



Original article

Soluble receptor for advanced glycation end-products inhibits ischemia/reperfusion-induced myocardial autophagy via the STAT3 pathway

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ARTICLE INFO

Keywords:

I/R
Myocardium
Autophagy
SRAGE
STAT3

ABSTRACT

The pathogenesis of myocardial ischemia/reperfusion (I/R) is poorly understood, but recent evidence suggests that autophagy plays crucial roles in I/R injuries. Soluble receptor for advanced glycation end-products (sRAGE) exerts protective effects during I/R by decreasing cardiac apoptosis, which is mediated via increasing the ubiquitin proteasome system (UPS) and signal transducer and activator of transcription 3 (STAT3). The present study examined the effects and mechanisms of sRAGE on I/R-triggered cardiac autophagy. I/R was performed in mice or primary neonatal cardiomyocytes with or without sRAGE administration or overexpression. Cardiac function and infarct size were detected in mouse hearts. Apoptosis, autophagy and autophagy-related signaling pathways were detected in mouse hearts and cardiomyocytes. The results demonstrated that sRAGE significantly improved cardiac function and reduced infarct size during I/R in mice. sRAGE inhibited I/R-induced apoptosis, which correlated with a reduction in autophagy-associated proteins, including ATG7, Beclin-1 and microtubule-associated protein 1 light chain 3 (LC3). sRAGE reduced autophagosome formation during I/R *in vivo* and *in vitro*. sRAGE significantly activated STAT3, but not mammalian target of rapamycin (mTOR), during I/R *in vivo* and *in vitro*, and suppression of STAT3 abolished the sRAGE inhibition of autophagy during I/R *in vitro*. Activation of autophagy using ATG7 overexpression with an adenovirus significantly abolished the sRAGE-induced reduction of cardiac apoptosis during I/R. These results suggest that sRAGE inhibits I/R injuries in the heart via a decrease in autophagy, a process that is dependent on STAT3 activation.

1. Introduction

Myocardial ischemia/reperfusion (I/R) injury is defined as the exacerbation of tissue injury following the reperfusion of an ischemic heart. I/R is a complex process that involves oxidative stress, mitochondrial dysfunction and the induction of autophagy dysfunction [1–3]. Autophagy is an important process for the degradation of long-

lived proteins and organelles [4]. Basic autophagy in normal cells is essential to maintain normal protein and organelle functions in heart [5,6], and a high AMP/ATP ratio, inhibition of mTOR [7,8] and the up-regulation of Beclin-1 in I/R condition further activates autophagy [7], which is critical for cell survival via the reuse of material (free fatty acids and amino acids) [9]. However, autophagy during I/R is not always protective, and excessive autophagy promotes the progressive

Abbreviations: 3-MA, 3-methyladenine; AAR, Area at risk; ADAM10, A disintegrin and metalloproteases 10; AGEs, Advanced glycation end-products; AKT, Protein kinase B; AMP, Adenosine monophosphate; ATG, Autophagy related; ATP, Adenosine triphosphate; Bcl-2, B-cell lymphoma 2; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle medium; EF, Ejection fraction; FOXO1, Forkhead box O1; FS, Fraction shortening; I/R, Ischemia/reperfusion; JAK2, Janus kinase 2; JNK, c-Jun N-terminal kinase; LAD, Left anterior descending; LC3, Microtubule-associated protein 1 light chain 3; mTOR, Mammalian target of rapamycin; PBS, Phosphate buffer saline; RAGE, Receptor for advanced glycation end-products; sRAGE, Soluble receptor for advanced glycation end-products; STAT3, Signal transducer and activator of transcription 3; TTC, 2,3,5-Triphenyltetrazolium chloride; TEM, Transmission electron microscopy; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; UPS, Ubiquitin proteasome system

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Received 6 August 2018; Received in revised form 20 October 2018; Accepted 22 October 2018

Available online 25 October 2018

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consumption of cellular constituents and leads to autophagic cell death [10]. Therefore, suppression of detrimental autophagy may be a target for myocardial I/R injuries.

sRAGE is the soluble form of RAGE, which is expressed on the surface of many cells, such as endothelial cells [11], pulmonary alveoli cells [12] and cardiomyocytes [11]. sRAGE is generated via ADAM10 cleavage of the extracellular regions of RAGE or direct release from the cells [13]. I/R induces RAGE and its ligands, such as AGEs, in cardiomyocytes, which leads to severe myocardial injuries via activation of signaling pathways downstream of RAGE, such as JNK [14,15]. However, sRAGE binds to the ligands of RAGE as a decoy and inhibits activation of the RAGE axis [14,15]. Our previous studies reported that sRAGE exerted protective effects during I/R in mouse heart via inhibition of myocardial apoptosis and inhibition of cytochrome *c* release [16], activation of the JAK2/STAT3 signaling pathway [17] and up-regulation of UPS [18]. Inhibition of autophagy suppresses cardiomyocyte apoptosis during I/R [19,20], which may be mediated via activation of STAT3 and UPS [21,22]. Therefore, sRAGE may protect hearts against I/R injuries via inhibition of autophagy in cardiomyocytes, which promotes cell survival and inhibits apoptosis.

The present study examined infarct size and apoptosis in I/R mice treated with sRAGE. I/R-induced autophagy was also examined before and after sRAGE treatment *in vivo* and *in vitro*. The results demonstrated that sRAGE markedly attenuated I/R-induced infarct size, cardiac dysfunction and cardiac apoptosis, which were accompanied with reduced autophagy. Activation of autophagy significantly abolished the protective effect of sRAGE during myocardial I/R. *In vitro* studies further demonstrated that the mechanisms underlying these actions were associated with STAT3 activation rather than mTOR signaling pathways.

2. Materials and methods

2.1. I/R injury model in mice

Adult male C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and randomly divided into four groups: sham group, which received saline injections into the myocardium without LAD ligation; sRAGE group, which received sRAGE injections into the myocardium without LAD ligation; I/R group, which received saline injections into the myocardium and LAD ligation; and I/R + sRAGE group, which received sRAGE injections into the myocardium and LAD ligation. A recombinant protein was purified from *E. coli* expressing the human sRAGE gene. Recombinant sRAGE was injected into 5 regions of the myocardium in the peri-infarct zone using a syringe attached to a 30-gauge needle just prior to ligation at a dose of 1 µg/10 g body weight [17]. I/R injuries were performed in mice as previously described [23]. Briefly, mice were anesthetized with isoflurane, and the heart was exposed from the fourth intercostal space. The LAD was ligated using 7–0 silk for 30 min (ischemia) and released for 24 h (reperfusion). Mice in the sham group underwent the same surgery without ligation. Mice were sacrificed after reperfusion, and the hearts were preserved at –80 °C for further analysis. The Institutional Animal Care and Use Committee of Capital Medical University approved all investigations, which conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2. I/R induction and adenovirus infection in cardiomyocytes

Sprague-Dawley rats born within 48 h were used for the culture of primary neonatal cardiomyocytes. The rats were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The methods for cardiomyocyte isolation were performed as described previously [24]. Recombinant adenovirus expressing GFP or a sequence of the extracellular domain of human RAGE was used to infect cardiomyocytes. Isolated cardiomyocytes were infected with adenovirus for

at least 24 h prior to stimulation with I/R. Cultured cardiomyocytes were treated with an “ischemia buffer” (pH 6.3), which included (mmol L⁻¹) 118 NaCl, 24 NaHCO₃, 1.0 NaH₂PO₄, 2.5 CaCl₂·2H₂O, 1.2 MgCl₂, 20 sodium lactate, 16 KCl and 10 deoxyglucose, for 2 h in an atmosphere of 5% CO₂ and 95% N₂ at 37 °C (ischemia). The “ischemia buffer” was removed, and the cells were washed once using PBS. DMEM was added to the cells at the onset of reperfusion and incubated with cells for 24 h in an atmosphere of 5% CO₂ and 95% O₂ at 37 °C.

2.3. Echocardiographic measurement of cardiac function in mice

Cardiac function was measured using the Vevo 2100 imaging system (Visual Sonics Inc., Toronto, Ontario, Canada). Mice were anesthetized using tribromoethanol via an intraperitoneal injection (100 mg kg⁻¹, Sigma-Aldrich, St. Louis, MO, USA). EF% and FS% were calculated at M-mode to evaluate cardiac function.

2.4. Infarct size assessment

Myocardial infarct size was determined using Evans-Blue/TTC (Sigma-Aldrich, St. Louis, MO, USA) double staining. Evans-Blue (1%, wv⁻¹) (Sigma-Aldrich, St. Louis, MO, USA) was injected from the end of the aortic arch into the coronary arteries. The heart was incubated in a 1% (wv⁻¹) TTC solution in 37 °C surroundings for 3–5 min and fixed in 4% (wv⁻¹) paraformaldehyde for 20 min. TTC and Evans-Blue were dissolved in PBS. The area of blue coloring represented viable tissue, and white represented the infarct size. The area of red plus white represented AAR. The hearts were sectioned transversely, and photographs were obtained using a digital camera. Image J software was used to quantify the area of each region on every section. Each region (red, white or blue) of every heart was summed, and the infarct size is expressed as a percentage of the area of AAR.

2.5. Determination of autophagy flux in cardiomyocytes

Adenovirus expressing mRFP-GFP-LC3 was used to assess the autophagy flux in cardiomyocytes during I/R, as described previously [25]. Briefly, yellow and red dots indicated the cellular autophagosome and autophagolysosome, respectively, and green dots indicated the total LC3 expressed in the cells. Cardiomyocytes were infected with mRFP-GFP-LC3 adenovirus for at least 24 h prior to I/R stimulation with or without sRAGE treatment.

As 900-ng/mL dose of sRAGE produced a significantly protective effect during I/R in cardiomyocytes without cytotoxicity [17]. Therefore, recombinant sRAGE protein was added to the medium (at a dose of 900 ng mL⁻¹) 30 min prior to I/R stimulation. Cardiomyocytes were fixed at the end of reperfusion using 4% (wv⁻¹) paraformaldehyde for 15 min and counterstained with DAPI to identify nuclei. Confocal fluorescence microscopy (TCS SP8 MP, Leica, Buffalo groove, Illinois, USA) was used to observe the LC3 puncta in cells. Red spots (autophagosomes) and yellow spots (autolysosomes) were analyzed using Image J software (NIH). The results are expressed as the number of red spots and yellow spots in cells, and at least 1000 cells were analyzed.

2.6. Evaluation of autophagy using TEM

Sample preparation for TEM was described previously [26]. Briefly, heart tissues or cell pellets were fixed using 3% (v⁻¹) glutaraldehyde (dissolved in PBS) for at least 48 h at 4 °C and post-fixed in osmium tetroxide. Samples were dehydrated in a graded series of alcohol concentrations, embedded in epoxy resins and sectioned. Sections were observed, and photographs were obtained using a TEM (H7650; Hitachi, Tokyo, Japan).

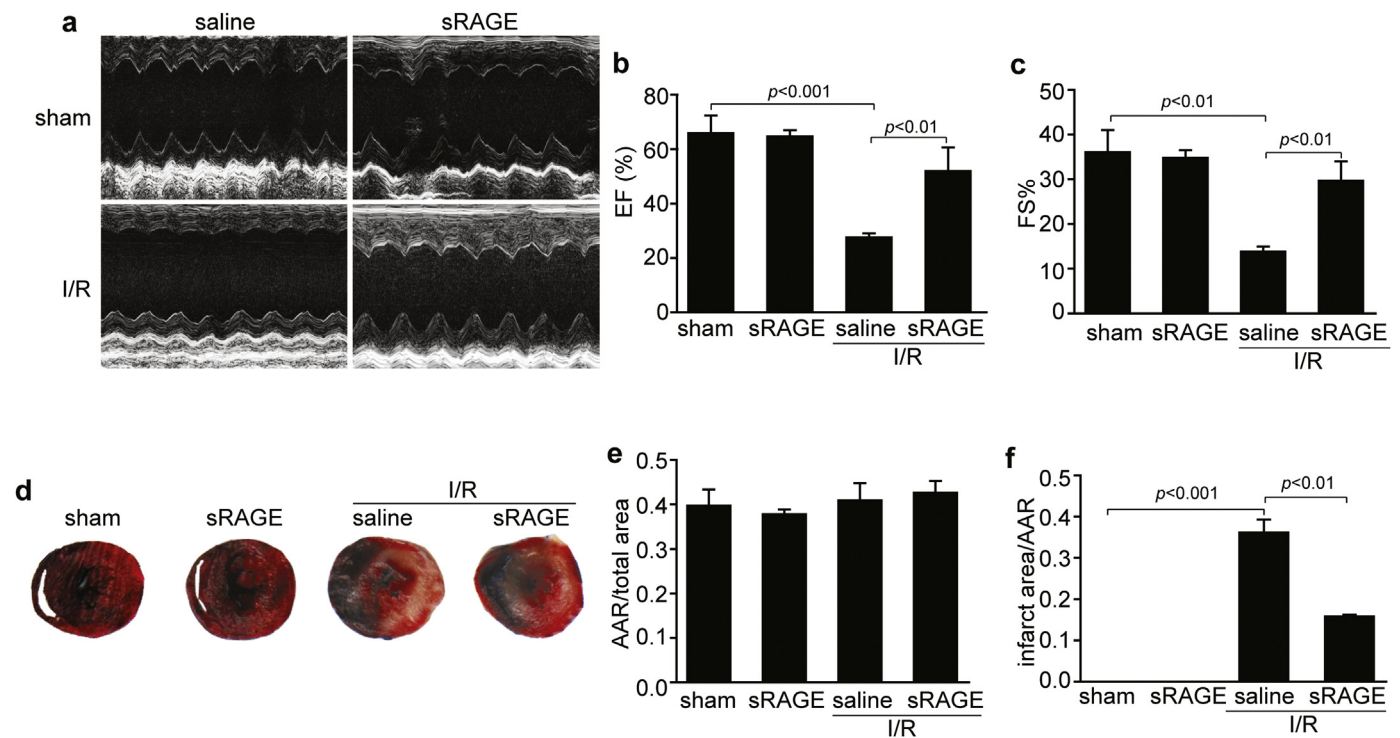


Fig. 1. sRAGE attenuated cardiac dysfunction and reduced infarct size in I/R mice. Mice received ischemia for 30 min and reperfusion for 24 h. Recombinant sRAGE protein (1 $\mu\text{g}/10\text{ g}$) or saline was injected into the myocardium prior to ischemia. Mouse cardiac function was measured using echocardiography. Infarct size was measured using TTC and Evans-Blue double staining. (a) The M-mode of echocardiography for each group. (b) Quantification of the left ventricular ejection fraction (EF%) for each group. (c) Quantification of fractional shortening (FS%) for each group. (d) Images of TTC and Evans-Blue double staining for each group. (e) Quantification of the AAR/total area ratio for each group. (f) Quantification of the infarct area/AAR ratio for each group. $n = 6/\text{group}$. Values are expressed as the mean $\pm$ s.e.m.

2.7. Evaluation of apoptosis using the TUNEL assay

Hearts were fixed in formalin overnight. Tissue was dehydrated in a graded series of alcohol concentrations and saturated and embedded with paraffin. Tissues were sectioned at a thickness of 5 μm . Sections were incubated in 0.1% (v/v^{-1}) Triton X-100 for 15 min at 4 $^{\circ}\text{C}$ and blocked using 3% (w/v^{-1}) bovine serum albumin for 1 h at room temperature. TUNEL staining was performed using the In Situ Cell Death Detection Kit, TMR red (Roche, Mannheim, German) according to manufacturer's instructions. Briefly, each section was incubated in 50 μL of a TUNEL mixture that included 5 μL of TUNEL enzyme and 45 μL of TUNEL labeling containing fluorescein isothiocyanate-conjugated dUTP at 37 $^{\circ}\text{C}$ for 60 min. The cytoskeleton was stained using an α -actinin antibody (Sigma-Aldrich, St. Louis, MO, USA) at 4 $^{\circ}\text{C}$ overnight. Sections were counterstained using DAPI. Photographs were obtained using an Olympus BX51 fluorescence microscope (Olympus America Inc., Center Valley, PA, USA) at 20X magnification. The number of TUNEL-positive cells and total cells were counted in each field using Image J software (NIH). The percentage of TUNEL-positive cells to total cells was calculated.

2.8. Western blot analyses

Heart tissue or cardiomyocytes were lysed using lysis buffer. Total proteins (40 μg) were separated using SDS-PAGE and transferred to PVDF membranes. Membranes were blocked using 5% (w/v^{-1}) skim milk at room temperature for 60 min and incubated with primary antibodies at 4 $^{\circ}\text{C}$ overnight. Membranes were incubated with a horseradish peroxidase-conjugated IgG antibody for 1 h at room temperature and exposed to a super signal chemiluminescence detection kit (Thermo Scientific, Waltham, MA, USA). The immunoblots were visualized using Chemiluminescent and Fluorescent Imaging Systems (ChangChem; SAGECREATION, Beijing). Image J software (NIH) was used for densitometry. GAPDH and β -actin were used to verify equal protein loading.

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2.9. Antibodies and reagents

Antibodies against ATG5, ATG7, caspase-3, Bcl-2, Beclin-1, phosphorylated AKT, AKT, phosphorylated mTOR, mTOR, phosphorylated STAT3, and STAT3 were purchased from Cell Signaling, Inc. (Danvers, MA, USA). The LC3A/B antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant adenoviruses expressing GFP alone (Ad-GFP), sRAGE (Ad-sRAGE) and ATG7 (Ad-ATG7) were purchased from Hanbio Co., Ltd. (Shanghai, China). Adenoviruses expressing mRFP-GFP-LC3 was purchased from Hanbio Co., Ltd. (Shanghai, China). Recombinant sRAGE protein was purchased from Aidi Bo biological Ltd. (Beijing, China). Stattic for STAT3 inhibition was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.10. Statistics analysis

The number of experiments is shown in the figure legends. All values are expressed as the mean $\pm$ s.e.m. The data were analyzed using the parametric test one-way analysis of variance (ANOVA) and least significant difference t -tests in SPSS v20.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered significant.

3. Results

3.1. sRAGE inhibited myocardial injuries during I/R

To explore the effects of sRAGE on cardiac injuries during myocardial I/R, cardiac function, infarct size and apoptosis were measured.

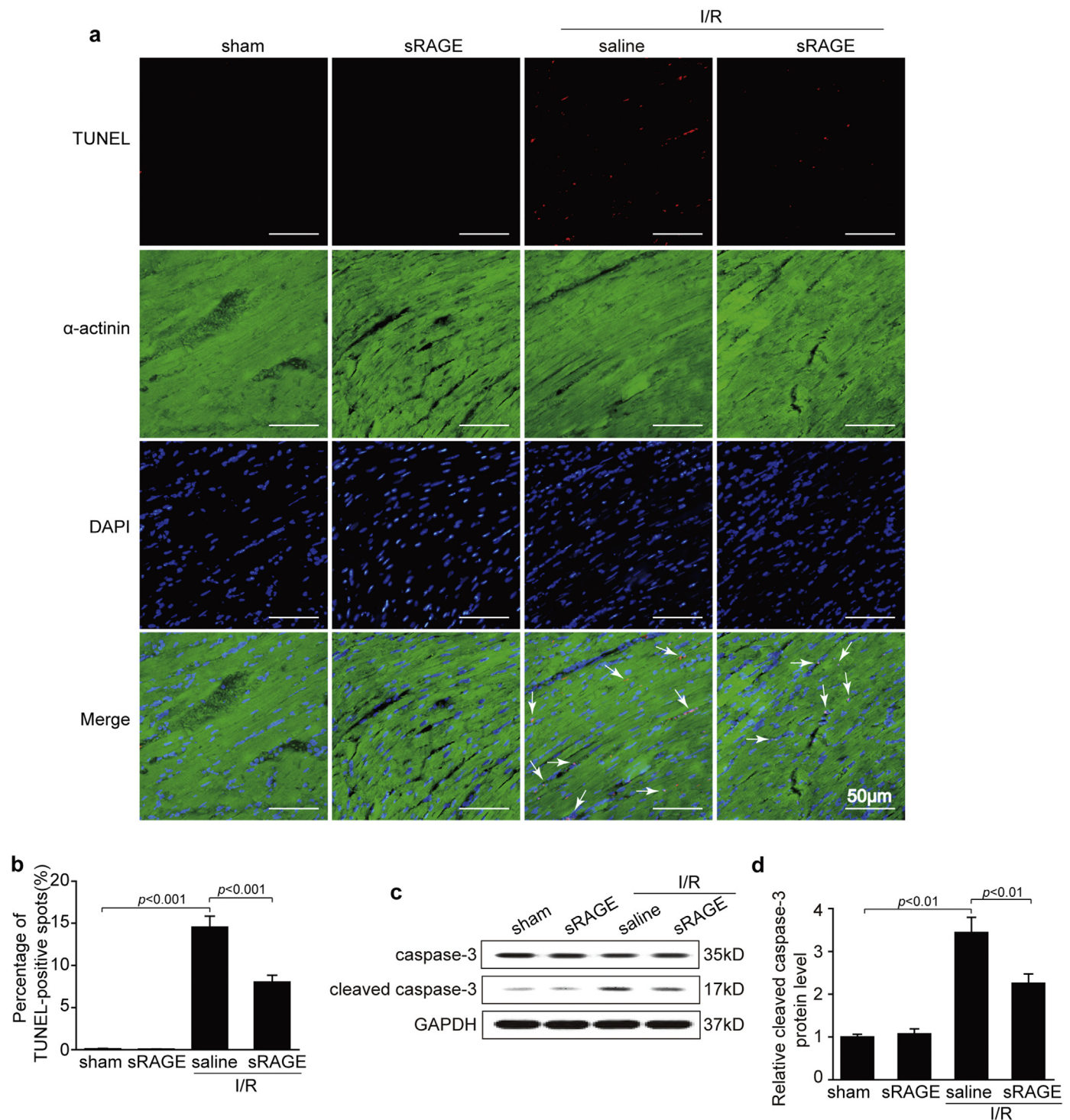


Fig. 2. sRAGE inhibited I/R-induced myocardial apoptosis in mice. Mice were treated as in Fig. 1a. (a) Representative image of TUNEL staining in each group. The red spots (TUNEL) indicate TUNEL-positive spots. The blue (DAPI) fluorescence indicates nuclei. The green fluorescence indicates α -actinin antibody staining. The white arrows indicate apoptosis spots (at $\times 20$ magnification). Scale bar: 50 μ m. (b) Quantitative analyses of the percentage of TUNEL-positive spots in each group. (c) Representative images of Western blots of caspase-3 and cleaved caspase-3 in heart tissues of each group. (d) Relative expression of cleaved caspase-3 in I/R mice with or without sRAGE treatment. $n = 6$ /group. All values are expressed as the mean $\pm$ s.e.m.

EF% and FS% decreased in I/R mice compared to sham mice. sRAGE significantly increased EF% from $27.60 \pm 1.47\%$ to $52.06 \pm 8.72\%$ and increased FS% from $13.82 \pm 1.41\%$ to $29.65 \pm 4.39\%$ during I/R ($p < 0.01$, Fig. 1a–c). sRAGE reduced the infarct size/AAR ratio from 0.36 ± 0.03 – 0.15 ± 0.04 in I/R mice ($p < 0.01$, Fig. 1d, f). TUNEL-positive cells and cleaved caspase-3 increased during I/R, and sRAGE reduced these increases (Fig. 2). These results indicate that sRAGE

significantly inhibited I/R-induced cardiac injuries.

3.2. sRAGE inhibited myocardial autophagy during I/R in vivo

Autophagy-associated proteins, including ATG5, ATG7, Beclin-1 and LC3-II, were detected using Western blotting to investigate the effects of sRAGE on autophagy during I/R *in vivo*. The expression of

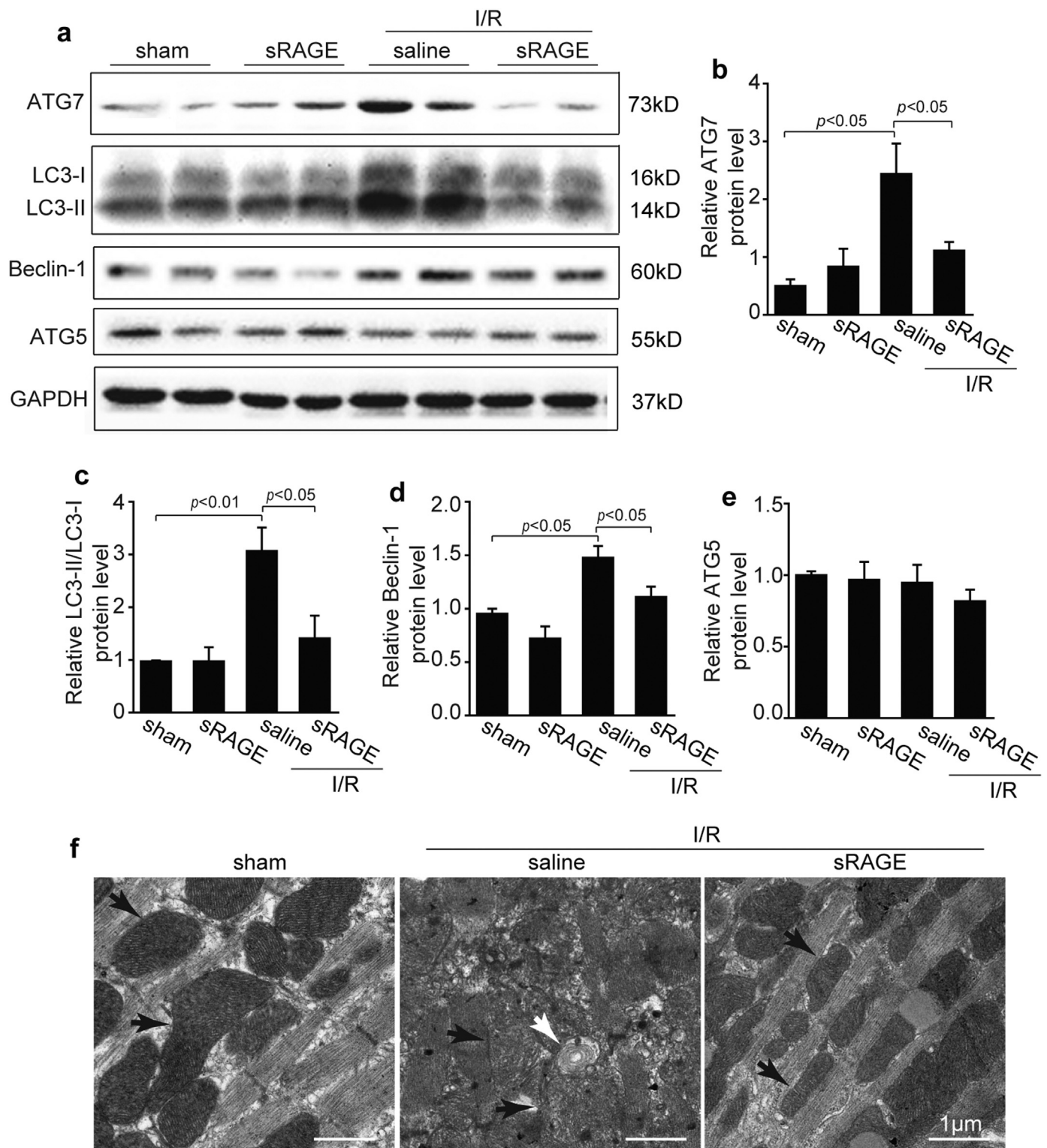


Fig. 3. sRAGE inhibited I/R-induced autophagy *in vivo*. Mice were treated as in Fig. 1a. (a) Representative images of Western blots of ATG5, ATG7, Beclin-1 and LC3-II in mouse hearts of each group. (b) Relative expression of ATG7 in I/R mice with or without sRAGE treatment. (c) Relative expression of LC3-II/LC3-I in I/R mice with or without sRAGE treatment. (d) Relative expression of Beclin-1 in I/R mice with or without sRAGE treatment. (e) Relative expression of ATG5 in I/R mice with or without sRAGE treatment. (f) Representative images of transmission electron microscope (TEM) detecting the ultrastructure of I/R mouse heart with or without sRAGE treatment. The black arrows indicate mitochondria. The white arrows indicate autophagosomes. $n = 6/\text{group}$. All values are expressed as the mean $\pm$ s.e.m.

ATG7, Beclin-1 and LC3-II increased during I/R compared to sham mice, and sRAGE decreased these increases ($p < 0.05$, Fig. 3a–d). However, these results indicated that sRAGE did not affect ATG5 expression ($p > 0.05$, Fig. 3a, e). TEM revealed that autophagosomes

(white arrow) and swelling mitochondria (black arrows) in I/R mouse hearts (Fig. 3f). However, sRAGE significantly decreased the appearance of autophagosomes and deformed mitochondria in I/R-treated hearts (Fig. 3f). These results suggest that sRAGE inhibited I/R-induced

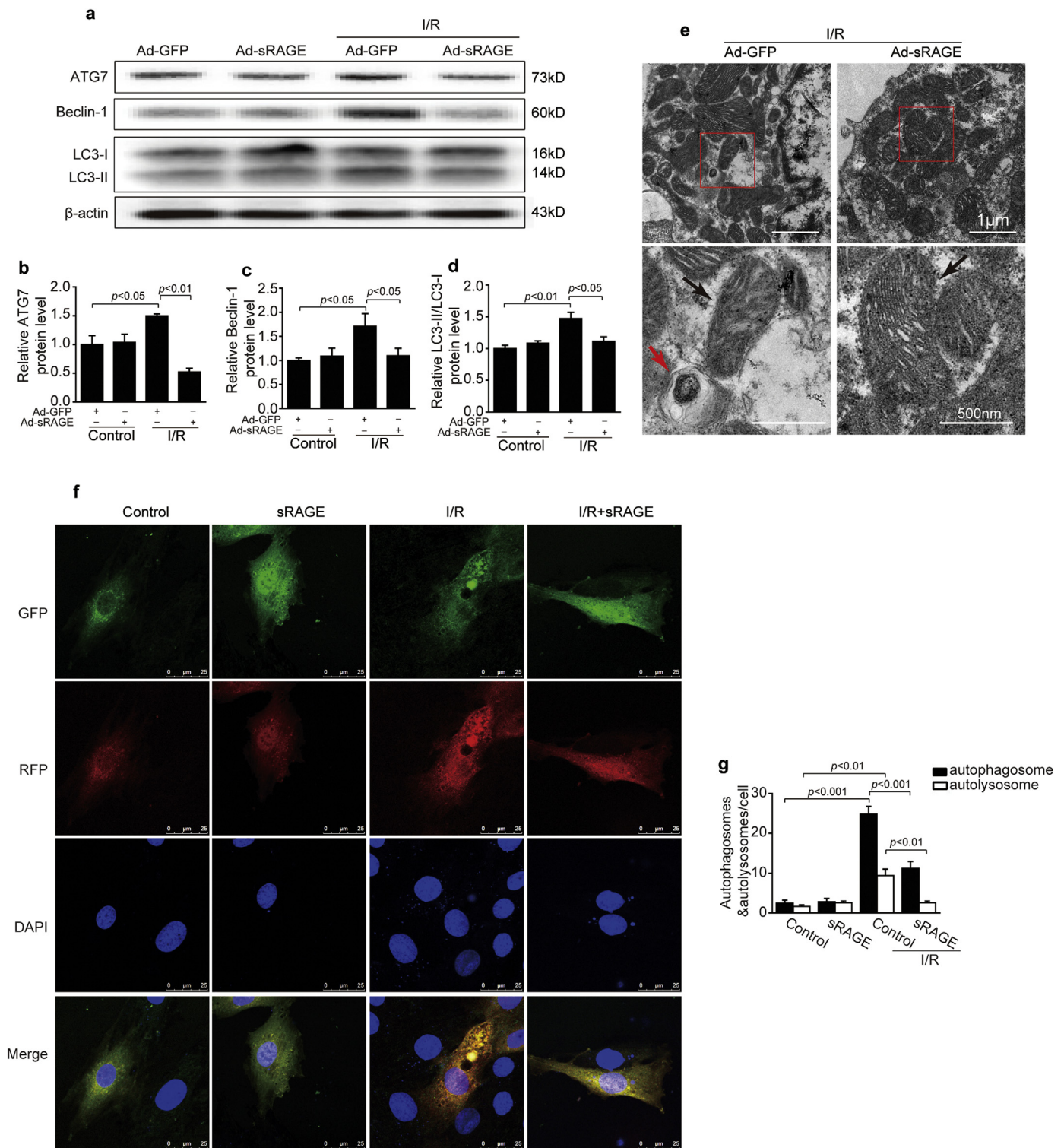


Fig. 4. sRAGE inhibited I/R-induced autophagy *in vitro*. Neonatal rat ventricular cardiomyocytes were infected with adenovirus control (Ad-GFP) or sRAGE (Ad-sRAGE) for 24 h and stimulated with I/R for 24 h. (a) Representative images of Western blots of ATG7, Beclin-1 and LC3-II in cardiomyocytes of each group. (b) Relative expression of ATG7 in I/R cardiomyocytes with or without sRAGE overexpression. (c) Relative expression of Beclin-1 in I/R cardiomyocytes with or without sRAGE overexpression. (d) Relative expression of LC3-II/LC3-I in I/R cardiomyocytes with or without sRAGE overexpression. (e) Representative images of transmission electron microscope (TEM) detecting the ultrastructure of I/R cardiomyocytes with or without sRAGE overexpression. The black arrows indicate mitochondria. The red arrows indicate autophagosomes. (f) Representative images of autophagy flux in each group. Cardiomyocytes were transfected with mGFP-RFP-LC3 adenovirus for 24 h. Recombinant sRAGE protein (900 ng mL^{-1}) was added to the media 30 min before I/R. The red spots indicate autophagosomes. The yellow spots indicate autolysosomes. (g) Quantitative analyses of the number of red (autophagosomes) and yellow (autolysosomes) spots in cardiomyocytes. $n = 5/\text{group}$. All values are expressed as the mean $\pm$ s.e.m.

autophagy in mouse hearts.

3.3. sRAGE inhibited myocardial autophagy during I/R *in vitro*

Neonatal rat cardiomyocytes were used to confirm the effects of sRAGE on autophagy during I/R *in vitro*. Cardiomyocytes were infected with adenovirus expressing sRAGE (Ad-sRAGE) or control (Ad-GFP) and treated with I/R. The results demonstrated that I/R increased ATG7, Beclin-1 and LC3-II in cardiomyocytes compared to Ad-GFP control, and sRAGE overexpression dramatically suppressed I/R-induced ATG7, Beclin-1 and LC3-II expression in cardiomyocytes (Fig. 4a–d). TEM indicated that sRAGE overexpression reduced the formation of autophagosomes (red arrow) during I/R in cardiomyocytes. sRAGE overexpression also improved the I/R-induced swollen and vesiculated mitochondria (black arrows) in cardiomyocytes (Fig. 4e). Autophagy flux was detected in cardiomyocytes during I/R using an mRFP-GFP-LC3 adenovirus. The quantity of red fluorescence spots (representing autolysosome formation) and yellow fluorescence spots (representing autophagosome formation) increased during I/R, and sRAGE overexpression inhibited these increases (Fig. 4f, g). These results indicate that sRAGE overexpression inhibited I/R-triggered autophagy in cardiomyocytes.

3.4. sRAGE did not affect activation of the mTOR signaling pathway *in vivo* or *in vitro*

mTOR negatively modulates autophagy, and AKT positively regulates mTOR [27]. Therefore, mTOR and AKT were detected in hearts using Western blotting. AKT phosphorylation increased during I/R compared to sham mice, and sRAGE reduced this increase ($p < 0.05$, Fig. 5a, b). mTOR was activated during I/R in mouse heart, but sRAGE did not affect mTOR activation during I/R ($p > 0.05$, Fig. 5a, c). Phosphorylated STAT3 decreased significantly in I/R mice compared to sham mice, and sRAGE dramatically reversed this decrease ($p < 0.01$, Fig. 5a, d). The AKT/mTOR signaling pathway was also detected during I/R in cardiomyocytes before and after sRAGE overexpression. The results indicated that sRAGE overexpression abolished I/R-induced AKT phosphorylation ($p < 0.05$, Fig. 5e, f). However, sRAGE overexpression did not reverse the reduction of phosphorylated mTOR in I/R cardiomyocytes ($p > 0.05$, Fig. 5e, g). These results suggest that sRAGE does not influence mTOR activation during I/R *in vivo* or *in vitro*.

3.5. The STAT3 inhibitor stattic abolished sRAGE inhibition of autophagy during I/R

sRAGE significantly activated STAT3 during I/R. Therefore, a STAT3 inhibitor was used to further confirm the function of STAT3 on the preventative effects of sRAGE on I/R-induced autophagy. The STAT3 inhibitor stattic was added to the media ($2 \mu\text{mol L}^{-1}$) 30 min before I/R stimulation. Stattic significantly inhibited sRAGE overexpression-induced STAT3 activation during I/R in cardiomyocytes ($p < 0.05$, Fig. 6a, b). Stattic abolished the sRAGE overexpression-induced reduction of ATG7, Beclin-1 and LC3-II in cardiomyocytes during I/R (Fig. 6a, c–e). I/R inhibited Bcl-2 in cardiomyocytes, and sRAGE enhanced Bcl-2. However, stattic pretreatment reversed the increase in Bcl-2 following sRAGE overexpression during I/R ($p < 0.01$, Fig. 6a, f). TEM revealed more autophagosomes and deformed mitochondria in I/R cells treated with stattic and sRAGE overexpression compared to I/R cells with sRAGE overexpression alone (Fig. 7a). Stattic pretreatment of I/R cells overexpressing sRAGE significantly induced more autophagosomes and autolysosomes in cardiomyocytes compared to I/R cells treated with sRAGE overexpression alone (Fig. 7b, c). These results suggest that the inhibition of STAT3 abolished the sRAGE suppression of I/R-induced autophagy in cardiomyocytes.

3.6. Activation of autophagy abolished the protective effect of sRAGE on I/R-induced myocardial apoptosis

ATG7 was overexpressed in cardiomyocytes using adenovirus ATG7 (Ad-ATG7) to activate autophagy and to examine whether the cardioprotective effects of sRAGE on I/R injuries was related to autophagy inhibition. Ad-ATG7 dramatically up-regulated ATG7 expression ($p < 0.001$, Fig. 8c, d). ATG7 overexpression significantly abolished the sRAGE-induced reduction of TUNEL-positive cardiomyocytes during I/R ($p < 0.001$, Fig. 8a, b). ATG7 overexpression also reversed sRAGE inhibition of cleaved caspase-3 during I/R ($p < 0.05$, Fig. 8c, e). These data indicate that activation of autophagy abolished the protective effects of sRAGE on I/R-induced apoptosis.

4. Discussion

The present study demonstrated that sRAGE inhibited I/R-triggered myocardial autophagy *in vivo* and *in vitro*. sRAGE reduced the I/R-induced expression of ATG7 and Beclin-1 and the conversion of LC3-I to LC3-II in mouse hearts and in cultured neonatal rat cardiomyocytes. sRAGE also improved mitochondrial morphology and reduced the formation of autophagosomes during I/R in cardiomyocytes and mouse hearts. However, sRAGE inhibited myocardial autophagy via the STAT3, rather than mTOR, signaling pathway during I/R. sRAGE increased STAT3 phosphorylation, and the inhibition of STAT3 using stattic abolished the sRAGE-induced suppression of ATG7, Beclin-1 and LC3-II in cardiomyocytes during I/R. Stattic inhibition of STAT3 reversed the sRAGE inhibition of autophagic structures, mitochondrial swelling and autophagy flux during I/R. The present study is the first report that sRAGE antagonizes I/R injuries in heart via the inhibition of autophagy, which was dependent on the STAT3 pathway.

Previous studies reported a complicated crosslink between apoptosis and autophagy [28,29], which was responsible for irreversible myocardial damage following I/R in myocardial cells [30]. The present study demonstrated that sRAGE improved the I/R-induced cardiac dysfunction, infarct size and apoptosis induced *in vivo* and *in vitro* (Figs. 1 and 2), which is consistent with previously reported results [17,18]. Notably, sRAGE also inhibited autophagy after 24-h reperfusion *in vivo* and *in vitro* (Figs. 3 and 4), which was demonstrated as reductions in ATG7, Beclin-1 and LC3-II expression. Therefore, sRAGE may protect heart from I/R injuries via autophagy reduction. Numerous studies reported that the activation of autophagy protected heart from I/R-induced apoptosis [31,32], but autophagy is a double-edged sword in the pathology of I/R [33,34]. Excessive autophagy is often observed in dying cells, which may be a trigger for cardiomyocyte apoptosis [35,36]. Inhibition of autophagy using 3-MA or Beclin-1 siRNA during I/R suppressed cardiomyocytes death [35], which indicated that the vigorous autophagy in I/R was critical to the induction of myocardial injuries in ischemia heart diseases.

Notably, the effects of autophagy on I/R injuries are dependent on the time window of ischemia [37]. Rapamycin attenuated apoptosis in cardiomyocytes during 6 h of sustained anoxia, but this protective effect of autophagy gradually weakened and was reversed when the anoxia time was prolonged to 12 or 24 h. Disruption of autophagy exhibited a cardioprotective effect on mouse heart during 45 min of ischemia and 24-h reperfusion [38]. These studies suggest that autophagy enhancement during a late stage of reperfusion contributes to the reperfusion injuries. The present study used 24-h reperfusion of mouse heart, which may explain the detrimental effect of autophagy. Therefore, the inhibition of this vigorous autophagy using sRAGE promoted cell survival during this process.

Many signaling pathways mediate autophagy, and mTOR is a classical inhibiting factor [27]. The present study detected mTOR activation *in vivo* and *in vitro*. However, the results of mTOR in mice and cardiomyocytes were conflicting. mTOR phosphorylation increased after I/R treatment in mouse heart (Fig. 5a, c) but decreased in cultured

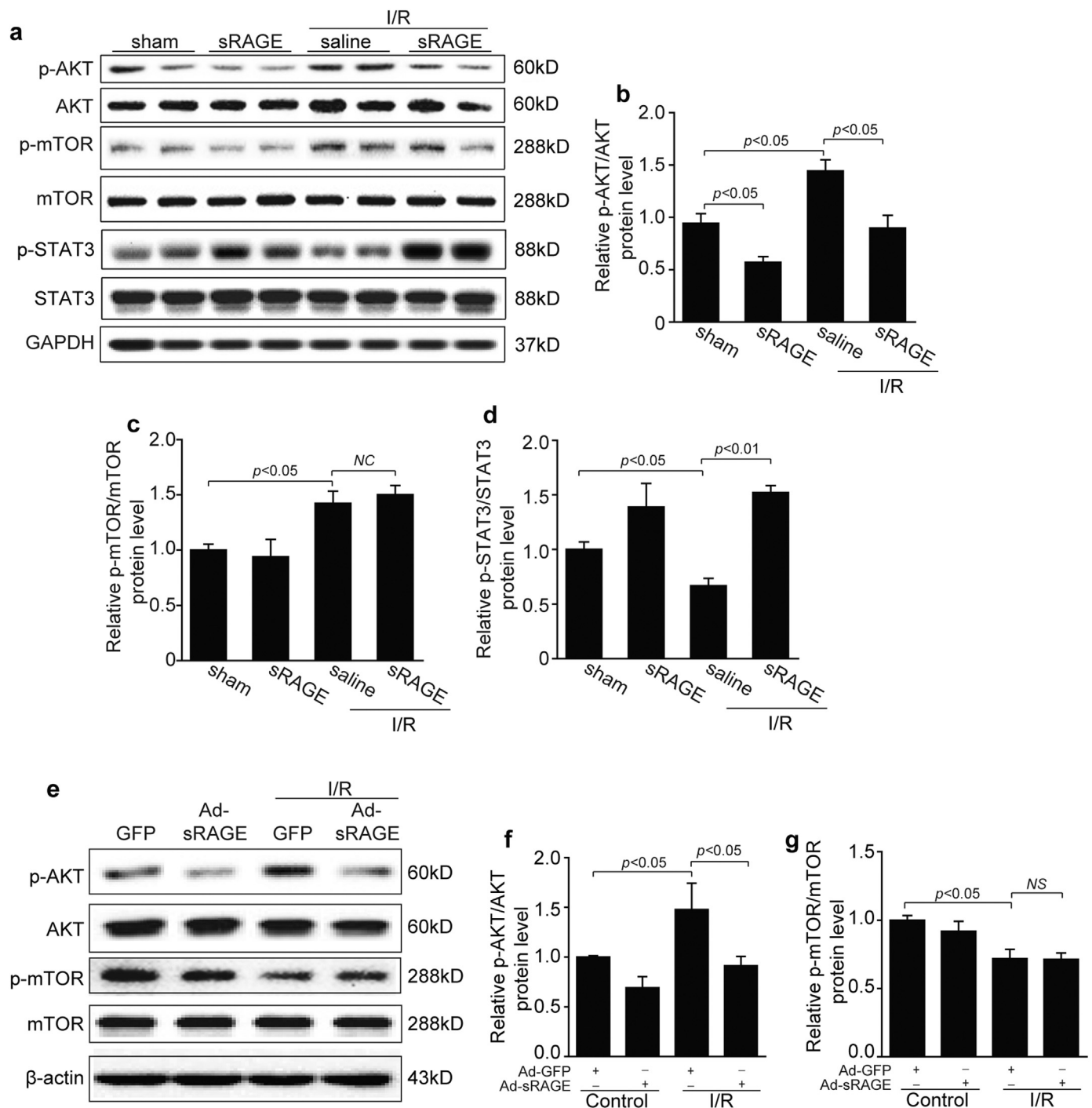


Fig. 5. Effects of sRAGE on the signaling pathways mediating autophagy during I/R *in vivo* and *in vitro*. (a) Representative images of Western blots of signaling pathway proteins in heart tissues of each group. (b) Relative expression of phosphorylated AKT/AKT in I/R mice with or without sRAGE treatment. (c) Relative expression of phosphorylated mTOR/mTOR in I/R mice with or without sRAGE treatment. (d) Relative expression of phosphorylated STAT3/STAT3 in I/R mice with or without sRAGE treatment. $n = 6$ /group (Fig. a-d). (e) Representative images of Western blots of AKT and mTOR in cardiomyocytes of each group. (f) Relative expression of phosphorylated AKT/AKT in I/R cardiomyocytes with or without sRAGE overexpression. (g) Relative expression of phosphorylated mTOR/mTOR in I/R cardiomyocytes with or without sRAGE overexpression. $n = 5$ /group (Fig. e-g). All values are expressed as the mean $\pm$ s.e.m.

cardiomyocytes (Fig. 5e, g). This difference between heart tissue and cultured cells may be related to the complex composition of cells in heart tissues, which might exhibit different trends in autophagy and mTOR phosphorylation during I/R (as suggested by the data in supplemental Fig. S1). However, sRAGE did not influence mTOR phosphorylation after I/R treatment in mouse heart or cultured cells, which suggested that sRAGE modulated autophagy in a mTOR-independent pathway in cardiomyocytes. Sufficient evidence supports mTOR

regulation of I/R-induced autophagy in I/R injuries in heart [31,32,39], but various mTOR-independent autophagy pathways were reported [40,41].

STAT3 is a cardiac protective factor [25], and sRAGE significantly activated the STAT3 signaling pathway during myocardial I/R in hearts and cultured cardiomyocytes in the present study. Notably, sRAGE increased Bcl-2 [17], and an inhibitor of STAT3 reversed this increase in the present study. Bcl-2 inhibited Beclin-1-dependent autophagy [42],

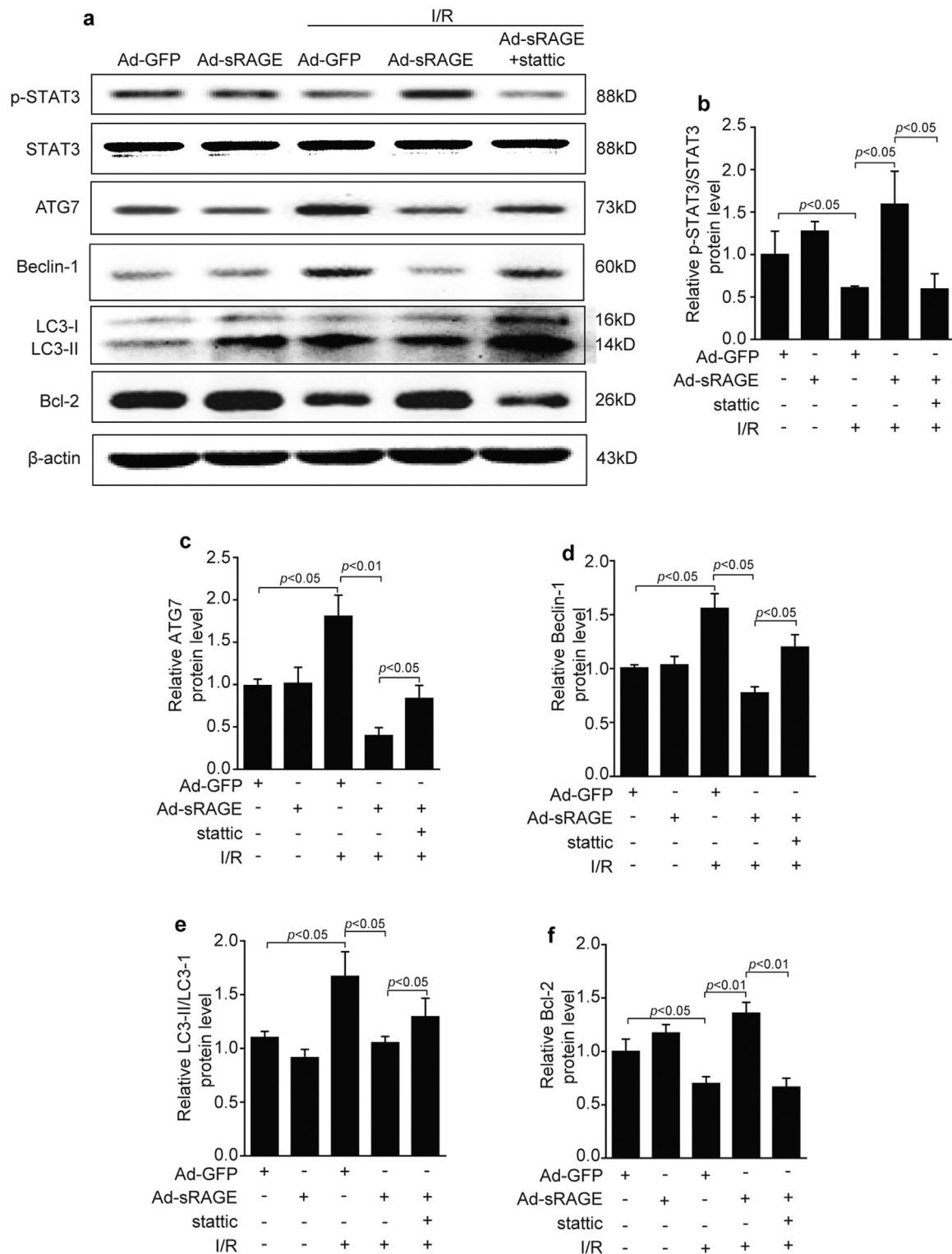


Fig. 6. STAT3 inhibitor abolished sRAGE-induced inhibition of I/R-induced autophagy. Cardiomyocytes were treated as in Fig. 4a, and the STAT3 inhibitor static (2 $\mu\text{mol L}^{-1}$) was added to the media 30 min prior to I/R. Static was also added to the “ischemia buffer” or normal media during ischemia and reperfusion. (a) Representative images of Western blots of ATG7, Beclin-1, LC3-II, Bcl-2 and STAT3 in cardiomyocytes of each group. (b) Relative expression of phosphorylated STAT3/STAT3 in cardiomyocytes of each group. (c) Relative expression of ATG7 in I/R cardiomyocytes of each group. (d) Relative expression of Beclin-1 in cardiomyocytes of each group. (e) Relative expression of LC3-II/LC3-I in cardiomyocytes of each group. (f) Relative expression of Bcl-2 in cardiomyocytes of each group. $n = 5/\text{group}$. All values are expressed as the mean $\pm$ s.e.m.

and sRAGE decreased Beclin-1 in this study. Therefore, sRAGE may reduce autophagy in cardiomyocytes during I/R via the fixation of Beclin-1 with Bcl-2, which was increased by activated STAT3, or decreased transcription of Beclin-1, which was reported previously [43].

Previous studies demonstrated that STAT3 reduced ATG7 expression to inhibit autophagy via inhibition of oxidative stress and FOXO1 in the cytoplasm during I/R [44,45]. Therefore, STAT3 may inhibit autophagy via a reduction in Beclin-1 and ATG7 in I/R. The results of the present

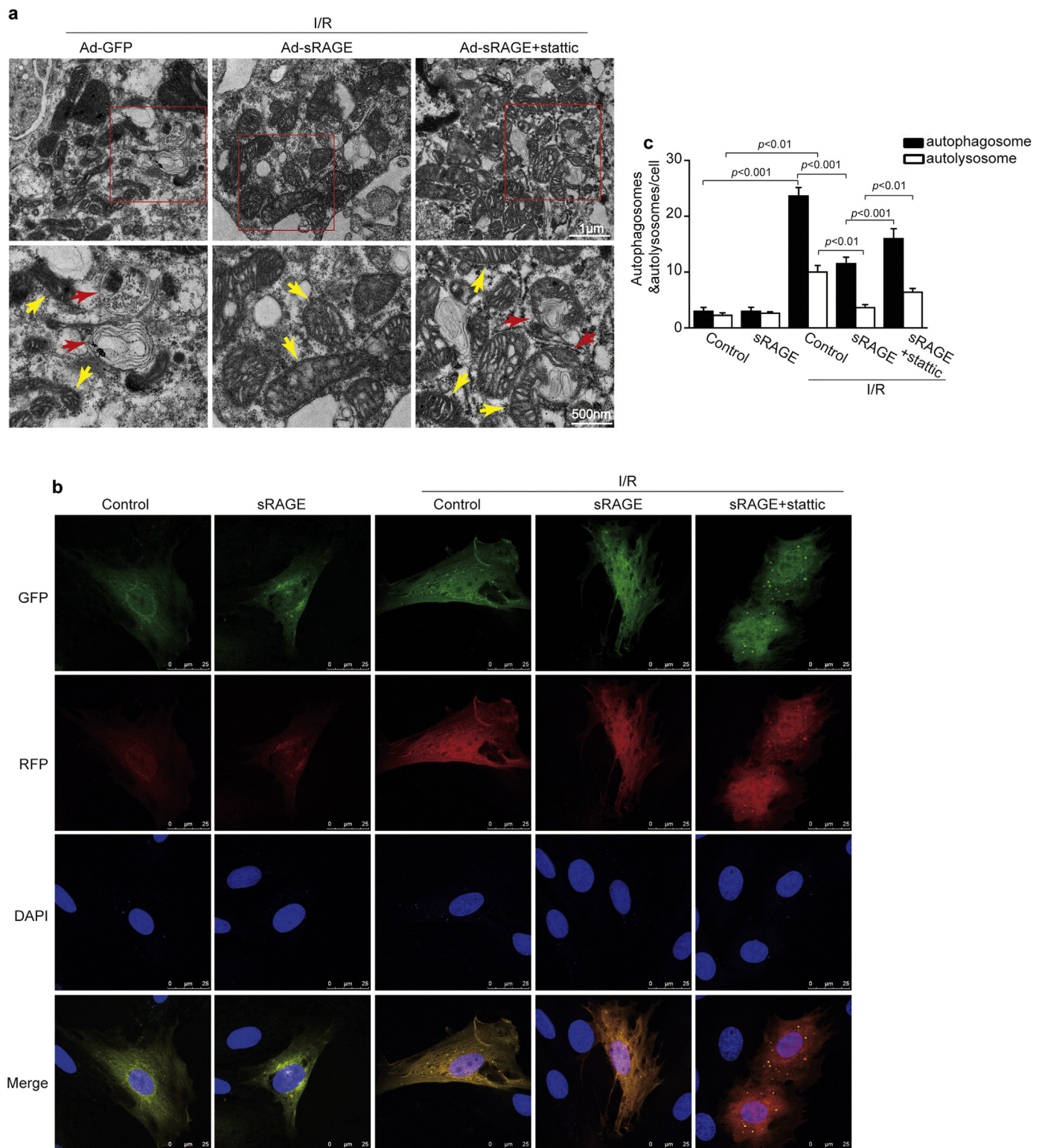
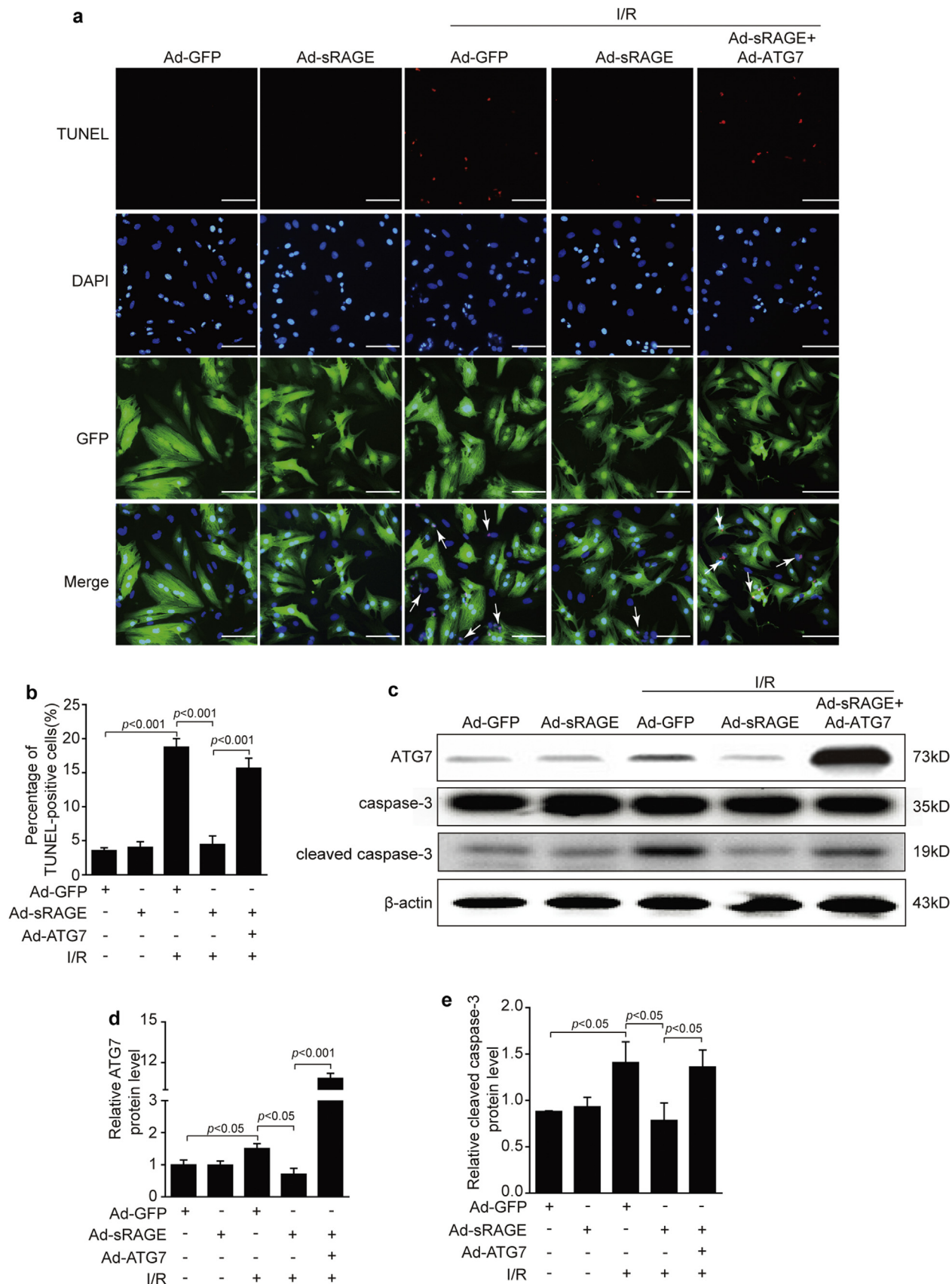


Fig. 7. STAT3 inhibitor abolished the sRAGE-induced reduction of I/R-induced autophagosomes. Cardiomyocytes were treated as Fig. 7a. (a) Representative images of transmission electron microscope (TEM) detecting the ultrastructure of cardiomyocytes of each group. The yellow arrows indicate mitochondria. The red arrows indicate autophagosomes. (b) Representative images of autophagy flux in cardiomyocytes of each group. The cardiomyocytes were treated as Fig. 4f. Static (2 $\mu\text{mol L}^{-1}$) was added to the media 30 min before I/R. The red spots indicate autophagosomes. The yellow spots indicate autolysosomes. (c) Quantitative analysis of the number of red (autophagosomes) and yellow (autolysosomes) spots in cardiomyocytes. $n = 5/\text{group}$. All values are expressed as the mean $\pm$ s.e.m.

study demonstrate that sRAGE increased STAT3 activation during I/R, which was accompanied with an increase in Bcl-2 and reduction in Beclin-1 and ATG7. However, a STAT3 inhibitor abolished the sRAGE-induced inhibition of Beclin-1 and ATG7 during myocardial I/R. STAT3

inhibition also suppressed the sRAGE-induced up-regulation of Bcl-2 during I/R. These results indicate that sRAGE inhibits I/R-provoked autophagy via a STAT3-dependent, rather than an mTOR-dependent, pathway.



(caption on next page)

Fig. 8. ATG7 overexpression abolished the sRAGE-induced inhibition of I/R-induced cardiomyocyte apoptosis. ATG7 was overexpressed in cardiomyocytes using adenovirus ATG7 (Ad-ATG7). (a) Representative images of TUNEL staining in each group. The red spots (TUNEL) indicate TUNEL-positive spots. The blue (DAPI) fluorescence indicates nuclei. The green fluorescence (GFP) indicates the myoneme. The white arrows indicate apoptosis spots (at $\times 20$ magnification). Scale bar: 50 μ m. (b) Quantitative analysis of the percentage of TUNEL-positive spots in each group. (c) Representative images of Western blots of ATG7, caspase-3 and cleaved caspase-3 in each group. (d) Relative ATG7 expression in each group. (e) Relative expression of cleaved caspase-3 in each group. $n = 3/\text{group}$. All values are expressed as the mean $\pm$ s.e.m.

Sufficient evidence confirms an impaired autophagy flux during I/R, which is considered “ineffective” autophagy [3,46]. This “ineffective” autophagy is indicated as an accumulation of autophagosomes due to the dysfunction of autolysosomes formation, which results in an accumulation of presumably undigested cargo and further induces cell death [3]. The present study demonstrated a remarkable increase in autophagosomes and autolysosomes after I/R in cardiomyocytes, which suggests a strong stimulation of autophagy flux during this process. However, overexpression of sRAGE decreased both processes and reduced cardiomyocyte apoptosis. This result suggests that sRAGE restores “ineffective” autophagy into “adequate” autophagy during its attenuation of I/R injuries. ATG7 is a crucial factor in the generation of autophagosomes. ATG7 acts as an E1-like activating enzyme and promotes the activation of ATG12 and LC3, which are essential in the initiation and expansion of autophagosomal membranes [47]. The results of the present study demonstrated that sRAGE significantly inhibited ATG7 expression in heart (Fig. 3a, b) and cardiomyocytes (Fig. 4a, b). Therefore, sRAGE may protect the heart from I/R-induced apoptosis via a reduction in ATG7 expression and inhibition of vigorous autophagy. Therefore, adenovirus ATG7 was used to overexpress ATG7 and stimulate autophagy in cardiomyocytes. ATG7 overexpression abolished the sRAGE-induced inhibition of I/R-induced cardiomyocyte apoptosis, which suggests that sRAGE inhibits I/R-induced cardiac apoptosis via a reduced autophagy that is modulated by ATG7.

sRAGE protects the heart from I/R injuries via binding to AGEs, which are ligands of RAGE, to inhibit the signaling pathways that cause cardiac damage [11]. The results of the present study demonstrated that sRAGE inhibited autophagy via a STAT3-dependent pathway, but failed to eliminate the ligand-binding effects of sRAGE. However, a previous study also reported that sRAGE decreased the delayed-type hypersensitivity response in RAGE knockout mice [48], which indicates that sRAGE plays roles via a RAGE-independent manner. Therefore, the sRAGE inhibition of autophagy during I/R may occur via RAGE-dependent and STAT3-dependent pathways. sRAGE interacts with integrins on cell membranes [49] to activate STAT3 [50], which protects cardiomyocytes from I/R injuries [51]. sRAGE produced similar activating effects of STAT3 in mouse heart and cultured cardiomyocytes, with few AGEs present in cultural medium. Therefore, the present study hypothesized that sRAGE activation of STAT3 was mediated via interaction with integrins on cardiomyocytes.

In conclusion, the present study reported, for the first time, that sRAGE protected the myocardium from I/R-induced apoptosis via the inhibition of autophagy. The mechanism for sRAGE inhibition of autophagy was STAT3-dependent, rather than mTOR-dependent. This finding expands the role of sRAGE in autophagy and may help elucidate the function of sRAGE in myocardial injuries during I/R.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant no. 30801217, 81370313, 81570321, 81870265), Beijing Nova Program (Grant no. 2010B050) and Beijing Health System High Level Health Technical Personnel Training Program (Grant no. 2013-3-046), China Young and Middle-aged Clinical Research Foundation (2017CCA-VG045).

Conflict of interest

None declared.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2018.10.437.

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