#### ARTICLE





# Interferon-y mediates the protective effects of soluble receptor for advanced glycation end-product in myocardial ischemia/reperfusion

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Received: 14 January 2018 / Revised: 29 May 2018 / Accepted: 1 June 2018 / Published online: 8 August 2018 © United States & Canadian Academy of Pathology 2018

#### Abstract

The ubiquitin–proteasome system (UPS) is essential for protein degradation and plays critical roles in myocardial ischemia/ reperfusion (MI/R) injuries. Previous studies have demonstrated that the soluble receptor for advanced glycation end-product (sRAGE) inhibited MI/R-induced apoptosis by upregulating proteasome subunits. However, the mechanism remains unknown. An MI/R model was established by left anterior descending (LAD) coronary artery ligation in mice. Recombinant sRAGE protein or saline was injected intramyocardially with or without neutralizing interferon- $\gamma$  (IFN- $\gamma$ ) antibody injected intraperitoneally before ligation. In cardiomyocytes, ischemia was simulated with "ischemia buffer" and sRAGE was overexpressed by adenovirus. Adenovirus expressing the interference RNA of  $\beta 5i$  was used to knockdown  $\beta 5i$  in cardiomyocytes. IFN-y was induced by sRAGE both in sham and MI/R mice. Blockade of IFN-y using IFN-y antibody abolished the rescue effects of sRAGE for cardiac dysfunction, infarct size and apoptosis provoked by MI/R. Blockade of IFN- $\gamma$  reversed the upregulation of  $\beta$ 1i and  $\beta$ 5i expression induced by sRAGE during MI/R in heart, accompanied by decreasing chymotrypsin-like proteasome activity. In addition, IFN-y antibody abolished the suppressing effect of sRAGE on MI/R-induced p38 and c-Jun N-terminal kinase (JNK) activation, as well as p53 expression, both in vivo and in vitro. However, knockdown of  $\beta$ 5i abolished the antiapoptosis effect of sRAGE during hypoxia/reoxygenation (H/R) in vitro, accompanied by decreased degradation of p53. Our data suggest a novel mechanism for sRAGE in preventing MI/R-induced apoptosis in heart: sRAGE inhibits MI/R-induced apoptosis in cardiomyocytes by degrading p53 by 65i subunit that is increased via upregulation of IFN- $\gamma$ .

# Introduction

The soluble receptor for advanced glycation end-product (sRAGE) is the soluble form of the receptor for advanced

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**Electronic supplementary material** The online version of this article (https://doi.org/10.1038/s41374-018-0102-z) contains supplementary material, which is available to authorized users.

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glycation end-product (RAGE), a cell surface molecule that is expressed on a wide variety of tissues such as heart, lung and blood vessels [1, 2]. The extracellular region of RAGE could be separated from the cell membrane by disintegrin metalloprotease 10 or encoded by a mRNA modified from RAGE mRNA [3]. sRAGE acts as a competing factor to inhibit signaling pathways of RAGE that might induce tissue damage [4]. Several lines of evidence demonstrated that sRAGE, used as a pharmacological blocking agent against RAGE, reduced myocardial ischemia/reperfusion (MI/R)

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injuries in mice [5–7]. Our previous studies showed that administration of recombinant sRAGE protein ameliorated MI/R-induced apoptosis in mice that was mediated by activation of Janus kinase2/signal transductor and activator transcription3 pathway [8], inhibition of the mitochondriamediated apoptosis pathway [9] and upregulation of the ubiquitin–proteasome system (UPS) [10]. However, the mechanism by which sRAGE upregulates proteasome expression and contributes to the inhibition of cardiac injuries during MI/R remains to be elucidated.

UPS is a critical system in regulating protein homeostasis involving degradation of proteins by the 26s proteasome after conjugation with ubiquitins [11]. The 26s proteasome is composed of a 19s regulatory subunit and a 20s catalytic subunit. The 20s subunit includes three constitutive catalytic subunits:  $\beta$ 1,  $\beta$ 2 and  $\beta$ 5 in the inner  $\beta$ -rings that can be replaced by inducible  $\beta$ -subunits, including  $\beta$ 1i (LMP2 or PSMB9),  $\beta$ 2i (MECL-1 or PSMB10) and  $\beta$ 5i (LMP7 or PSMB8) [12]. It was demonstrated that UPS was involved in many cardiovascular diseases, including cardiac hypertrophy [13], angiotensin II-induced abdominal aortic aneurysm [14] and MI/R injuries [15]. Our previous studies showed that sRAGE protected the heart against MI/R injuries by increasing the expression of  $\beta$ 1i and  $\beta$ 5i subunits [10], although the mechanism is still not understood.

It was reported that interferon- $\gamma$  (IFN- $\gamma$ ) was a crucial factor that induced the expression of the immunoproteasome by increasing the levels of mRNA encoding  $\beta$ -subunits [16]. Studies on endogenous activity and properties of sRAGE showed that sRAGE dose dependently induced the production of cytokines, including interleukin-6, tumor necrosis factor- $\alpha$  and IFN- $\gamma$  in spleen cells that interact with  $\beta$ 2-integrin on macrophages to activate nuclear factor kappa B [17–19]. Due to the induction of inflammatory cytokines by sRAGE, it was postulated that the increase in the expression of proteasome subunits mediated by sRAGE during MI/R might be related to IFN-y. To prove this hypothesis, the expression of IFN-y was measured with or without the existence of sRAGE during MI/R, and anti-IFN- $\gamma$  antibody was used to observe the effects of sRAGE on MI/R injuries and the expression of proteasome subunits after IFN-y was neutralized. Meanwhile, the effects of proteasome subunits on MI/R injuries in cardiomyocytes were evaluated as well.

# Materials and methods

### Animals and treatment

Eight- to 10-week-old adult male C57BL/6 mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The mice were randomly divided into five groups: the sham + saline group that was intramyocardially injected with saline without left anterior descending (LAD) ligation (n = 7); the sham + sRAGE group that was intramyocardially injected with recombinant sRAGE protein (2 µg/mouse, Aidi Bo biological Ltd. Beijing, China) without LAD ligation (n = 7); the MI/R + saline group that was intramyocardially injected with saline with LAD ligation (n = 8); the MI/R + sRAGE group that was intramyocardially injected with recombinant sRAGE protein  $(2 \mu g/mouse)$  with LAD ligation (n = 8); and the  $MI/R + sRAGE + anti-IFN-\gamma$  group that was intraperitoneally injected with anti-IFN- $\gamma$  antibody (1 µg/g, R&D System, Minneapolis, MN, USA) and intramyocardially injected with recombinant sRAGE protein (2 µg/mouse) with LAD ligation (n = 8). The model of MI/R is described below. Recombinant sRAGE protein or saline was injected into mice heart just before LAD ligation. Saline or recombinant sRAGE protein in a total of 20 µl was injected into five regions of the muscle in the peri-infarct zone using a syringe attached to a 30-gauge needle. The anti-IFN-y antibodies were injected intraperitoneally 30 min before LAD ligation. The mice were offered standard laboratory raising conditions in Capital Medical University Laboratory of Animal Experiments. All investigations were approved by the Institutional Animal Care and Use Committee of Capital Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

#### MI/R in mice

The model of MI/R in mice was established by ligating the LAD as described previously [20]. Briefly, the mice were anesthetized with 2% isoflurane, and then the heart was exposed from the left fourth intercostal space. The LAD was ligated with 7–0 silk at the initial site. The slipknot was released after 30 min, and reperfusion persisted for 24 h or 14 days. Mice in the sham group underwent the same operation without ligation. After reperfusion, the mice were sacrificed under anesthesia with tribromoethanol (100 mg/kg, Sigma-Aldrich, St Louis, MO, USA). Hearts were quickly removed for further analysis.

#### MI/R in neonatal cardiomyocytes

Cardiomyocytes were isolated from hearts of 1–2-day-old Sprague-Dawley rats purchased from Vital River Laboratory Animal Technology Co. Ltd. The method was described previously [21, 22]. The hypoxia/reoxygenation (H/R) model in cardiomyocytes was established with "ischemia buffer" (pH 6.3) including (mmol/l): 118 NaCl, 24 NaHCO<sub>3</sub>, 1.0 NaH<sub>2</sub>PO<sub>4</sub>, 2.5 CaCl<sub>2</sub>-2H<sub>2</sub>O, 1.2 MgCl<sub>2</sub>, 20 sodium lactate, 16 KCl and 10 deoxyglucose.

Cardiomyocytes were incubated with "ischemia buffer" for 2 h and then transferred to normal medium for 24 h.

# **Adenovirus infection**

Adenovirus-expressing sRAGE (Ad-sRAGE) and control adenovirus-expressing GFP (Ad-GFP) were purchased from Hanbio Co. Ltd. (Shanghai, China). The adenovirus-expressing  $\beta$ 5i shRNA (Ad-si $\beta$ 5i) and  $\beta$ 1i shRNA (Ad-si $\beta$ 1i) were purchased from Genechem Co. Ltd. (Shanghai, China). The RNA interference shRNA sequences of  $\beta$ 1i and  $\beta$ 5i were 5'-ACCATCATGGCTGTGGAAT-3' and 5'-GG AATGCAGGCTATACTAT-3', respectively. Adenovirus transinfection in cardiomyocytes was as previously described [10]. Briefly, cardiomyocytes were transfected with adenovirus for 6–8 h in serum-free medium, and then were transferred to normal medium with 5% fetal bovine serum and were incubated for at least 24 h.

### **ELISA** assay

The level of IFN- $\gamma$  in the serum of mice was measured using mouse IFN-y ELISA Kit (CSB-E04578m; CUSABIO, Wuhan, China). The experiments were performed as previously described according to the manufacturer's instructions [23]. Briefly, 100 µl of serum for each sample was added to the plate and incubated for 2 h at 37 °C. Then, the plate was incubated with biotin-labeled antibody for 60 min at 37 °C. Next, avidin-conjugated horseradish peroxidase was added to each plate and incubated for 60 min at 37 °C. Finally, the plate was incubated at 37 °C for 15 min with substrate solution. The reaction was stopped with stopping buffer and the color was measured with a microplate reader (Eppendorf, Hamburg, Germany) at 450 nm of each sample. A standard curve for quantification was created with purified IFN-y in a concentration range from 0 to 1000 ng/mL. The levels of each sample were calculated using the standard curve.

#### **Evaluation of cardiac function**

After reperfusion for 24 h, the cardiac function of the mice was measured by the Vevo 2100 imaging system (Visual-Sonics Inc., Toronto, Ontario, Canada). The mice were anesthetized by intra-peritoneal injection with tribromoethanol (100 mg/kg, Sigma-Aldrich, St Louis, MO, USA). M-mode echocardiography was used to evaluate cardiac function.

# **Evaluation of cardiac infarction**

Evans blue and 2'3'5-triphenyltetrazolium chloride(TTC) double staining were used to determine the myocardial

infarct size. The protocol was described in detail previously [24]. The LAD was retied and the heart was removed quickly. The heart was perfused with 1% (wv<sup>-1</sup>) TTC (Sigma-Aldrich, St Louis, MO) from the aortic arch and was incubated for 5 min at 37 °C. The heart was fixed with 4% (wv<sup>-1</sup>) paraformaldehyde overnight after injection with 1% (wv<sup>-1</sup>) Evans blue (Sigma-Aldrich, St Louis, MO, USA) from the aortic arch. The next day, the heart was sectioned transversely (1 mm) and was imaged with a digital camera. The blue region represented viable tissue, whereas the white region represented as area at risk (AAR). The area of each region was quantified by ImageJ software (NIH). The infarct size was expressed as the percentage of the white region in AAR.

# **TUNEL staining**

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were used to evaluate apoptosis of cardiomyocytes in hearts. TUNEL staining was performed with the In Situ Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) according to manufacturer's instructions. Briefly, hearts were embedded with paraffin and sectioned to slices of 5 µm. After incubation at 4 °C in 0.1% (vv<sup>-1</sup>) Triton X-100 for 15 min, the sections were incubated with 50 µl TUNEL mixture including 5 µl of TUNEL enzyme and 45 µl of fluorescein isothiocyanateconjugated dUTP at 37 °C for 60 min. Then, the sections were stained with  $\alpha$ -actinin (Sigma-Aldrich, St Louis, MO, USA) and 4',6-diamidino-2-phenylindole (DAPI). The  $\alpha$ -actinin-positive cells indicated the cardiomyocytes. An Olympus BX63 Fluorescence Microscope (Olympus America Inc., Center Valley, PA) was used to capture the photographs. The number of TUNEL-positive cells and the  $\alpha$ -actinin-positive cells was counted with Image J (NIH) software. The results were expressed as the percentage of TUNEL-positive cells in the  $\alpha$ -actinin-positive cells.

#### Quantitative real-time PCR analysis

Quantitative real-time polymerase chain reaction (real-time PCR) was performed as described previously [25]. Approximately  $1-2 \mu g$  of total mRNA was reverse-transcribed to complementary DNA (cDNA) using GoScriptTM reverse transcription system (S1000 Thermal Cycler, California, USA). Real-time PCR of each sample was performed as described below with  $2 \mu l$  of cDNA sample,  $1 \mu l$  of forward primer,  $1 \mu l$  of reverse primer,  $10 \mu l$  of SYBR Premix Ex Taq (Takara, Dalian, China) and  $6 \mu l$  of DNA-free water. The 7500 Real-Time PCR system (Applied Biosystems) was used to perform the experiments. The mouse primers were as follows: IFN- $\gamma$ : 5'-GGTTG

TCTCCTGCGACTTCA-3' (forward), 5'-GGTGGTCCAG GGTTTCTTACTC-3' (reverse), GAPDH: 5'-ATGCCT CGGGAATGGAAAGG-3' (forward), 5'-TCTAGCGGCA TGAAGCACTC-3' (reverse).

# Proteasome activity assays

The proteasome activity assay was determined as previously described [15, 26]. Briefly, heart tissues were lysed with HEPES buffer (pH 7.5) containing 50 mmol/l HEPES, 20 mmol/l KCl, 5 mmol/l MgCl<sub>2</sub> and 1 mmol/l DLdithiothreitol. The proteins were quantified with a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The caspase-like, trypsin-like and chymotrypsin-like activities were measured with the synthetic fluorescent substrates Z-LLE-AMC (45 mM), Ac-RLR-AMC (40 mM) and Suc-LLVY-AMC (18 mM), respectively. Finally, proteasome activity was quantified using a PerkinElmer 2030 Multilabel Microplate Reader with 380 nm excitation wavelength and 480 nm emission wavelength.

#### Ubiquitination of p53

Cardiomyocytes were lysed with lysis buffer containing 20 mmol/L Tris-Hcl (pH 7.5), 0.1% NP-40, 5 mmol/L MgCl<sub>2</sub>, 10 mmol/L NaF, 10 mmol/L Na-pyrophosphate, 10 mmol/L β-glycerophosphate, 20% glycerol, 1 mmol/L DL-dithiothreitol and 1 mmol/L phenylmethanesulfonyl fluoride (Sigma-Aldrich, St Louis, MO, USA). The supernatants were collected after the lysates were centrifuged at 12,500 rpm for 15 min at 4 °C. Dynabeads Protein G (Invitrogen, USA) was incubated with anti-p53 antibody (9282 s, Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature and were then washed three times with washing buffer including 50 mmol/L Tris-Hcl (pH 7.5), 0.5% NP-40 and 150 mmol/L NaCl. Then, the beads were incubated with the supernatants obtained from cell lysates at 4 °C overnight. Precipitated p53 protein was eluted using sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and was measured using anti-ubiquitin antibody.

#### Immunohistochemistry

The embedded hearts were sectioned into 5  $\mu$ m slices. The sections were incubated with IFN- $\gamma$  (AF-585-NA; Novus, Littleton, USA) or CD68 antibodies (ab125212, Abcam, Cambridge, UK) overnight at 4 °C after antigen retrieval in citrate buffer and antigen blocking with 3% bovine serum albumin. Finally, IFN- $\gamma$  or CD68 in the heart were detected by the horseradish peroxidase-DAB detection method. An Olympus BX63 Microscope (Olympus America Inc., Center Valley, PA) was used to capture the photographs.

#### Western blot

Western blot was performed as described previously [10]. Briefly, heart tissues or cardiomyocytes were lysed in lysis buffer (Solarbio, Beijing, China) containing 1 mM phenylmethanesulfonyl fluoride at 4 °C. The concentration of the protein was measured using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Equivalent masses of proteins in each group were separated by 6-15% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride filter membranes by electroblotting. The membranes were blocked with 5% skim milk for 60 min and were incubated with primary antibodies overnight at 4 °C. The membranes were developed with a chemiluminescence detection kit (Millipore, Billerica, MA, USA) and were captured with Chemiluminescent and Fluorescent Imaging Systems (ChampChemi; SAGECREATION, Beijing, China) after incubation with horseradish peroxidaseconjugated IgG antibody for 60 min at room temperature. Densitometry was calculated by ImageJ software (NIH). GAPDH or  $\beta$ -actin was used to verify equal loading.

Antibodies are as follows: c-Jun N-terminal kinase 1/2 (JNK1/2; 9252s, Cell Signaling Technology, Danvers, MA, USA); phosphorylated JNK1/2 (9255s, Cell Signaling Technology); p53 (9282s, Cell Signaling Technology); p38 (8690s, Cell Signaling Technology); phosphorylated p38 (4511s, Cell Signaling Technology); caspase-3 (9662s, Cell Signaling Technology);  $\beta$ -actin (4970s, Cell Signaling Technology); GAPDH (5174s, Cell Signaling Technology);  $\beta$ 1i (ab3328, Abcam, Cambridge, UK);  $\beta$ 2i (ab77735, Abcam);  $\beta$ 5i (ab3329, Abcam); ubiquitin (662056, Millipore, Billerica, MA, USA); horseradish peroxidaseconjugated IgG antibody (sc-2313, Santa Cruz Biotechnology, CA, USA).

### **Statistical analysis**

All data were expressed as the mean  $\pm$  SEM. SPSS v17.0 (SPSS Inc., USA) was used to perform the statistical tests. The parametric test one-way analysis of variance (ANOVA) was used to determine significance among groups. The least significant difference test was used to evaluate the significant differences between two groups. Statistical significance difference was defined as p < 0.05.

# Results

# sRAGE increased the expression of IFN- $\gamma$ during MI/R in mice

To determine whether sRAGE regulated the expression of IFN- $\gamma$  in mice during MI/R, protein levels of IFN- $\gamma$  in the

Fig. 1 sRAGE increased expression of IFN-y in MI/R mice. **a** Levels of IFN- $\gamma$  in the serum of mice with or without sRAGE in sham- and MI/Rtreated mice. b IFN-y mRNA levels in hearts of sham- and MI/ R-treated mice with or without sRAGE. c Representative immunohistochemistry staining of IFN-y in hearts of sham- and MI/R-treated mice with or without sRAGE treatment (magnification:  $\times$  20). Scale bar: 50 µm. d Representative quantification of IFN-y-positive cells in heart tissue with or without sRAGE treatment during MI/R. Scale bar: 20 µm. Values are expressed as the mean  $\pm$  SEM (n = 6 mice per group) \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001



serum of mice and mRNA levels of IFN-y in mouse hearts were measured with or without sRAGE treatment. IFN- $\gamma$ in serum significantly increased from  $119.7 \pm 7.6$  to 371.2 $\pm$  38.7 pg/ml (p < 0.01) during MI/R and further increased to  $730.0 \pm 58.2$  pg/ml after sRAGE treatment (p < 0.01, Fig. 1a). In addition, sRAGE increased IFN-y in sham mice serum from  $119.7 \pm 7.6$  to  $360.1 \pm 54.8$  pg/ml (p < 0.01, Fig. 1a). The real-time PCR results showed that the mRNA of IFN-y in mice heart increased 85.5% during MI/R over levels in sham mice (p < 0.01, Fig. 1b), whereas sRAGE increased IFN-y mRNA expression by 106.5% in sham mouse hearts (p < 0.01) and by 69.6% in MI/R mouse hearts (p < 0.01, Fig. 1b). Immunohistochemistry of IFN- $\gamma$  in mouse hearts showed that IFN- $\gamma$ increased 3.2-fold (p < 0.01) in sham mice and 2.6-fold (p < 0.001) in MI/R mice after treatment with sRAGE. In addition, IFN-y immunohistochemistry showed that MI/R increased IFN-y expression 1.1-fold over levels in sham mice (p < 0.05, Fig. 1c, d)

# Neutralization of IFN-γ abolished the protective effect of sRAGE on MI/R-induced cardiac dysfunction

To explore the effects of IFN- $\gamma$  on the improvement of cardiac function induced by sRAGE during MI/R, anti-IFN- $\gamma$  antibodies were injected intraperitoneally before LAD ligation. Echocardiography revealed that MI/R significantly

reduced LVEF from  $65.6 \pm 4.64\%$  to  $23.3 \pm 4.41\%$  (p < 0.01) and reduced FS from  $35.6 \pm 3.52\%$  to  $12.0 \pm 2.00\%$ (p < 0.001). However, sRAGE significantly increased LVEF from  $23.3 \pm 4.41\%$  to  $46.9 \pm 8.50\%$  (p < 0.05) and increased FS from  $12.0 \pm 2.00\%$  to  $30.6 \pm 5.71\%$  (*p* < 0.001) during MI/R. Moreover, blockade of IFN-y reduced LVEF from  $46.9 \pm 8.50\%$  to  $30.1 \pm 4.80\%$  (p < 0.05) and reduced FS from  $30.6 \pm 5.71\%$  to  $12.2 \pm 1.61\%$  (p < 0.01) in mice treated with sRAGE and MI/R (Table 1). In addition, MI/R increased LVEDV from  $63.2 \pm 3.01$  to  $88.6 \pm 4.76 \,\mu\text{l}$  (p < 0.05) and increased LVESV from  $23.8 \pm 2.04$  to  $65.6 \pm$ 7.16  $\mu$ l (p < 0.01). However, sRAGE reduced LVEDV from  $88.6 \pm 4.76$  to  $60.8 \pm 9.52 \,\mu\text{l} \ (p < 0.05)$  and reduced LVESV from  $65.6 \pm 7.16$  to  $28.4 \pm 9.13 \,\mu\text{l} \ (p < 0.01)$  during MI/R. Moreover, pretreatment of IFN-y antibody increased LVEDV from  $60.8 \pm 9.52$  to  $105.8 \pm 9.71 \,\mu\text{l} \ (p < 0.001)$  and increased LVESV from  $28.4 \pm 9.13$  to  $95.8 \pm 11.1 \,\mu\text{l}$  (p < 0.001) in mice treated with sRAGE and MI/R (Table 1).

# Neutralization of IFN-γ reversed the inhibiting effect of sRAGE on MI/R injuries

To explore the role of IFN- $\gamma$  in reducing infarct size by sRAGE during MI/R, infarct size was measured by Evans blue/TTC staining after IFN- $\gamma$  was neutralized. No significant differences in AAR/total ratio were observed among groups (Fig. 2a, b). Infarct size/AAR ratio was

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Table1Echocardiographyresults for each group

	sham	sRAGE	MI/R	MI/R + sRAGE	MI/R + sRAGE + anti-IFN-γ
HR	541.1 ± 65.93	527.8 ± 17.05	558.6 ± 28.24	$571.0 \pm 12.73$	$551.0 \pm 34.78$
LVEF (%)	$65.5 \pm 4.63$	$65.2 \pm 3.95$	$23.3 \pm 4.41^{**}$	$46.9 \pm 8.50^{\#}$	$30.1 \pm 4.80^{\&}$
FS (%)	$35.6 \pm 3.52$	$35.0 \pm 2.94$	$12.0 \pm 2.00^{***}$	$30.6 \pm 5.71^{\#\#}$	$12.2 \pm 1.61^{\&\&}$
CO (mL/min)	$20.0\pm2.43$	$18.7 \pm 1.03$	$13.6 \pm 1.39^*$	$17.0 \pm 2.40$	$13.9 \pm 2.10$
SV (µL)	$36.9 \pm 1.43$	$35.6 \pm 3.10$	$24.4 \pm 2.53*$	$30.2 \pm 4.81$	$22.7 \pm 2.27$
LVM (mg)	$63.3 \pm 3.86$	$66.9 \pm 1.09$	$68.0 \pm 2.15$	$72.1 \pm 4.07$	$67.4 \pm 5.86^{\&}$
LVEDV (µL)	$63.2 \pm 3.01$	$56.8 \pm 1.46$	$88.6 \pm 4.76^{*}$	$60.8 \pm 9.52^{\#}$	105.8 ± 9.71 <sup>&amp;&amp;&amp;</sup>
LVESV (µL)	$23.8 \pm 2.04$	$20.0\pm0.01$	$65.6 \pm 7.16^{**}$	$28.4 \pm 9.13^{\#\#}$	95.8±11.1 <sup>&amp;&amp;&amp;</sup>
LVEDD (mm)	$4.0 \pm 0.24$	$3.6 \pm 0.04$	$4.2\pm0.29$	$3.8 \pm 0.27$	$4.3 \pm 0.29$
LVESD (mm)	$2.9 \pm 0.33$	$2.3 \pm 0.08$	$3.9 \pm 0.20$	$2.9 \pm 0.36$	$3.8 \pm 0.34$

All the values were expressed as mean ± SEM and calculated from at least five mice in each group

*MI/R* myocardial ischemia/reperfusion, *HR* heart rate, *LVEF* left ventricular ejection fraction, *FS* fractional shortening, *CO* cardiac output, *SV* stroke volume, *LVM* left ventricular mass, *LVEDV* left ventricular end-diastolic volume, *LVESV* left ventricular end-systolic volume, *LVEDD* left ventricular end-diastolic diameter, *LVESD* left ventricular end-systolic diameter

\*p < 0.05 vs sham; \*\*p < 0.01 vs sham; \*\*\*p < 0.001 vs sham; #p < 0.05 vs MI/R; #p < 0.01 vs MI/R; ##p < 0.01 vs MI/R; \*p < 0.05 vs MI/R + sRAGE; \*p < 0.01 vs MI/R + sRAGE

significantly reduced by 58.2% with sRAGE treatment during MI/R (p < 0.05, Fig. 2a–c), whereas blockade of IFN- $\gamma$  before sRAGE treatment increased infarct size/AAR ratio by 156.7% compared with mice treated with sRAGE alone during MI/R (p < 0.05, Fig. 2a–c).

To test the role of IFN- $\gamma$  in inhibiting apoptosis by sRAGE during MI/R, TUNEL staining and caspase-3 expression were measured in mouse hearts after IFN- $\gamma$  was blocked. MI/R led to an increase of TUNEL-positive cells and cleaved caspase-3 in mouse hearts, whereas sRAGE reduced TUNEL-positive cells by 48.0% (p < 0.05, Fig. 2d, e) and reduced cleaved caspase-3 by 32.5% (p < 0.05, Fig. 2d–g) after MI/R. However, blockade of IFN- $\gamma$  before sRAGE treatment induced a 204.7% increase of TUNEL-positive cells (p < 0.001, Fig. 2d, e) and a 79.1% increase of cleaved caspase-3 (p < 0.05, Fig. 2d–g) compared with the mice treated with sRAGE during MI/R.

# Neutralization of IFN-γ inhibited the upregulating effect of sRAGE on proteasomes during MI/R

To explore whether IFN- $\gamma$  influenced the upregulation of proteasome subunits induced by sRAGE during MI/R, the expression of immunoproteasome subunits and proteolytic activities of proteasome were tested after blockade of IFN- $\gamma$ . The expression of immunoproteasome subunits, including  $\beta$ 1i,  $\beta$ 2i and  $\beta$ 5i were decreased by 39.0% (p < 0.05), 29.5% (p < 0.05) and 44.6% (p < 0.05) during MI/R compared with sham mice, respectively, whereas sRAGE markedly increased the expression of  $\beta$ 1i by 27.2% (p < 0.05) and of

β5i by 92.1% (p < 0.01) after MI/R (Fig. 3a–d). However, sRAGE had no effect on the expression of β2i during MI/R (p > 0.05, Fig. 3a–c). Blockade of IFN-γ using anti-IFN-γ antibody before sRAGE treatment reduced expression of β1i, β2i and β5i by 40.7% (p < 0.05), 38.7% (p < 0.05) and 49.6% (p < 0.01), respectively, during MI/R (Fig. 3a–d). In addition, blockade of IFN-γ before sRAGE treatment also reduced chymotrypsin-like activity by 15.8% (p < 0.05) during MI/R but did not change caspase-like and trypsinlike proteolytic activities (Fig. 3e).

# Neutralization of IFN- $\gamma$ abolished the signaling pathways induced by sRAGE

To determine whether IFN- $\gamma$  was involved in mediating the signaling pathways regulated by UPS during MI/R, p53, p38 and JNK1/2 were measured by western blot after IFN- $\gamma$ was neutralized. Phosphorylated p38, JNK1 and JNK2, as well as the expression of p53, were increased 1.56-fold (p <0.001), 3.51-fold (p < 0.001), 1.39-fold (p < 0.001) and 0.67-fold (p < 0.001), respectively, during MI/R, whereas sRAGE reduced phosphorylated p38, JNK1 and JNK2, as well as the expression of p53, by 69.5% (p < 0.001), 62.2% (p < 0.01), 26.9% (p < 0.01) and 56.1% (p < 0.001),respectively, after MI/R (Fig. 4a-e). Blockade of IFN-y before sRAGE treatment significantly decreased phosphorylated p38 and JNK1 and the expression of p53 by 93.8% (p < 0.05), 72.4% (p < 0.05) and 104.2% (p < 0.001), respectively, compared with mice treated with sRAGE and MI/R (Fig. 4a–e). However, IFN- $\gamma$  antibody had no effects



Fig. 2 Neutralization of IFN- $\gamma$  reversed the inhibiting effects of sRAGE on MI/R-induced injuries. **a** Representative images of Evans blue and TTC staining in hearts. The blue area indicates viable tissue. The white area indicates infarct tissue. The area of red plus white indicates AAR. **b** Quantification of AAR/total area ratio in each group. **c** Quantification of infarct area/AAR ratio in each group. **d** Representative image of TUNEL and  $\alpha$ -actinin staining in mouse hearts with

on the phosphorylation of JNK2 that was inhibited by sRAGE during MI/R (p > 0.05, Fig. 4d).

# Knockdown of $\beta$ 5i attenuated the antiapoptotic effects of sRAGE during H/R

To further explore the role of  $\beta$ 1i and  $\beta$ 5i subunits in sRAGE-inhibited apoptosis in H/R-treated cardiomyocytes, Ad-si $\beta$ 1i and Ad-si $\beta$ 5i were transfected into H/R-treated cardiomyocytes. Knockdown of  $\beta$ 5i reversed the anti-apoptosis effects of sRAGE in H/R-treated cardiomyocytes: TUNEL-positive cells increased by 93.6% (p < 0.01, Fig. 5a, b) and cleaved caspase-3 increased by 84.4% (p < 0.05, Fig. 5c, d) compared with sRAGE and H/R-treated cardiomyocytes. However, knockdown of  $\beta$ 1i did not affect the antiapoptosis effect of sRAGE during H/R (supplementary Figure S4).

To elucidate the molecular mechanism of  $\beta$ 5i in mediating the inhibiting effects of sRAGE on H/R-induced apoptosis, apoptosis signaling pathways, including p38, JNK1/2 and p53 were examined after knockdown of  $\beta$ 5i in

or without sRAGE and anti-IFN- $\gamma$  antibody treatment. Magnification: × 20. Scale bar: 50 µm. **e** Quantification of TUNEL-positive cells in fold-change over sham mice in each group. **f** Representative image of western blot for caspase-3 in mouse hearts with or without sRAGE and anti-IFN-antibody treatment. **g** Quantification of the relative expression of caspase-3 in mouse hearts. Values are expressed as the mean ± SEM (n = 7 mice per group). \*p < 0.05; \*\*\*p < 0.001

cardiomyocytes. Knockdown of  $\beta$ 5i with Ad-si $\beta$ 5i in cardiomyocytes increased expression of p53 144.3% compared with sRAGE- and H/R-treated cardiomyocytes (p < 0.05, Fig. 6a–c). However, knockdown of  $\beta$ 5i did not affect phosphorylated p38, JNK1 and JNK2 that were inhibited by sRAGE during H/R (Fig. 6a–e). Furthermore, to confirm whether p53 decreased due to increased degradation by  $\beta$ 5i that was upregulated by overexpression of sRAGE in cardiomyocytes, the ubiquitination of p53 was measured with or without Ad-sRAGE or Ad-si $\beta$ 5i. Overexpression of sRAGE reduced the accumulation of ubiquitinated p53 during H/R, whereas knockdown of  $\beta$ 5i markedly inhibited this effect (Fig. 6f).

#### sRAGE inhibited long-term injuries induced by MI/R

To evaluate whether or not the protective effects of sRAGE were sustained, heart function, cardiac apoptosis and morphological changes in hearts were observed after reperfusion for 14 days. Consistent with previous studies, sRAGE meliorated cardiac dysfunction (supplementary Table 1) and



**Fig. 3** Neutralization of IFN- $\gamma$  abolished the upregulating effect of sRAGE on proteasome subunits in MI/R-treated hearts. **a** Representative images of western blot for  $\beta$ 1i,  $\beta$ 2i and  $\beta$ 5i in mouse hearts with or without sRAGE and anti-IFN-antibody treatment. **b** Quantification of the relative expression of  $\beta$ 1i in mouse hearts. **c** Quantification of relative expression of  $\beta$ 2i in mouse hearts. **d** Quantification of

significantly alleviated remodeling and apoptosis in hearts (supplementary Figure S5A-D) after reperfusion for 14 days. These results suggested that the cardioprotection of sRAGE was sustained not only for 24 h but also for longer terms.

# Discussion

This work demonstrated that protective effects of sRAGE were mediated by IFN- $\gamma$  during MI/R, associated with upregulation of immunoproteasome subunits in mouse heart. The results showed that sRAGE increased the expression of IFN- $\gamma$  in mouse hearts during MI/R and blocking IFN- $\gamma$  significantly abolished the improvement of cardiac function, as well as the inhibition of apoptosis induced by sRAGE during MI/R. Moreover, blockade of IFN- $\gamma$  suppressed the upregulation of proteasome induced by sRAGE after MI/R, confirmed by the decreased

the relative expression of  $\beta 5i$  in mice hearts. **e** Quantification of the relative caspase-like, trypsin-like and chymotrypsin-like activities in mouse hearts with or without sRAGE and anti-IFN-antibody treatment. Values are expressed as the mean  $\pm$  SEM (n = 6 mice per group). \*p < 0.05; \*\*p < 0.01

expression of immunoproteasome subunits and chymotrypsin-like activity. In addition, deficiency of  $\beta 5i$ , rather than of  $\beta$ 1i, reversed the inhibition of apoptosis and the promotion of p53 degradation induced by sRAGE during H/R in cardiomyocytes. Furthermore, sRAGE displayed long-term protective effects on MI/R injuries in the heart in addition to the short-term effects. Thus, this work demonstrated that IFN- $\gamma$  was involved in the protective effects of sRAGE on MI/R-induced injuries, correlating with the increased assembly of the immunoproteasome and resulting in the degradation of p53, possibly promoting the translation of sRAGE into clinical medicine.

Previous studies demonstrated that sRAGE induced production of interleukin-6, tumor necrosis factor- $\alpha$  and IFN- $\gamma$ in mouse splenocytes [18, 19]. In the present study, intramyocardial injection of recombinant sRAGE protein increased levels of IFN- $\gamma$  both in heart tissues and in mice serum during MI/R (Fig. 1). To confirm the effect of sRAGE on the expression of IFN- $\gamma$ , human serum albumin was used



Fig. 4 Neutralization of IFN- $\gamma$  reversed the inhibiting effects of sRAGE on signaling molecules in MI/R-treated hearts. **a** Representative image of western blot for phosphorylated p38, p38, phosphorylated JNK1/2, JNK1/2 and p53 in mouse hearts with or without sRAGE and anti-IFN-antibody treatment. **b** Quantification of the relative expression of phosphorylated p38/p38 in mice hearts.

**c** Quantification of the relative expression of phosphorylated JNK1/ JNK1 in mouse hearts. **d** Quantification of the relative expression of phosphorylated JNK2/JNK2 in mouse hearts. **e** Quantification of the relative expression of p53 in mouse hearts. Values are expressed as the mean  $\pm$  SEM (n = 6 mice per group). \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001

as a vehicle to measure the expression of IFN- $\gamma$  in mice serum and IFN-y mRNA in mouse hearts (because the recombinant sRAGE protein in this study was from a human source) before and after MI/R. Expression of IFN- $\gamma$  in mice serum and heart tissue did not show any significant difference between mice treated with human serum albumin or saline (supplementary Figure S1). These results suggested that the increase in IFN- $\gamma$  was attributed to the effects of sRAGE but not those of serum albumin. IFN- $\gamma$ , the only member of the type II family of IFNs, was secreted by inflammatory cells including macrophages, lymphocytes and neutrophils [27]. The results in this work showed that sRAGE induced macrophages in the heart to produce IFN-y, as shown by increased numbers of IFN- $\gamma^+$ /CD68<sup>+</sup> macrophages compared with IFN- $\gamma^+$ /CD3<sup>+</sup> lymphocytes and IFN- $\gamma^+$ /Ly6G<sup>+</sup> neutrophils in double-immunofluorescent staining in hearts (Fig. 1 and supplementary Figure S2). Contrary to our expectations, the results also indicated that sRAGE increased the infiltration of macrophages, appearing to play a pro-inflammatory role during MI/R. Macrophages were derived from monocytes and differentiated into two phenotypes: classically activated macrophages (M1) and alternatively activated macrophages (M2) [28]. M1-macrophages were thought to be pro-inflammatory, dominating the early phase of myocardial ischemia processes [29–31], whereas M2-macrophages were proved to prevent heart or kidney from ischemia/reperfusion injuries [32, 33]. Previous studies demonstrated that sRAGE directly bound to monocytes and promoted their differentiation to macrophages [34]. To analyze the phenotype of macrophages recruited by sRAGE in mouse hearts, immunohistochemistry of iNOS and CD206 was used to reveal the M1-macrophages and M2-macrophages, respectively. Both M1-macrophages and M2-macrophages were increased during MI/R. However, M2-macrophages were further increased, whereas M1macrophages were reduced after sRAGE treatment in MI/ R mice hearts (supplementary Figure S3). Therefore, it could be supposed that sRAGE might facilitate differentiation of monocytes into M2-macrophage to protect the heart against injuries during MI/R, a finding that should be verified in future investigations.

It was reported that IFN- $\gamma$  was a critical factor in modulating the proteasome: the constitutive subunits in the 20s proteasome were replaced by the three inducible subunits, including  $\beta$ 1i,  $\beta$ 2i and  $\beta$ 5i when IFN- $\gamma$  was exposed to the cells [16, 35]. In the present study, the increase of  $\beta$ 1i and  $\beta$ 5i was accompanied by increased IFN- $\gamma$  in the serum and heart of mice with sRAGE treatment, whereas no such effects were observed in MI/R mice without sRAGE treatment (Fig. 3). These results suggested that IFN- $\gamma$  was not the only factor inducing expression of proteasome subunits; there are still undefined factors accompanying IFN- $\gamma$  to mediate the effects of sRAGE on MI/R mice.



Fig. 5 Knockdown  $\beta$ 5i abolished the inhibiting effects of sRAGE on H/R-induced apoptosis in cultured cardiomyocytes. Neonatal rat cardiomyocytes were infected with adenovirus expressing GFP (Ad-GFP), sRAGE (Ad-sRAGE) or  $\beta$ 5i shRNA (Ad-si $\beta$ 5i) for at least 24 h and then stimulated H/R. **a** Representative image of TUNEL staining in cardiomyocytes in each group. Magnification: × 20. Scale bar: 50

To confirm the effects of IFN- $\gamma$  on the expression of the proteasome in sRAGE-treated hearts, the neutralizing antibody of IFN- $\gamma$  was applied to mice. The effects of sRAGE on increasing  $\beta$ 1i and  $\beta$ 5i during MI/R were abolished by the antibody (Fig. 3), consistent with a previous report where there was a reduction of  $\beta$ 5i in IFN- $\gamma^{-/-}$  mice hearts [36]. Interestingly, neutralization of IFN- $\gamma$  only led to the reduction of chymotrypsin-like activity rather than to caspase-like and trypsin-like activities, in agreement with the report that both  $\beta$ 1i and  $\beta$ 5i subunits exerted chymotrypsin-like activity [12].

UPS was the major nonlysosome system for degradation of misfolded proteins and it played an important role in MI/ R. Substantial evidence has supported the notion that proteasome insufficiency during MI/R led to severe tissue damage [15, 37, 38], whereas enhancement of cardiac proteasome proteolytic activities resulted in increased cellular survival rates in MI/R in mice [39]. Consistent with our previous study [10], the present work confirmed that sRAGE abolished the reduction of  $\beta$ -subunits in the

μm. **b** Quantification of TUNEL-positive cells in fold-change over the control group. **c** Representative image of western blot for caspase-3 in cardiomyocytes for each group. **d** Quantification of the relative expression of caspase-3 in cardiomyocytes. Values are expressed as the mean ± SEM from three independent experiments. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001

proteasome and ameliorated MI/R injuries in hearts. Blockade of IFN- $\gamma$  reversed the upregulation of proteasome proteolytic activities induced by sRAGE during MI/R, accompanied by abolition of the protective effects of sRAGE on MI/R-induced injuries (the deterioration of cardiac function and exacerbation of infarct size and apoptosis, Table 1 and Fig. 2). Therefore, proteasome activities induced by IFN- $\gamma$  might mediate the protective effects of sRAGE during MI/R.

During MI/R, the proteasome was involved in degradation not only of misfolded and damaged proteins but also of cell signaling factors including JNK, p38 and p53 that would lead to apoptosis in cells [40]. In the present study, sRAGE decreased phosphorylation of p38, as well as the expression of p53 during MI/R, which was abolished when proteasome activities were downregulated by the blockade of IFN- $\gamma$ . sRAGE also inhibited MI/Rinduced phosphorylation of JNK1 and JNK2. However, blockade of IFN- $\gamma$  reversed the inhibiting effects of sRAGE on JNK1 phosphorylation during MI/R, but not



on JNK2 phosphorylation (Fig. 4c, d). These results suggested that decreased p38, p53 and JNK1 induced by sRAGE in MI/R injuries in heart might be mediated by

IFN- $\gamma$ -associated proteasome expression. As opposed to the pro-apoptotic effects of JNK1, JNK2 showed no such effects during H/R in vitro [41]; therefore, JNK2 may not Fig. 6 Knockdown of β5i abolished the inhibiting effects of sRAGE on signaling molecules in H/R-treated cardiomyocytes. a Representative image of western blot for the β5i subunit, phosphorylated p38, p38, phosphorylated JNK1/2, JNK1/2 and p53 in cardiomyocytes. b Quantification of the relative expression of β5i in cardiomyocytes. c Quantification of the relative expression of p53 in cardiomyocytes. d Quantification of the relative expression of phosphorylated p38/p38 in cardiomyocytes. e Quantification of the relative expression of phosphorylated p38/p38 in cardiomyocytes. e Quantification of the relative expression of phosphorylated JNK1/JNK1 in cardiomyocytes. f Quantification of the relative expression of phosphorylated JNK2/JNK2 in cardiomyocytes. g Representative images of ubiquitinated p53 in each group. Values are expressed as the mean ± SEM from three independent experiments. \*p < 0.05</p>

be involved in the antiapoptotic effects of sRAGE in MI/R injuries.

Previous studies reported that either  $\beta_{1i}$  or  $\beta_{5i}$  deficiency aggravated MI/R injuries in animals [38, 42, 43]. The present study showed that sRAGE significantly increased the expression of *β*1i and *β*5i, contributing to increased chymotrypsin-like proteasome activity in MI/R hearts. A question remained as to which subunit was involved in the protective effect of sRAGE on cardiomyocytes during MI/ R. A deficiency of  $\beta 5i$  in cardiomyocytes abolished the inhibiting effects of sRAGE on apoptosis in H/R-treated cardiomyocytes (Fig. 5), whereas deficiency of  $\beta$ 1i did not show such effects (supplementary Figure S4). Furthermore, deficiency of 65i significantly increased the expression of p53 in H/R-treated cardiomyocytes other than JNK and p38 with or without sRAGE (Fig. 6). These results suggested that β5i mediated the protective effects of sRAGE on MI/Rinduced apoptosis in cardiomyocytes by inducing p53 degradation.

To verify that sRAGE decreased p53 by degrading ubiquitinated p53, p53 ubiquitination was measured in cardiomyocytes. Overexpression of sRAGE reduced the accumulation of ubiquitinated p53 in cardiomyocytes during H/R, an effect that was abolished by the absence of  $\beta$ 5i. These results indicated that p53 was degraded by the  $\beta$ 5i immunoproteasome that was induced by sRAGE to decrease apoptosis in H/R cardiomyocytes.

In addition, sRAGE alleviated cardiac dysfunction, apoptosis and fibrosis after reperfusion for 14 days, suggesting that the protective effects of sRAGE were both short-term and long-term effects.

For the first time, this study demonstrated that sRAGE protected the heart against MI/R injuries by increasing the expression of IFN- $\gamma$  that facilitated expression of immunoproteasome subunit  $\beta$ 5i. In addition,  $\beta$ 5i might be the crucial subunit mediated by sRAGE to promote the degradation of p53, resulting in decreased apoptosis in MI/R-treated hearts. These findings provided a novel mechanism of sRAGE in inhibiting cardiac apoptosis induced by MI/R, which is possibly beneficial for accelerating the use of sRAGE in clinical therapeutics.

Acknowledgements This work was supported by the National Natural Science Foundation of China (grant no. 81570321, 81370313, 30801217), the Beijing Health System High Level Health Technical Personnel Training Program (grant no. 2013-3-046) and the Beijing Nova Program (grant no. 2010B050).

#### **Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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