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Integrinβ3 Mediates the Protective Effect of Soluble Receptor for Advanced Glycation End-products During Myocardial Ischemia/Reperfusion

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Research Article

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Abstract

Soluble receptor for advanced glycation end-product (sRAGE) was reported to protect myocardial ischemia/reperfusion (I/R) injuries via interacting with AGEs (the ligands of RAGE). Besides, sRAGE was also reported to interact with myocardial cell membranes to protect myocardial I/R injuries. However, the specific molecular of the interaction between sRAGE and myocardial cell membrane is not clearly defined. Integrins were the major adhesion receptors expressed on myocardial cells. The present study showed sRAGE affected the expression of integrin β 3 around integrin β 1 to β 5 in I/R treated cardiomyocytes. Consequently, it was supposed that integring3 might be related to the protective effects of sRAGE on myocardial ischemia-reperfusion injuries. The results in this study showed that the protective effects of sRAGE on cardiac function, cardiac infracting size and apoptosis in mice were cancelled by cilengitide (the inhibitor of integrinβ3). Myocardial apoptosis and autophagy decreased by sRAGE in I/R treated cardiomyocytes were showed to be increased by the inhibition of integrinß3. Mechanistically, the inhibition of integrin β 3 decreased the phosphorylation of Akt and STAT3 but did not affect the phosphorylation of FAK, PTEN, and MAPKs (P38 MAPK, JNK, Erk) in OGD/R and sRAGE treated cardiomyocytes. In addition, the phosphorylation of STAT3 was significantly downregulated by the inhibition of Akt (LY294002,10µM) in OGD/R and sRAGE treated cardiomyocytes. The present study demonstrated integrinß mediated the protective effects of sRAGE on myocardial I/R injuries through Aktinduced STAT3 signaling pathway thus may provide a novel molecular therapy strategy for ameliorating I/R injury.

Introduction

Cardiovascular diseases are the leading cause of death worldwide. Myocardial ischemia/reperfusion (I/R) injuries and aggravation of tissue injury after blood perfusion of the ischemic myocardium are considered risk factors for cardiac function impairment.[1]·[2] I/R injury is characterized by the outbreak of reactive oxygen species (ROS),[3] mitochondrial dysfunctions, apoptosis, autophagy, and increased infarct area[3, 4] Previous studies have shown that soluble receptors for advanced glycation end-products (sRAGE) have a protective role in I/R injury by inhibiting myocardial apoptosis and autophagy.[5] sRAGE is produced by proteolytic cleavage or alternative RNA splicing in the extracellular area of the cell surface receptor.[6] It acts as a natural antagonist to RAGE signaling for isolating RAGE ligands, such as AGEs to inhibit RAGE-dependent cellular responses.[7] However, whether sRAGE depended on RAGE or other membrane receptors on cells to protect I/R myocardium remains unclear.

Integrins, which have been reported as a major adhesion receptor on the myocardium,[8] can promote mitochondrial function through STAT3 and lead to cell survival.[9] Previous studies have shown that RAGE can interact with integrins to mediate leukocyte adhesion.[10] Also, sRAGE might interact with integrins to induce the protective effects on cardiomyocytes during I/R injuries.

Integrinβ3 promotes cardiomyocyte proliferation and reduces hypoxia-induced apoptosis.[11] Yet, the role of integrinβ3 in sRAGE protecting cardiomyocytes from I/R injuries has not been reported so far. In this

Materials And Methods

Animals

C57bL/6 adult male mice, 8 ~ 10 weeks old, were purchased from the Experimental Animal Laboratory, Capital Medical University. All the animals were housed in an environment with a temperature of 22 ± 1 °C, relative humidity of $65 \pm 5\%$, and a light/dark cycle of 12/12 hr, having free access to food and water. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Capital Medical University institutional animal care and conducted according to the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) and the IACUC (Institutional Animal Care and Use Committee) guidelines. The present study was approved by the Ethics Committee of Capital Medical University.

Treatment and grouping

The recombinant adenovirus vector was purchased from Shandong Veizhen Biotechnology Co. LTD(China). Two weeks before the acute myocardial infarction/reperfusion operation, an adeno-associated virus with GFP or sRAGE ($10^{11}v.g/kg$) (Veizhen Co., Ltd. Shandong. China) was injected into mice through the tail vein. The mice were then randomly divided into five groups: (1) Sham group: animals received GFP adeno-associated virus injection without undergoing left anterior descending (LAD) ligation; (2) sRAGE group: sRAGE adeno-associated virus was injected intravenously ($10^{12}v.g./kg$) without LAD ligation; (3) I/R group: GFP adeno-associated virus was injected intravenously and the LAD ligation (n = 8) was performed; (4) I/R + sRAGE group: sRAGE adeno-associated virus was intravenously injected with cilengitide ($75mg\cdotkg^{-1}$)[22] (ApexBio technology LLC. Houston. USA) via intraperitoneal injection 2h before LAD ligation surgery.

I/R operation

The mice were subjected to I/R operation as previously described.[23] Briefly, the hearts of isofluraneanesthetized mice were treated with 6 – 0 silk thread 30min after coronary artery occlusion and 24h reperfusion, after which the hearts were exposed through the fourth intercostal space.[5]

All animals were anesthetized by pentobarbital sodium $(50 \text{ mg} \cdot \text{kg}^{-1})$ and then euthanized by cervical dislocation. The hearts were collected and stored in the – 80°C refrigerator for further experiments. **Echocardiography**

Twenty-four hours after reperfusion, mice were anesthetized with an intraperitoneal injection of tribromoethanol (100 mg/kg, T48402; Sigma-Aldrich, USA). An echocardiogram was then performed using the Vevo 2100 imaging system (Visual Sonics Inc., Toronto, Ontario, Canada) to measure Left

ventricular ejection fraction (LVEF), left fractional ventricular shortening (LVFS), left ventricular endsystolic volume (LVESV), and left ventricular end-diastolic volume (LVEDV) as previously described.[24] Vevo LAB 3.3.1.was used to calculate the data.

2,3,5-triphenyl tetrazolium chloride (TTC) staining

Hearts were immediately removed after refilling with 1%TTC (Sigma-Aldrich St. Louis. MO. USA) solution. [25] Hearts were incubated in 1%TTC solution at 37°C for 3–5min and fixed in 4% (wv⁻¹) paraformaldehyde for 5min. They were then cut vertically and photographed with a digital camera. Image J software (NIH) was used to quantify the area of infarction and normal sections. The data were expressed as the percentage of the infarction area and total area.

Immunofluorescence staining

Myocardial tissue was fixed with 10% buffered formalin and embedded in paraffin, after which it was cut into 4µm thick slices. The sections were incubated in 0.1% (vv^{-1}) Triton X-100 for 8min and blocked with normal horse serum for 30min at room temperature. The sections were then incubated with cleaved-caspase3 antibody (1:600, 9664, Cell Signaling, Inc. Danvers, MA, USA) and α -actinin (1:600, A7811, Sigma-Aldrich, St Louis, MO, USA) at 4°C overnight,[26] and then with corresponding fluorescent secondary antibody for 1.5h at room temperature in dark. The sections were then stained with DAPI and observed by Olympus BX51 fluorescence microscope (Olympus America Inc., Center Valley, PA, USA). ImageJ software (NIH) was used to calculate the positive area.

TUNEL assay

TUNEL staining was performed in accordance with the instructions from TMR Red (Roche, Mannheim, Germany).[27] Tissues were paraffin-embedded and cut into 4μm thick slices. The myocardial cells were washed twice with PBS and fixed for 15min at room temperature. The slices or cells were incubated in 0.1% (VV-1) Triton X-100 for 8min and sealed with 3% (WV-1) bovine serum for 1h at room temperature. Each section was incubated with TUNEL conjugated dUTP at 37°C for 60min. After staining with α-actin and 4 ', 6-diamino-2-phenylindoles (DAPI), sections were stored in a refrigerator at -20°C. Images were obtained using an Olympus BX51 fluorescence microscope (Olympus America Inc., Center Valley, PA, USA) at 20× magnification. Image J (NIH) was used to calculate the number of TUNEL-positive cells and the total cells in each field.

Isolation and culture of the myocardium

Neonatal rat ventricular cardiomyocytes were isolated from the hearts of 1-to 2-day-old Sprague-Dawley rats with 0.25% trypsin as previously described.[28] Rats were purchased from Viton Lever Laboratory Animal Technology Co., LTD. (Beijing, China). Cell pellets were resuspended in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and cultured in an incubator of 5%CO₂ and 21%O₂ at 37°C for 1.5h to remove fibroblasts. Then, 1×10⁶ cells/well were cultured in DMEM/F12 medium containing 0.3g/l glutamine, 4.5g/l glucose, 10% FBS, and 1% penicillin/

streptomycin at an atmosphere of 5% CO_2 and 21% O_2 at 37°C for 48h. After the cardiomyocytes adhered to the wall, the subsequent operation for further experiments was carried out.

Adenovirus transfection and OGD/R model simulation in cardiomyocytes

The recombinant adenovirus vector was purchased from Hanheng Biotechnology Co Ltd (Shanghai, China). Myocardial cells were transfected by recombinant adenovirus expressing GFP or a sequence of the extracellular domain of human RAGE for 24h before stimulation with I/R injury. Cells were then treated with an "ischemia buffer" (pH 6.3), which included 118 NaCl, 24 NaHCO₃, 1.0 NaH₂PO₄, 2.5 CaCl₂·2H2O, 1.2 MgCl₂, 20 sodium lactate, 16 KCl, and 10 deoxyglucose (mM) for 2h in 1%O₂ and 5%CO₂ at 37°C (ischemia).[29] Subsequently, DMEM/F12 was added to the cells in an atmosphere of 5%CO₂ and 21%O₂ at 37°C. In the I/R and sRAGE group, cilengitide (1µM)[30] was added to the medium 30 min before and during the ischemia and reperfusion.

Autophagic flux assessment

As mentioned before,[9] an adenovirus expressing mRFP-GFP-LC3 (Hanheng Biotechnology Co Ltd., Shanghai, China) was transfected into cultured cardiomyocytes for 24 hours to assess autophagic flux. Recombinant sRAGE protein (900 μ M)[31](Aidi Bo biological Ltd. Beijing. China) was then applied to the medium 30minutes before I/R stimulation. After reperfusion, cardiomyocytes were fixed with 4% (wv⁻¹) paraformaldehyde for 15min, and the nucleus was stained by DAPI. Twenty images were collected using confocal fluorescence microscopy (TCS SP8 MP, Leica, Buffalo Groove, Illinois, USA) to observe LC3 spots in cells.

Real-time PCR

Real-time polymerase chain reaction (real-time PCR) was performed as previously described.[32] Approximately 1–2μg of total mRNA was reverse-transcribed to complementary DNA (cDNA) using Go Script TM reverse transcription system (S1000 Thermal Cycler, California, USA). Expression of integrins was analyzed via q-PCR using SYBR-Green PCR Master Mix (RR420A; TaKaRa Bio) with a 7500 Real-Time PCR System. The mouse primers were as follows: Integrinβ1: forward primer: 5'-

GACCTGCCTTGGTGTCTGTGC-3', reverse primer: 5'-AGCAACCACCAGCTACAAT-3'; Integrinß2: forward primer: 5'-TTCGGGTCCTTCGTGGACA-3', reverse primer: 5'-ACTGGTTGGAGTTGTTGGTCA-3'; Integrinß3: forward primer: 5'-ACCGGGGAAAGCTACATGAA-3', reverse primer: 5'-TAGGTACAGTTCACCGCGTT-3'; Integrinß4: forward primer: 5'-GAGGGTGTCATCACCACTTGAA-3', reverse primer: 5'-

CCAGCGACTACACTATTGGAT-3'; Integrinβ5: forward primer: 5'-CCGGTTGGGATTTGGGTATT-3', reverse primer: 5'-CTCAGGGGCATCTCGGTTC-3'; GAPDH: forward primer: 5'-CAAGTTCAACGGCACAGTCA-3', reverse primer: 5'-CCCCATTTGAT GTTAGCGGG-3'.

Western blot

Cardiac proteins of the same amounts (40µg) were separated by SDS-PAGE (6-15%) and transferred to PVDF membranes. After being blocked by 5% skim milk at room temperature for 1 hour, the membranes were incubated in primary antibodies anti-phospho-STAT3 (1:1000; 9131; Cell Signaling Technology, MA, USA), anti-STAT3 (1:1000; 4904; Cell Signaling Technology), anti-phospho-FAK (1:1000; 3283; Cell Signaling Technology), anti-FAK (1:1000; 3285; Cell Signaling Technology), anti-integrinß3 (1:1000; ab119992; Abcam), anti-P62 (1:1000; 23214; Cell Signaling Technology), anti-LC3A/B (1:1000; ARG55799; Arigo), anti-Beclin-1 (1:1000; 3738; Cell Signaling Technology), anti-cleaved-caspase3 (1:1000; 9664; Cell Signaling Technology), anti-phospho-Akt (1:1000; 9271; Cell Signaling Technology), anti-Akt (1:1000; 4691; Cell Signaling Technology), anti-phospho-p38 MAPK (1:1000; 4511; Cell Signaling Technology), anti-p38 MAPK (1:1000; 8690; Cell Signaling Technology), anti-phosphoErk1/2 (1:2000; 4370; Cell Signaling Technology), anti-Erk1/2 (1:1000; 4695; Cell Signaling Technology), anti-phospho-JNK(1:1000;9251; Cell Signaling Technology), anti-JNK (1:1000; 4668; Cell Signaling Technology) and anti-GAPDH (1:1000; 5174; Cell Signaling Technology) at 4°C overnight. The PVDF membranes were then balanced for 20min at room temperature and incubated with a horseradish peroxidase-conjugated secondary antibody anti-rabbit IgG (1:2000; 7074; Cell Signaling Technology) for 1 hour. Specific bands were exposed to ultrafiltration HRP substrate (WBKLS0500, Millipore, Billerica, MA, USA). Fluor Chem FC3 (Protein Simple, Wallingford, CT) was used to capture the images. The density was determined by Image J software (NIH). Loading differences were controlled by probing the blot with an antibody against GAPDH.

Statistical analysis

Data were expressed as the mean \pm SEM. SPSS V25.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A nonparametric test was used when data deviated from a normal distribution. Significance between three groups or more was determined by the parametric test of one-way ANOVA. The least significant difference test (LSD) was used to evaluate the significant difference between the two groups. A *p* < 0.05 was considered statistically significant.

Results

sRAGE upregulated the expression of integrin β 3 after I/R treatment

To decide which subunit of integrins was influenced by I/R and sRAGE, integrin β 1, β 2, β 3, β 4, and β 5 in cardiomyocytes were detected by real-time PCR. The results showed that I/R decreased relative integrin β 3 mRNA levels, which were reversed by sRAGE in cardiomyocytes (p < 0.05) (Fig. 1c); these data were consistent with Western blot results, which revealed that I/R decreased integrin β 3 protein expression, which was reversed by sRAGE treatment (p < 0.05) (Fig. 1f and 1g). However, I/R or sRAGE had no effects on the expression of other integrins (Fig. 1a, b, d, and **e**). These results suggested that integrin β 3 might be involved in the antagonistic effects of sRAGE on I/R injuries in cardiomyocytes.

Integrinβ3 inhibition reversed the antagonistic effects of sRAGE on cardiac I/R injury

To identify the effects of integrin β 3 on sRAGE and I/R treated hearts, cilengitide, an integrin β 3 inhibitor, was applied. The results showed that after integrin β 3 was inhibited, a cardiac function decreased: LVEF decreased from 40.8%±3.84% (I/R and sRAGE treated mice) to 30.48%±1.35% (sRAGE, I/R and cilengitide treated mice, n = 6–8, *p* < 0.05. Figure 2a, b); LVFS decreased from 25.8%±0.74% (I/R and sRAGE treated mice) to 15.34%±0.67% (sRAGE, I/R, and cilengitide treated mice) (n = 6–8, *p* < 0.01. Figure 2c); LVESV decreased from 29.19±1.55% (I/R and sRAGE treated mice) to 23.7±1.34% (sRAGE, I/R and cilengitide treated mice) (n = 6–8, *p* < 0.05. Figure 2d) and LVEDV decreased from 77.29±0.20% (I/R and sRAGE treated mice) to 72.31±0.06% (sRAGE, I/R, and cilengitide treated mice) (n = 6–8, *p* = 0.42. Figure 2e). In addition, results from TTC staining showed that the myocardial infarction size was enlarged from 19.98–29.92% after inhibition of integrin β 3 by cilengitide (n = 6–8, *p* < 0.05. Figure 2f, g). These results suggested that integrin β 3 mediated the antagonistic effects of sRAGE on cardiac function and myocardial infarction size of hearts during I/R injuries.

The inhibition of integrin β 3 abolished the antagonistic effects of sRAGE on myocardial apoptosis after I/R injury

The results from the TUNEL assay, which was used to determine myocardial apoptosis in the marginal zone of myocardial infarction, showed that cilengitide increased the ratio of TUNEL-positive cells from $0.0462 \pm 1.23\%$ (I/R and sRAGE treated mice) to $0.3140 \pm 2.88\%$ (sRAGE, I/R, and cilengitide treated mice) (n = 6-8, *p* < 0.05, Fig. 3a, c), which was remarkably decreased by sRAGE in I/R-treated hearts. In addition, cleaved-caspase3 immunofluorescent staining was used to detect the distribution of apoptotic cardiomyocytes. The results showed that after integrin β 3 was inhibited by cilengitide, the relative cleaved-caspase3 positive area was increased from 11.13%±0.83% (I/R and sRAGE treated mice) to 37.33% ±0.88% (sRAGE, I/R, and cilengitide treated mice) (n = 6-8, *p* < 0.05, Fig. 3b, d). These results indicated that integrin β 3 mediates the antagonistic effects of sRAGE on myocardial apoptosis during I/R injuries.

The inhibition of integrin β 3 diminished the antagonistic effects of sRAGE on cardiomyocytes apoptosis after I/R injuries

Cardiomyocytes were subjected to hypoxia, and a TUNEL assay was used to determine the hypoxiainduced cardiomyocytes apoptosis. It was observed that after integrin β 3 was inhibited by cilengitide, the percentage of TUNEL-positive cells was significantly increased from 9.95%±0.01% (I/R and sRAGE treated cardiomyocytes) to 24.51%±0.02% (sRAGE, I/R, and cilengitide treated cardiomyocytes) (n = 6–10, p<0.05, Fig. 4a, b). Meanwhile, Western blotting was performed to detect the expression of cleavedcaspase3. After integrin β 3 was inhibited by cilengitide, the expression of cleaved-caspase3 was significantly increased from 0.89 folds (I/R and sRAGE treated cardiomyocytes) to 1.76 folds (sRAGE, I/R, and cilengitide treated cardiomyocytes) (n = 3, p<0.05, Fig. 4c, d). These results confirmed that integrin β 3 influenced the antagonistic effects of sRAGE on cardiomyocytes' apoptosis during hypoxia/reperfusion stimulation.

The inhibition of integrin β 3 abolished the antagonistic effects of sRAGE on autophagy in myocardial cells after I/R injury

After cardiomyocytes were infected with mRFP-GFP-LC3 adenovirus for at least 24 hours, autophagy flow was detected. Yellow and red dots represented autophagosomes and autolysosomes, respectively.[12] Image J (NIH) was used to analyze the number of spots. The results revealed that after integrinβ3 was inhibited by cilengitide, the number of autophagosomes increased from 16.5 ± 0.76% (I/R and sRAGE treated cardiomyocytes) to 26.33 \pm 1.52% (sRAGE, I/R and cilengitide treated cardiomyocytes) (n = 6, p < 0.05, Fig. 5a, b) and the number of autolysosomes were not remarkably changed by the inhibition of integrin β 3 in I/R and sRAGE treated cardiomyocytes (n = 6–10, Fig. 5a, c). Besides, Western blot was performed to detect the expression of autophagy relative proteins. These results showed that after integrinβ3 was abolished by cilengitide, autophagy relative proteins such as LC3-II were activated from 1.19 folds (I/R and sRAGE treated cardiomyocytes) to 1.47 folds (sRAGE, I/R and cilengitide treated cardiomyocytes) (n = 5–6, p < 0.05, Fig. 5d, f) and Beclin-1 was activated from 1.11 folds (I/R and sRAGE treated cardiomyocytes) to 1.37 folds (sRAGE, I/R, and cilengitide treated cardiomyocytes) (n = 3-4, p =0.2, Fig. 5d, g). Meanwhile, after integrin β 3 was inhibited by cilengitide, the expression of p62 was downregulated from 1.62 folds (I/R and sRAGE treated cardiomyocytes) to 1.14 folds (sRAGE, I/R, and cilengitide treated cardiomyocytes) (n = 4-7, p < 0.05, Fig. 5d, e). Briefly, this data suggested that integrinβ3 mediated the reductive effect of sRAGE on excessive autophagy in I/R-treated cardiomyocytes.

AKT and STAT3 signalings are downstream molecules of integrin β 3 induced by sRAGE

To demonstrate the intracellular signaling of integrin β 3 induced by sRAGE in I/R treated cardiomyocytes, the potential molecules were detected after integrin β 3 was inhibited by cilengitide. Briefly, the expression of integrin β 3 was showed to be decreased from 1.75 folds (I/R and sRAGE treated cardiomyocytes) to 0.48 folds (sRAGE, I/R, and cilengitide treated cardiomyocytes) (n = 6–8, *p* < 0.05, Fig. 6a, b) in OGD/R treated cardiomyocytes after integrin β 3 was inhibited by cilengitide, suggesting that cilengitide inhibited the expression of integrin β 3 in cardiomyocytes during I/R inducement. In addition, phosphorylated STAT3 was downregulated from 3.7 folds (I/R and sRAGE treated cardiomyocytes) to 0.73 folds (sRAGE, I/R, and cilengitide treated cardiomyocytes) to 3.7 folds (I/R and sRAGE treated cardiomyocytes) to 0.73 folds (sRAGE, I/R, and cilengitide treated cardiomyocytes) to 3.7 folds (I/R and sRAGE treated cardiomyocytes) (n = 5–6, *p* < 0.05, Fig. 6a, d), which had been proved to be increased from 0.47 folds (I/R treated cardiomyocytes) to 3.7 folds (I/R and sRAGE treated cardiomyocytes) (n = 5–6, *p* < 0.05, Fig. 6a, d) by sRAGE in I/R treated cardiomyocytes. However, no changes in the expression of phosphorylated PTEN, and MAPKs (JNK, Erk, P38) were observed among the groups in cardiomyocytes (n = 3–6, *p* > 0.05, Fig. 6a, c, e **to i**). Additionally, the expression of phosphorylated Akt was decreased from 0.67 folds (I/R and sRAGE treated cardiomyocytes) to 0.42 folds (sRAGE, I/R, and cilengitide treated cardiomyocytes) (n = 4, *p* < 0.05, Fig. 6e, j). To sum up, these results suggested that FAK, PTEN, and MAPKs were not the downstream pathway of sRAGE-integrin β 3 in cardiomyocytes during

I/R injury, while STAT3 and Akt might be involved in the effects of integrinβ3 mediated function of sRAGE during cardiac I/R injuries.

To further explore the interaction between Akt and STAT3, LY294002 (an inhibitor of Akt) was adopted in I/R and sRAGE treated cardiomyocytes. The results showed that the phosphorylated STAT3 was downregulated after Akt was inhibited in I/R and sRAGE treated cardiomyocytes (n = 4, p < 0.05, Fig. 6k **to m**). These results suggested that Akt induced the activation of STAT3 signaling in I/R and sRAGE treated cardiomyocytes.

Discussion

The present study demonstrated that integrin β 3 mediated the protective effects of sRAGE on myocardial I/R injuries through Akt-induced STAT3 signaling pathway, which might be used as a potential molecular therapy strategy for ameliorating I/R injury in hearts.

Integrins are widely expressed on cell membranes, including cardiomyocytes.[13] The integrin family contains many subunits, including integrin β 1, 2, 3, 4, and 5.[14] This study showed that integrin β 3 was markedly decreased by I/R, which was reversed by sRAGE in cardiomyocytes (Fig. 1). Previous studies have revealed that the expression of integrin β 3 was upregulated by hypoxia inducement in H9C2 cells (cell line derived from embryonic BD1X rat heart tissue).[15] However, the results in this study revealed that oxygen and glucose deprivation/reperfusion reduced integrin β 3 expression, which was reversed by sRAGE. Due to the previous results that sRAGE protected hearts from ischemia/reperfusion injuries, it was supposed that integrin β 3 might be related to the protective effects of sRAGE on I/R cardiomyocytes.

sRAGE was reported to protect cardiac function and the heart infarction size induced by I/R.[16] The same as the previous reports, the results in this study showed that sRAGE protected cardiac function and the infarction size in I/R treated hearts. However, the protective effects of sRAGE were reversed after integrin β 3 was inhibited by cilengitide (Fig. 2), which suggested that integrin β 3 might mediated the antagonistic effects of sRAGE on I/R injuries in hearts. In addition, sRAGE was also reported to inhibit apoptosis induced by I/R in hearts besides the cardiac function and the heart infarction size.[5] Did integrin β 3 mediate the effects of sRAGE on apoptosis in the hearts as well?

Then,myocardial apoptosis in mice was examined by TUNEL assay and cleaved-caspase3 immunofluorescent staining. The results showed that myocardial apoptosis was decreased by sRAGE in I/R treated mice, which was increased after integrin β 3 was inhibited in I/R and sRAGE treated hearts (Fig. 3). These results suggested that integrin β 3 mediated the suppressive effects of sRAGE on apoptosis during I/R injuries in mice hearts. Then the question arising was, did sRAGE interact with integrin β 3 on myocardial cells directly?

Consequently, cardiomyocytes were isolated and treated with sRAGE and cilengitide (the inhibitor of integrin β 3). The results showed that myocardial apoptosis induced by I/R and inhibited by sRAGE were reversed by the inhibition of integrin β 3 in I/R and sRAGE treated cardiomyocytes (Fig. 4), which confirmed

that sRAGE interacted with integrinβ3 on myocardial cells directly to inhibit apoptosis in I/R treated cardiomyocytes. In addition, previous studies have reported that the inhibition of excessive autophagy resisted apoptosis induced by I/R in cardiomyocytes,[5] which contributed to the inhibiting effects on myocardial ischemia-reperfusion injuries.[17] Then, did the effects of sRAGE on apoptosis which mediated by integrinβ3 in cardiomyocytes also mediated via autophagy?

Therefore, autophagosomes and autolysosomes were detected by mRFP-GFP-LC3 virus transfection in cardiomyocytes. The results showed that the number of autophagosomes was increased, the expression of LC3-II and Beclin-1 were upregulated and P62 was downregulated after integrin β 3 was inhibited in I/R and sRAGE treated cardiomyocytes (Fig. 5). All the results suggested that integrin β 3 mediated the antagonistic effects of sRAGE on excessive autophagy in myocardial cells. The present study verified the assumption that integrin β 3 mediated the suppressive effects of sRAGE on excessive autophagy in I/R myocardium. Then, what was the intracellular signaling pathway of integrin β 3 to mediate the antagonistic effects of sRAGE on ischemia/reperfusion injuries in cardiomyocytes?

Integrin-FAK signaling was reported to enhance mitochondrial function[9] and decrease cell death[18] via STAT3 signaling. In this study, phosphorylation of STAT3 but not FAK was decreased after integrin β 3 was inhibited by cilengitide in I/R and sRAGE treated cardiomyocytes (Fig. 6a to d). Mechanistically, Akt signaling[19], MAPKs[20], JNK signaling[21] and PTEN[11] were reported to be affected by the activation of integrin in non-small cell lung cancer cells, human osteoarthritis chondrocytes, vascular endothelial cells, and cardiomyocytes, respectively. Nevertheless, the results in this study showed that the phosphorylation of PTEN, p38 MAPK,JNK, and Erk1/2 were not affected by the inhibitor of integrin β 3 in I/R and sRAGE treated cardiomyocytes (Fig. 6e to i). Interestingly, the phosphorylation of Akt was significantly upregulated after sRAGE administration in I/R treated cardiomyocytes, while it was downregulated by the inhibitor of Akt (LY294002) in I/R and sRAGE treated cardiomyocytes, which suggested that STAT3 was the downstream molecule of Akt pathway. Based on these findings, the present study concluded that integrin β 3 mediated the protective effects of sRAGE during I/R in cardiomyocytes via Akt and STAT3 signaling pathways.

In summary, the present study demonstrated that integrinβ3 mediated the suppressive effects of sRAGE on I/R-induced apoptosis and autophagy via the Akt and STAT3 signaling pathway in myocardial cells, which contributed to the antagonistic effects of sRAGE on cardiac function and the infarct size in hearts during I/R injuries.

Declarations

Ethics approval and Consent to participate

All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Capital Medical University institutional animal care and conducted according to the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) and

the IACUC (*Institutional Animal Care and Use Committee*) guidelines. The study was reported in accordance with **ARRIVE guidelines.** This study did not involve human participants.

Consent for publication

This work did not involve the identification images or other personal or clinical details of participants. The authors of this paper consent for publication.

Availability of data and materials

The data that support the findings of this study are not openly available and are available from the first author or the corresponding author upon reasonable request.

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Conflict of interest and authors' contributions

All authors contributed to the study conception and design. Material preparation was performed by X.G. and J.Z., data collection and analysis were performed by X.H., J.C. and L.C.. The first draft of the manuscript was written by X.H. and X.G.. X.Z., H.W. and F.D. commented on previous versions of the manuscript. C.G. provided the funding acquisition and supervised the study. All authors read and approved the final manuscript. The authors declare no potential conflict of interest.

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sRAGE increases the expression of integrin β 3 in I/R-treated cardiomyocytes. a, Relative integrin β 1 mRNA levels in cardiomyocytes. b, Relative integrin β 2 mRNA levels in cardiomyocytes. c, Relative integrin β 3 mRNA levels in cardiomyocytes. d, Relative integrin β 4 mRNA levels. e, Relative integrin β 5 mRNA levels. f, Representative images of Western blots for integrin β 3. g, The statistical chart of relative expression of integrin β 3/GAPDH in cardiomyocytes. n=3~8/group. All values are expressed as the mean± SEM.



sRAGE protects the cardiac function and decreases the myocardial infarction area after I/R injury, which is abolished by cilengitide. a, Images of M-mode echocardiography for each group. b, The statistical diagram of EF (%). c, The statistical diagram of FS (%). d, The statistical diagram of LVESV (μ I). e, The statistical diagram of LVEDV(μ I). f, Representative photographs from TTC staining of heart sections in each group. Scale bars are 1cm. g, The statistical chart of relative myocardial infarction area in each group. n=6~8/group. All values are expressed as the mean ± SEM. CLG = cilengitide, the inhibitor of integrin β 3.



sRAGE relieves myocardial apoptosis after myocardial infarction/reperfusion in C57bL/6 mice. Cilengitide reversed the protective effect of sRAGE on apoptosis. a, Representative images of TUNEL staining of cardiomyocytes (magnification: 20×). The red spots indicate TUNEL-positive spots; the blue ones indicate nuclei; green spots indicate α-actinin antibody staining. Scale bars are 50µm. b, Representative images of cleaved caspase-3 immunofluorescent staining in heart tissues for each group. c, The statistical image of the proportion of TUNEL-positive cells. d, The statistical image of the relative ratio of cleaved caspase-3 positive area. $n=6\sim8/group$. All values are expressed as the mean ± SEM. CLG = cilengitide, the inhibitor of integrin β 3.



Figure 4

sRAGE relieves myocardial apoptosis after I/R stimulation in primary cardiomyocytes, while cilengitide reverses the protective effect of sRAGE on apoptosis. a, Representative images of myocardial cells from TUNEL staining (magnification: 20×). Scale bars are 50 μ m. b, Statistical images for the percentage of TUNEL-positive cells in each group. c, Representative images from Western blots showing cleavedcaspase3 expression. d, The statistical image of the relative expression of cleaved-caspase3 with or without sRAGE treatment. All values are expressed as the mean± SEM. n=3-10/group. CLG = cilengitide, the inhibitor of integrin β 3.



sRAGE reduces the myocardial autophagy promoted by I/R, while cilengitide reverses this effect. a, Representative images of the autophagy flux dots per field. Scale bars are 50µm. b, The statistical images of autophagosomes. c, The statistical images of autolysosomes. All values are expressed as the mean ± SEM. n = 6-8/group. d, Representative pictures from immunoblot of cardiac protein levels about LC3-II, p62, and Beclin-1. e, The densitometric analysis of normalized LC3-II / LC3-I levels. f, The densitometric analysis of normalized p62 levels. g, The densitometric analysis of normalized Beclin-1 levels. n = 4-6/group. CLG = cilengitide, the inhibitor of integrin β 3. GAPDH = glyceraldehyde 3-phosphate dehydrogenase. GFP = green fluorescent protein.



Figure 6

Effect of sRAGE-integrin β 3 on intracellular pathways in cardiomyocytes during I/R analyzed by Western blot. a, Expression of integrin β 3, FAK, and STAT3 proteins in myocardial cells. b-d, Quantification of integrin β 3, the phosphorylated FAK and STAT3 in (a). All values are expressed as the mean ± SEM. n=5-

8/group. e, Expression of PTEN, MAPKs (JNK, Erk, P38), and Akt proteins in myocardial cells. f-j, Quantification of PTEN, MAPKs, and Akt in (e). Data are mean \pm SEM, n = 3-4/group. CLG = cilengitide, the inhibitor of integrin β 3. k, Expression of Akt and STAT3 proteins after Akt was inhibited by LY294002 in I/R and sRAGE treated cardiomyocytes. I to m, Quantification of Akt and STAT3 in (k). Data are mean \pm SEM, n = 4/group.

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