cell signaling, Inc., USA), p-JAK2 (1:1000; Cell signaling, Inc., USA), STAT3 (1:1000; Cell signaling, Inc., USA), p-STAT3 (1:1000; Cell signaling, Inc., USA), STAT5A (1:1000; Cell signaling, Inc., USA), p-STAT5A (1:1000; Cell signaling, Inc., USA), STAT6 (1:1000; Cell signaling, Inc., USA), p-STAT6 (1:1000; Cell signaling, Inc., USA), AKT (1:1000; Cell signaling, Inc., USA), p-AKT (1:1000; Cell signaling, Inc., USA), ERK 1/2 (1:1000; Cell signaling, Inc., USA), p-ERK 1/2 (1:1000; Cell signaling, Inc., USA), Bcl-2 (1:1000; Cell signaling, Inc., USA), p53 (1:800; Abcam), Bax (1:1000; Abcam) and β-actin (1:3000; Santa Cruz) overnight at 4 °C. The membrane was then washed with TBST and incubated with horseradish peroxidase-conjugated IgG antibody (1:3000) for 1 h at room temperature. As for the phosphorylated protein, we normalized the signal to the amount of total target protein and β-actin. The blots were developed with a super signal chemiluminescence detection kit (Thermo Scientific, USA). The immunoblotting was visualized with Chemiluminescent And Fluorescent Imaging Systems (ChampChemi; SAGECREATION, Beijing).

Statistical analysis

All values in the figures are presented as mean ± SE. Data were subjected to one-way ANOVA analysis followed by

LSD for multiple comparisons within treatment groups with use of SPSS v13.0 (SPSS Inc., Chicago, IL). $p < 0.05$ was considered statistically significant.

Results

sRAGE improved I/R-induced LV function injury

To investigate the effect of sRAGE on *heart function*, the EF and FS were measured following I/R with or without sRAGE. The benefits on EF and FS observed with the administration of sRAGE following I/R was demonstrated in Fig. 1. Compared with sham group, EF and FS were significantly *reduced* by 57 and 66 % in the I/R group ($p < 0.05$), but were significantly increased by 42 and 57 % in the I/R+sRAGE group compared to single I/R group ($p < 0.05$). The EF and FS in the IR+sRAGE group were lower than in sham group ($p < 0.05$). sRAGE alone had no effect on cardiac function as compared to sham group (Fig. 1a–c).

sRAGE reduced I/R-induced myocardial injury

To determine the impact of sRAGE on I/R-induced myocardial injury, the AAR and infarct size were determined. There was no difference in the measured AAR as a percentage of the total LV area among any groups (Fig. 2a, b). However, the infarct size in mice were pretreated with sRAGE following I/R was lowered compared to I/R alone group ($p < 0.05$; Fig. 2a, c). This represents a significant 52 % reduction of infarct size in hearts with sRAGE-treated following I/R.

sRAGE inhibited apoptosis induced by I/R in vivo

To evaluate the role of sRAGE in apoptosis, we next tested whether administration of sRAGE could influence the effect of I/R. In in vivo experiment, there were rare TUNEL-positive nuclei both in sham and sham+sRAGE group. The number of TUNEL-positive cardiomyocytes were increased by 153 % in I/R group compared to sham group ($p < 0.05$; Fig. 3a, b). The caspase-3 activity *rose* 66 % in

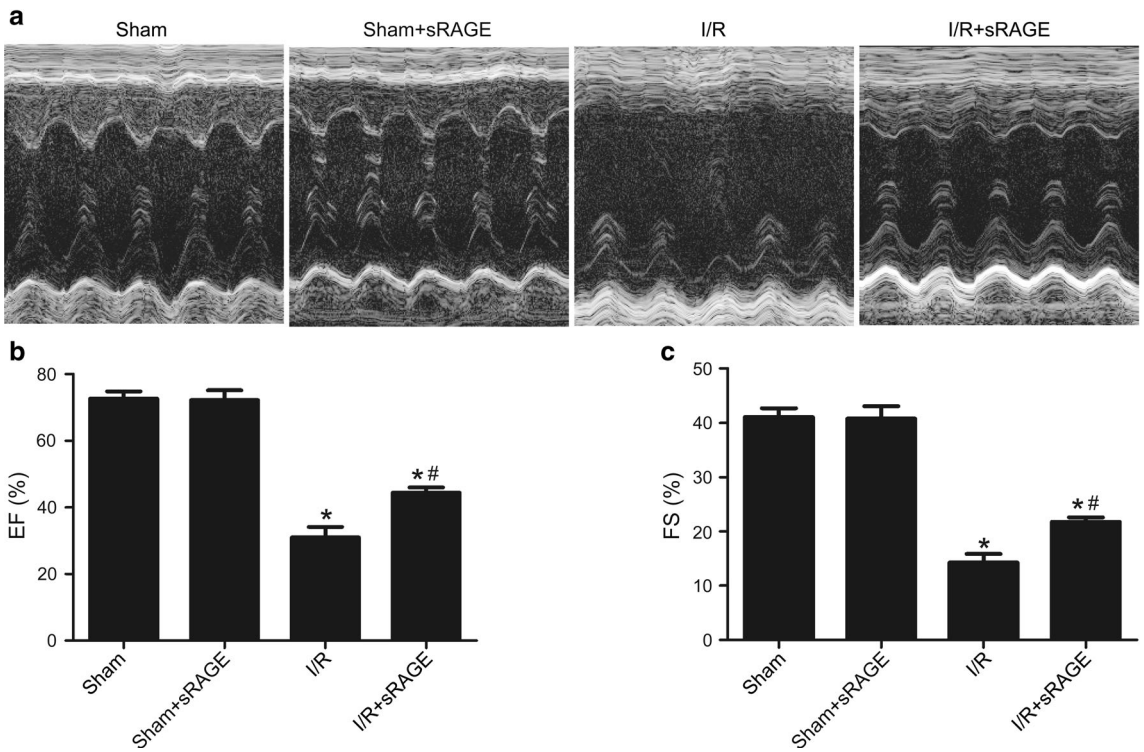


Fig. 1 Effects of sRAGE inhibition on reduction of cardiac function induced by I/R. **a** Representative M-mode echocardiography recordings from sham group, sham+sRAGE group, I/R group and I/R+sRAGE group, respectively. **b** Left ventricle ejection fraction (LVEF %) were assessed by echocardiography in sham group and experimental mice 24 h after I/R injury from different groups (n = 6–8). Values are expressed as mean ± SEM. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group. **c** Left ventricle fractional shortening (LVFS %) were assessed by echocardiography in sham group and experimental mice 24 h after I/R injury from different groups (n = 6–8). Values are expressed as mean ± SEM. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group

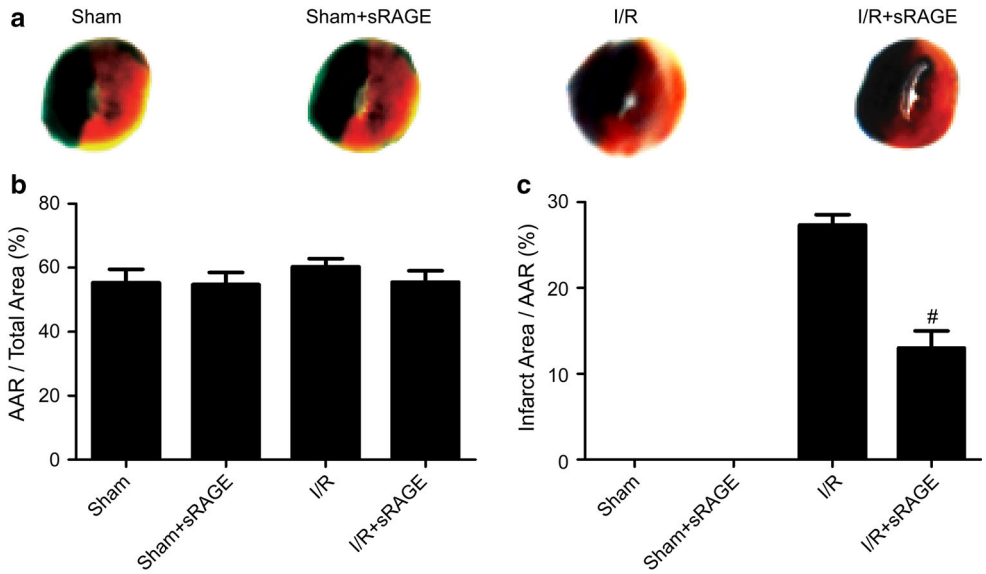


Fig. 2 sRAGE improved I/R-induced myocardial injury in mice. **a** Representative photographs of medial sections of cardiac tissues following Evan's blue and TTC staining of sham group, sham+sRAGE group, I/R group and I/R+sRAGE group, respectively. Evans blue stained areas (*black*) indicated non-ischemic/reperfused area; TTC stained areas (red staining) indicated ischemic but viable tissue; Evans blue/TTC staining negative areas indicated infarct myocardium. **b** Quantitative analyses of Evan's blue and TTC staining to determine the AAR areas was shown for different groups (n = 3–5). Values are expressed as mean ± SEM. **c** Quantitative analyses of Evan's blue and TTC staining to determine the infarct areas was shown for different groups (n = 3–5). Values are expressed as mean ± SEM. $^{\#}p < 0.05$ compared with I/R group

I/R group compared to sham group ($p < 0.05$; Fig. 3c). Compared with sham group, I/R significantly *elevated* p53 and the Bax/Bcl-2 ratio by 148 and 112 %, respectively ($p < 0.05$; Fig. 3d–f). However, the number of TUNEL-positive cardiomyocytes was markedly *reduced* by 66 % and caspase-3 activity was significantly decreased by 24 % in the I/R+sRAGE group compared with the I/R group ($p < 0.05$; Fig. 3a–c). Compared with I/R group, sRAGE-pretreated significantly decreased the I/R-induced increase in p53 by 29 % and ratio of Bax to Bcl-2 by 87 % ($p < 0.05$; Fig. 3d–f). The protein level of p53 *rose* 76 % and ratio of Bax to Bcl-2 were markedly *reduced* by 77 % in the I/R+sRAGE group compared with the sham group ($p < 0.05$; Fig. 3a–c). Furthermore, sRAGE alone *diminished* the Bax/Bcl-2 ratio by 75 % ($p < 0.05$; Fig. 3d, f). Compared with sham group, the ratios of p-JAK2/JAK2 and p-STAT3/STAT3 were markedly reduced by 60 and 44 % in I/R group ($p < 0.05$; Fig. 4a–c); the ratios of p-AKT/AKT and p-ERK/ERK were significantly *elevated* by 30 and 120 % in I/R alone group, respectively ($p < 0.05$; Fig. 4a, d, e); the ratios of p-STAT5A/STAT5A and p-STAT6/STAT6 *rose* 74 and 91 % in I/R alone group, respectively ($p < 0.05$; Fig. 4a, f, g). Compared with I/R group, the ratio of p-JAK2/JAK2 was significantly elevated by 92 % in the I/R+sRAGE group ($p < 0.05$; Fig. 4a, b), the ratios of p-STAT3/STAT3 was significantly *elevated* by 281 % and p-AKT/AKT was further increased by 31 % in the IR+sRAGE group ($p < 0.05$; Fig. 4a, c, d). There was no difference in the ratios of p-ERK/ERK, p-STAT5A/STAT5A and p-STAT6/STAT6 between the IR and IR+sRAGE groups ($p > 0.05$; Fig. 4a, e, f, g). Besides, compared with sham group, p-JAK2 protein level rose 50 % in the sham+sRAGE group ($p < 0.05$; Fig. 4a, b).

sRAGE inhibited apoptosis induced by I/R in vitro

To further clarify the role of sRAGE on apoptosis, the number of TUNEL-positive cardiomyocytes and caspase-3 activity were detected in vitro. The number of TUNEL-positive cardiomyocytes was markedly *promoted* by 243 % in the I/R group compared with the control group ($p < 0.05$; Fig. 5a, b), and the caspase-3 activity was increased by 68 % in the I/R group compared to the control group ($p < 0.05$; Fig. 5c). Compared with control group, I/R significantly *elevated* p53 and the Bax/Bcl-2 ratio by 34 and 62 %, respectively ($p < 0.05$; Fig. 6a–c). However, the percentage of TUNEL-positive cardiomyocytes was significantly decreased by 67 % in the I/R+sRAGE group compared with the I/R alone group ($p < 0.05$; Fig. 5a, b), and compared with I/R group, the caspase-3 activity was significantly *reduced* by 20 % in the I/R+sRAGE group ($p < 0.05$; Fig. 5c). p53 protein level and the Bax/Bcl-2 ratio were markedly decreased by 58 and 86 % in the I/R+sRAGE group compared with the I/R alone group ($p < 0.05$; Fig. 6a–c). Additionally, sRAGE alone *lowered* the Bax/Bcl-2 ratio by 29 % ($p < 0.05$; Fig. 6a, c). All of

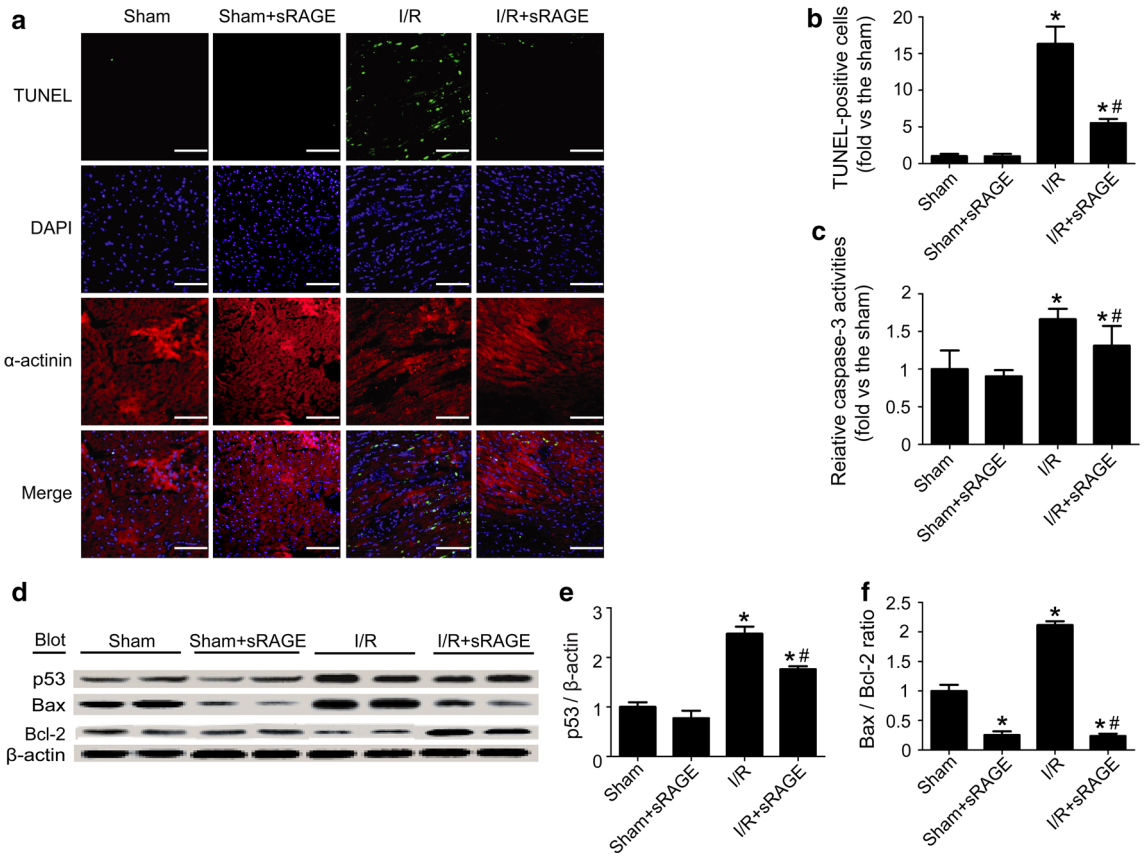


Fig. 3 Effect of sRAGE inhibition on I/R-induced apoptosis in mice. **a** Representative photomicrographs of TUNEL-stained myocardium sections from sham group, sham+sRAGE group, I/R group and I/R+sRAGE group. TUNEL-positive nuclei (*green*), myoglobin (*red*), and DAPI (*blue*). *Scale bar*, 50 μm. **b** Quantitative analysis of TUNEL-positive cells was shown for different groups (n = 3–5). Values are expressed as mean ± SEM. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group. **c** Quantitative analysis of caspase-3 activity was shown for different groups (n = 3–5). Values are expressed as mean ± SEM. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group. **d** Representative Western blots showed expression levels of p53, Bax and Bcl-2 proteins from sham group, sham+sRAGE group, I/R group and I/R+sRAGE group. A representative blot is shown for each condition. **e** The quantified intensity of protein bands was shown as the ratio of p53/β-actin for different groups (n = 3). Values are expressed as mean ± SEM. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group. **f** The quantified intensity of protein bands was shown as the ratio of Bax/Bcl-2 for different groups (n = 3). Values are expressed as mean ± SEM. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group (Color figure online)

these effects were blocked by the JAK2 inhibitor AG 490 in cardiomyocytes. Compared with I/R+sRAGE group, the number of TUNEL-positive cardiomyocytes, caspase-3 activity, p53 protein level and the Bax/Bcl-2 ratio were markedly *elevated* in I/R+sRAGE+AG 490 group ($p < 0.05$; Fig. 5a–c, 6a–c). In addition, there were no difference in the p53 protein level and Bax/Bcl-2 ratio between control and control+AG 490 groups ($p > 0.05$; Fig. 6a–c). The percentage of TUNEL-positive cardiomyocytes and the caspase-3 activity were significantly increased by 202 and 26 % in I/R+sRAGE+LY294002 group compared with I/R+sRAGE group ($p < 0.05$; Fig. 7a–c). But there was no difference in TUNEL-positive cardiomyocytes and caspase-3 activity between I/R+sRAGE+PD58098 group and I/R+sRAGE groups ($p > 0.05$; Fig. 8a–c).

Anti-apoptotic effect of sRAGE on the JAK2/STAT3 signaling pathway in vitro following I/R

To confirm the anti-apoptotic signaling pathway for sRAGE, the ratios of p-JAK2/JAK2 and p-STAT3/STAT3 were determined. Compared with control group, the ratios of p-JAK2/JAK2 and p-STAT3/STAT3 were significantly *reduced* by 22 and 59 % in I/R alone group, respectively ($p < 0.05$; Fig. 9a, d, e). Compared with I/R group, p-JAK2 and p-STAT3 levels were significantly *elevated* by 26 and 156 % when cardiomyocytes were subjected to sRAGE for 24 h in the I/R+sRAGE group ($p < 0.05$; Fig. 9a, d, e). In addition, compared with control group, p-JAK2 and p-STAT3 levels were significantly increased by 22 and 33 % in the control+sRAGE group ($p < 0.05$; Fig. 9a, d, e). Compared with the I/R+sRAGE group, the

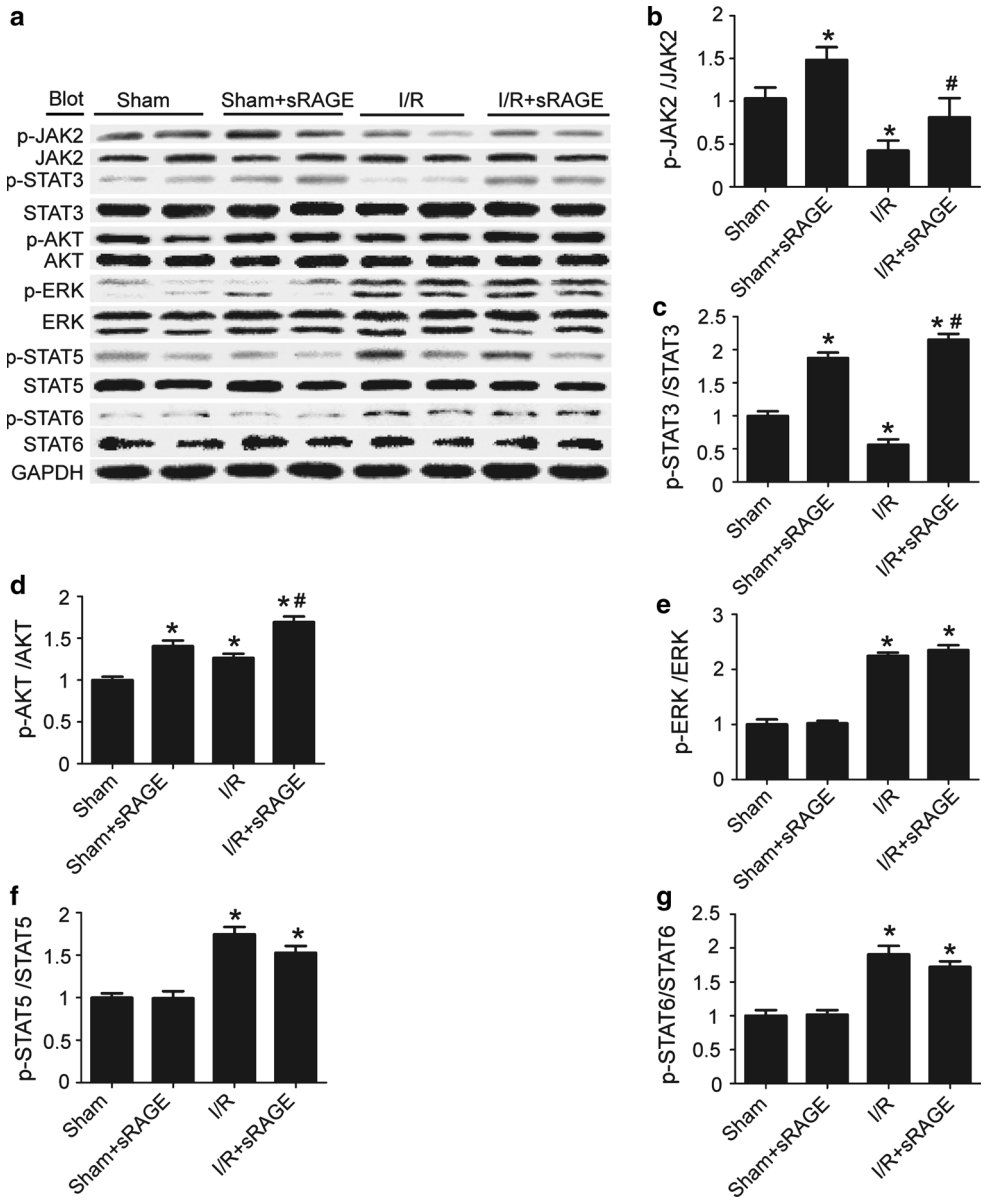


Fig. 4 Effect of sRAGE on the protein expression of phosphorylated JAK2, phosphorylated STAT3, phosphorylated AKT, phosphorylated ERK, phosphorylated STAT5A and phosphorylated STAT6 followed by I/R in mice. **a** Representative Western blots showed expression levels of total and phosphorylated JAK2, total and phosphorylated STAT3, total and phosphorylated AKT, total and phosphorylated ERK, total and phosphorylated STAT5A and total and phosphorylated STAT6 proteins from sham group, sham+sRAGE group, I/R group and I/R+sRAGE group. **b** The relative protein levels were quantified and was shown as the ratio of phosphorylated protein/total protein after normalized by GAPDH from three independent experiments. The quantified intensity of protein bands was shown as the ratio of p-JAK2/JAK2 for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group. **c** The relative protein levels were quantified and was shown as the ratio of phosphorylated protein/total protein after normalized by GAPDH from three independent experiments. The quantified intensity of protein bands was shown as the ratio of p-STAT3/STAT3 for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group. **d** The quantified intensity of protein bands was shown as the ratio of p-AKT/AKT for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group. **e** The quantified intensity of protein bands was shown as the ratio of p-ERK/ERK for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group. **f** The quantified intensity of protein bands was shown as the ratio of p-STAT5A/STAT5A for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group. **g** The quantified intensity of protein bands was shown as the ratio of p-STAT6/STAT6 for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with sham group, $^{\#}p < 0.05$ compared with I/R group

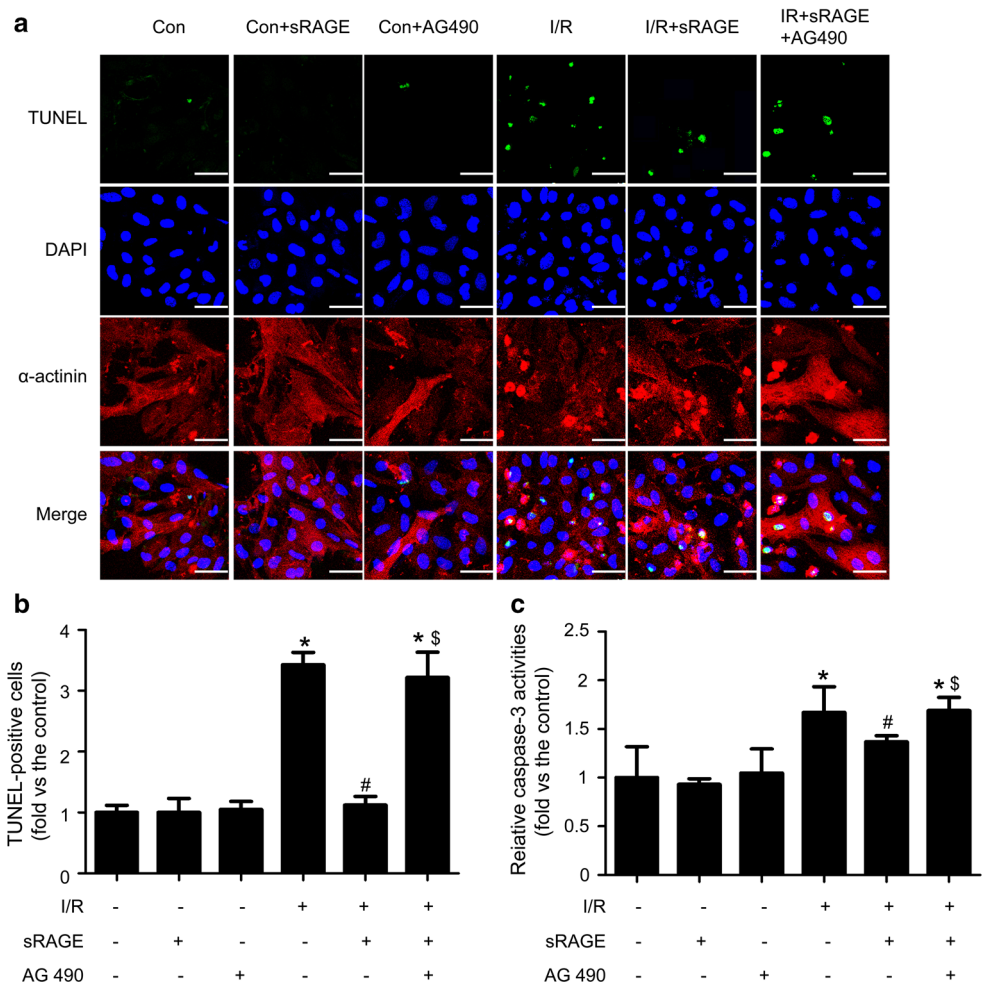


Fig. 5 Suppression of STAT3 blocks effect of sRAGE inhibition on I/R-induced apoptosis in cardiomyocytes. **a** Representative photomicrographs of TUNEL-stained cardiomyocytes from con, con+sRAGE, con+AG 490, I/R, I/R+sRAGE and I/R+sRAGE+AG 490. TUNEL-positive nuclei (*green*), myoglobin (*red*), and DAPI (*blue*). *Scale bar* 100 μm. **b** Quantitative analysis of TUNEL-positive cells was shown for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group, $^{\$}p < 0.05$ compared with I/R+sRAGE group. **c** Quantitative analysis of caspase-3 activity assay was shown for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group, $^{\$}p < 0.05$ compared with I/R+sRAGE group (Color figure online)

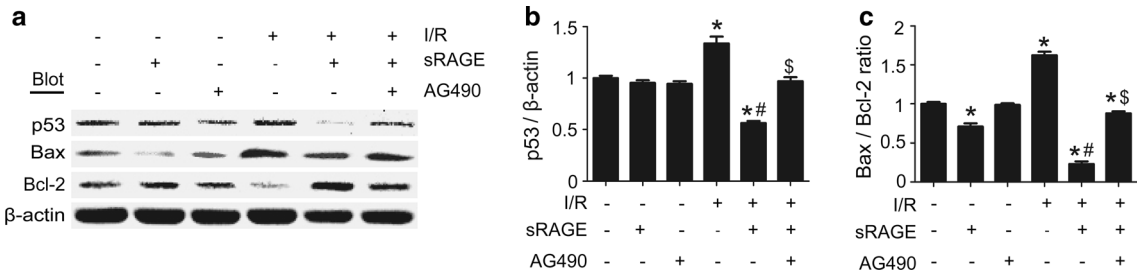


Fig. 6 Inhibition of STAT3 abolishes effect of sRAGE-induced elevation of p53 and Bax/Bcl-2 ratio induced by I/R in cardiomyocytes. **a** The protein levels of p53, Bax and Bcl-2 were examined by Western blotting from con, con+sRAGE, con+AG 490, I/R, I/R+sRAGE and I/R+sRAGE+AG 490. A representative blot is shown for each condition. **b** The quantified intensity of protein bands was shown as the ratio of p53/β-actin for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group, $^{\$}p < 0.05$ compared with I/R+sRAGE group. **c** The quantified intensity of protein bands was shown as the ratio of Bax/Bcl-2 for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group, $^{\$}p < 0.05$ compared with I/R+sRAGE group

level of p-JAK2 and p-STAT3 were significantly decreased by 29 and 48 % in the I/R+sRAGE+AG 490 group ($p < 0.05$; Fig. 9a, d, e). Compared with control group, the ratios of p-JAK2/JAK2 and p-STAT3/STAT3 were significantly reduced by 21 and 44 % in control+AG 490 group, respectively ($p < 0.05$; Fig. 9a, d, e).

The rationale to use LY294002 and PD98059 is that JAK-STAT signaling may provide upstream initiation of RISK pathway signaling via PI3K-Akt activation. Moreover, the RISK Pathway including Akt and Erk1/2, which confer powerful cardioprotection. Compared with control group, the protein expression of p-AKT was significantly

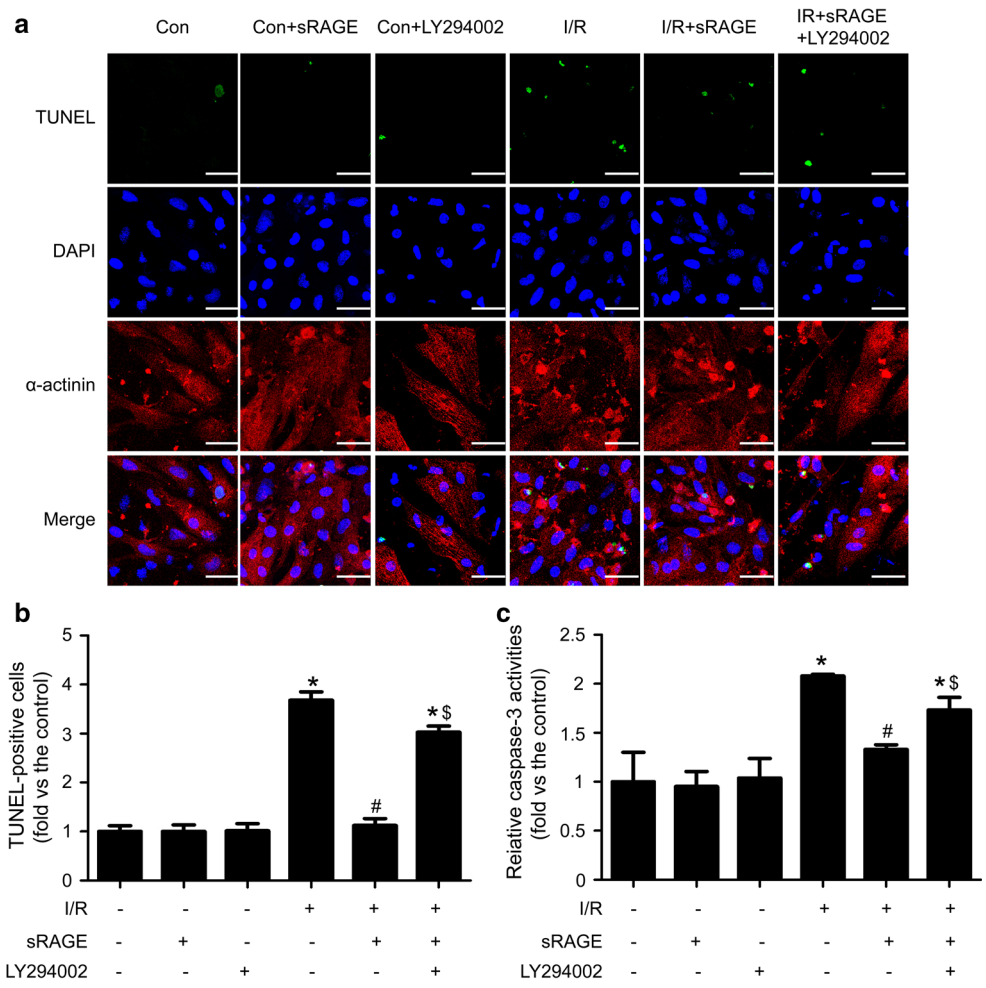


Fig. 7 Inhibition of PI3K abolishes effect of sRAGE inhibition on I/R-induced apoptosis in cardiomyocytes. **a** Representative photomicrographs of TUNEL-stained cardiomyocytes from con, con+sRAGE, con+LY294002, I/R, I/R+sRAGE and I/R+sRAGE+LY294002. TUNEL-positive nuclei (*green*), myoglobin (*red*), and DAPI (*blue*). *Scale bar*, 100 μm. **b** Quantitative analysis of TUNEL-positive cells was shown for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group, $^{\$}p < 0.05$ compared with I/R+sRAGE group. **c** The fold induction of caspase-3 activity relative to the control were analyzed to determine apoptosis for six groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group, $^{\$}p < 0.05$ compared with I/R+sRAGE group (Color figure online)

lowered by 50 % in the control+LY294002 group ($p < 0.05$; Fig. 9b, g); the protein expression of p-ERK was significantly decreased by 41 % in the control+PD95089 group ($p < 0.05$; Fig. 9c, i). Compared with I/R group, the ratios of p-AKT/AKT was significantly increased by 38 % in I/R+sRAGE group. Compared with I/R+sRAGE group, the ratio of p-AKT/AKT was *reduced* by 25 % in I/R+sRAGE+AG 490 group ($p < 0.05$; Fig. 9a, f). Compared with I/R group, the ratio of p-STAT3/STAT3 was significantly *elevated* by 150 % in I/R+sRAGE group. There was no difference in the ratio of p-STAT3/STAT3 between the I/R+sRAGE and I/R+sRAGE+LY294002 groups ($p > 0.05$; Fig. 9b, h).

Discussion

Myocardial metabolic disorders, contractile dysfunction, occurrence of arrhythmias, and structural remodeling are involved in the pathogenesis of myocardial I/R injury [32–34]. The present study showed that cardiac function was reduced, myocardial apoptosis was increased, and myocardial damage was aggravated following by I/R. However, this myocardial injury was significantly attenuated by sRAGE treatment. Our study showed that the protective effect of sRAGE on myocardial injury induced by I/R was associated with activation of the JAK2/STAT3 pathway, accompanied by a decrease in apoptosis. This mechanism of cardioprotection produced by sRAGE involving in the activation of the JAK2/STAT3 signaling pathway was shown by using the JAK2 inhibitor (AG 490), which eliminated the cardioprotective effect of sRAGE. To the best of our knowledge, this is the first report that links sRAGE cardioprotection with the JAK2/STAT3 signaling pathway.

Apoptosis is an important pathogenesis of myocardial I/R injury. Fliss et al. showed that ischemic and reperfused rat myocardium can undergo apoptosis and cell death [35]. These studies suggest that apoptosis is closely associated with myocardial I/R injury. Inhibition on apoptosis of cardiomyocytes can prevent the decrease of cells and attenuate cardiac injury induced by myocardial I/R. The

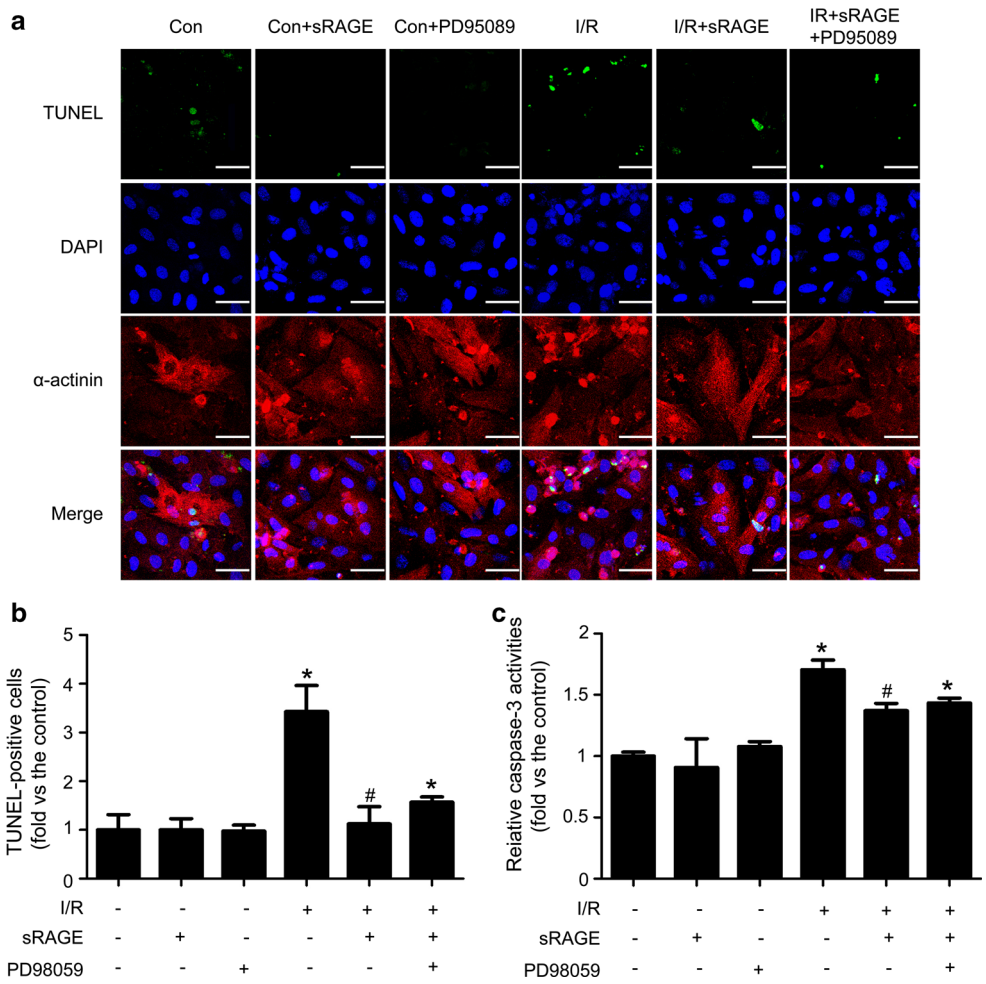
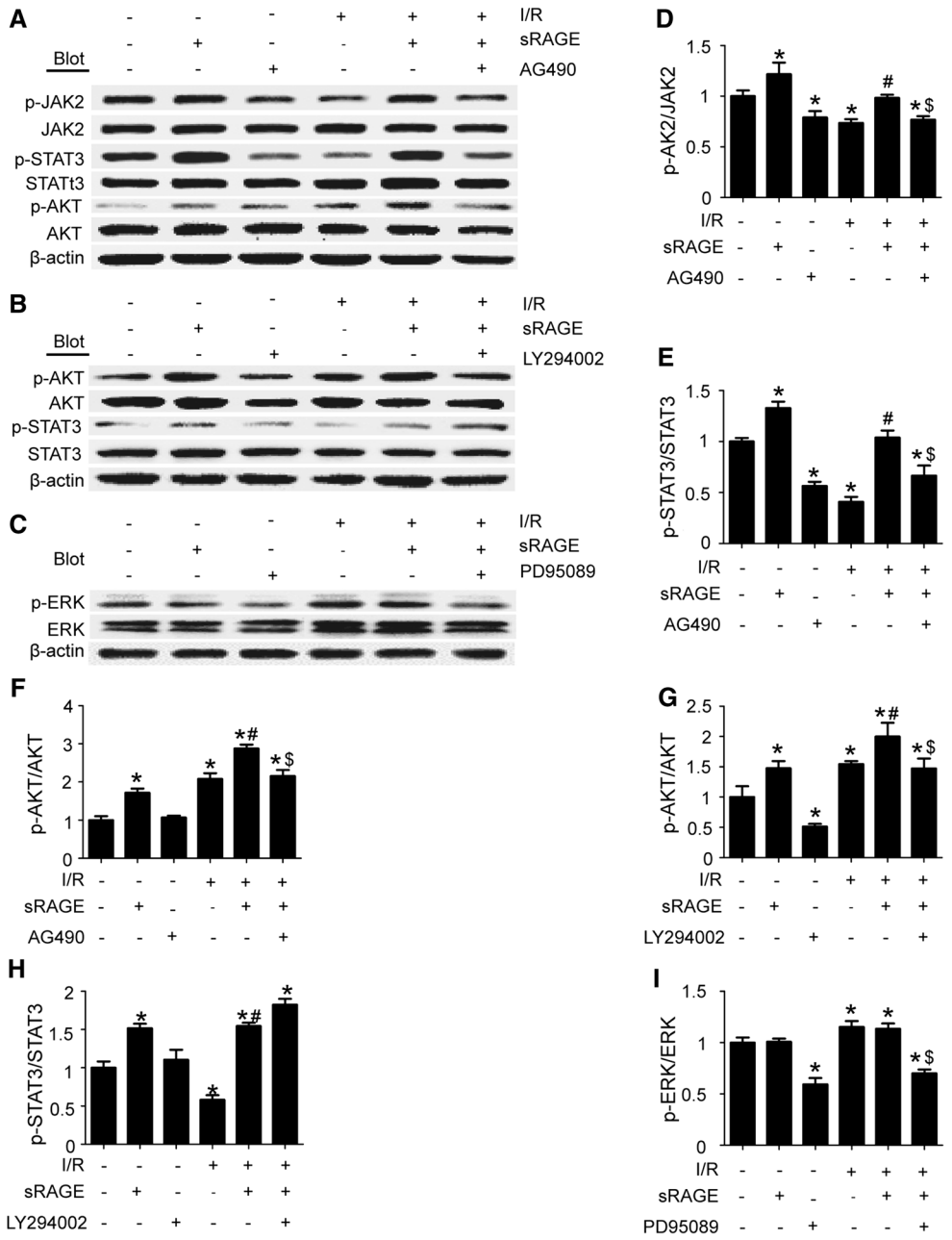


Fig. 8 Suppression of ERK 1/2 have no effect on sRAGE-induced reduction of apoptosis induced by I/R in cardiomyocytes. **a** Representative photomicrographs of TUNEL-stained cardiomyocytes from con, con+sRAGE, con+PD95089, I/R, I/R+sRAGE and I/R+sRAGE+PD98059. TUNEL-positive nuclei (*green*), myoglobin (*red*), and DAPI (*blue*). *Scale bar* 100 μm. **b** Quantitative analysis of TUNEL-positive cells was shown for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group. **c** The fold induction of caspase-3 activity relative to the control were analyzed to determine apoptosis for six groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group

present study showed that cardiac systolic dysfunction, an enlarged myocardial infarction area, an elevated number of TUNEL-positive cardiomyocytes and caspase-3 activity were induced by I/R. However, sRAGE treatment inhibited myocardial apoptosis, causing a decrease in the number of TUNEL-positive cardiomyocytes and caspase-3 activity. In addition, sRAGE improved cardiac function and attenuated myocardial infarction that was induced by I/R. These results indicated that the sRAGE-treated group was less vulnerable to I/R injury than the sham group.

The p53 protein occurs at low levels and inactive state under normal conditions in cells The expression of p53 is upregulated in the heart following by ischemia [36, 37]. Activation of p53 in response to ischemia is associated with an increase in its protein level and phosphorylation activity. Furthermore, Bax is involved in cytochrome c release from mitochondria and promotes apoptosis. Anti-apoptotic Bcl-2 disturbs mitochondrial cytochrome c release and suppress the progression of apoptosis [38, 39]. Therefore, the Bax/Bcl-2 ratio reflects the extent of apoptosis [40]. We further investigated the effect of sRAGE on p53, Bax, and Bcl-2 expression in vivo and in vitro after I/R injury. Western blotting showed that p53 and the Bax/Bcl-2 ratio was markedly increased by I/R, and this effect was inhibited by sRAGE. sRAGE significantly reduced the Bax/Bcl-2 ratio under normal conditions, indicating that sRAGE inhibited myocardial apoptosis partly via inhibiting p53 expression and Bax translocation to mitochondria, and upregulating Bcl-2 expression. Hattori and coworkers showed that the early phase of ischemic preconditioning is associated with JAK/STAT activation, accompanied by upregulation of Bcl-2 and downregulation of Bax [20, 41]. Activation of STAT3 is associated with anti-apoptotic signals through the induction of Bcl-2 [42]. Therefore, whether JAK2/STAT3 is involved in the anti-apoptotic effect of sRAGE needs to be investigated.

The JAK/STAT pathway plays a vital role in regulating the expression of genes by transducing signals received at the cell surface to the nucleus [43]. The present study showed that p-JAK2 and p-STAT3 protein levels were reduced in I/R alone group, however, the p-JAK2 and p-STAT3 protein levels were significantly increased in



sRAGE pretreatment I/R group accompanied by a reduced number of TUNEL-positive cardiomyocytes and caspase-3 activity. Blockade of an upstream regulator of STATs, by AG 490, resulted in these effects being inhibited and abolished. These data strongly support the notion that sRAGE mediates cardioprotection following by I/R, in part via the modulation of STAT3 phosphorylation in cardiomyocytes.

sRAGE functions as a "decoy" by binding RAGE ligands to abrogate cellular activation. In addition, sRAGE also has the capacity to regulate cytokines secretion, such as IFN-γ. The intracellular signaling pathways were activated by binding of the cytokines to cell surface receptor [44]. So we propose that the JAK2/STAT3 pathway was activated by sRAGE was mediated through upregulating cytokines secretion.

Some endogenous protein kinases that provide cardioprotection against myocardial I/R injury are increased at the early stage of myocardial reperfusion, including Akt and ERK 1/2 [45, 46]. In our study, as downstream effectors of JAK2/STAT3, LY294002 (PI3 K inhibitor) and PD98059 (ERK 1/2 inhibitor) were administrated to investigate the specific signaling pathway through which JAK2/STAT3 is activated. The data showed that the

◀**Fig. 9** The effect of AG 490, LY294002 and PD95089 on the protein expression of JAK2, STAT3, AKT and ERK. **a** Representative Western blots showed expression levels of total and phosphorylated JAK2, total and phosphorylated STAT3, total and phosphorylated AKT proteins from con, con+sRAGE, con+AG 490, I/R, I/R+sRAGE and I/R+sRAGE+AG 490. **b** Representative Western blots showed expression levels of total and phosphorylated AKT, total and phosphorylated STAT3 proteins from con, con+sRAGE, con+LY294002, I/R, I/R+sRAGE and I/R+sRAGE+LY294002. **c** Representative Western blots showed expression levels of total and phosphorylated ERK proteins from con, con+sRAGE, con+PD95089, I/R, I/R+sRAGE and I/R+sRAGE+PD95089. **d** The relative protein levels were quantified and was shown as the ratio of phosphorylated protein/total protein after normalized by β-actin from three independent experiments. The quantified intensity of protein bands was shown as the ratio of p-JAK2/JAK2 for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group, $^{\$}p < 0.05$ compared with I/R+sRAGE group. **e** The quantified intensity of protein bands was shown as the ratio of p-STAT3/STAT3 for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group, $^{\$}p < 0.05$ compared with I/R+sRAGE group. **f** The quantified intensity of protein bands was shown as the ratio of p-AKT/AKT for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group, $^{\$}p < 0.05$ compared with I/R+sRAGE group. **g** The quantified intensity of protein bands was shown as the ratio of p-AKT/AKT for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group, $^{\$}p < 0.05$ compared with I/R+sRAGE group. **h** The quantified intensity of protein bands was shown as the ratio of p-STAT3/STAT3 for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group. **i** The quantified intensity of protein bands was shown as the ratio of p-ERK/ERK for different groups. Values are expressed as mean ± SEM for three independent experiments. $^{*}p < 0.05$ compared with Con group, $^{\#}p < 0.05$ compared with I/R group, $^{\$}p < 0.05$ compared with I/R+sRAGE group

percentage of TUNEL-positive cardiomyocytes and the caspase-3 activity were significantly higher in I/R+sRAGE+LY294002 group than in I/R+sRAGE group, indicating that the protective effect of sRAGE against I/R injury was abolished in part by the PI3K inhibitor LY294002. However, PD98059 showed no effect of inhibition with sRAGE-induced cardioprotection. One possible interpretation for this finding is that the JAK/STAT pathway operates upstream of the endogenous protein kinases cascade. Evidence has suggested that JAK2 signaling regulates activation of the PI3K/Akt pathway [47], which is supported by the present study.

In summary, sRAGE performs cardioprotection via activation of endogenous phosphorylation of JAK2 and STAT3, which may in turn regulates the PI3K/Akt pathway. The cardioprotective effects of sRAGE are blocked in the presence of AG 490 and AKT inhibitor LY294002. This suggests that sRAGE protects cardiomyocytes from apoptosis induced by I/R in in vitro and in vivo models by activating the JAK2/STAT3 signaling pathway. This novel mechanism of sRAGE-induced cardioprotection may be beneficial for development of therapeutic application of this drug in limiting myocardial infarction and inhibiting apoptosis following I/R.

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Conflict of interest We have no competing interest and conflict interest.

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