CREG1 ameliorates myocardial fibrosis associated with autophagy activation and Rab7 expression

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ABSTRACT
In cardiomyocytes subjected to stress, autophagy activation is a critical survival mechanism that preserves cellular energy status while degrading damaged proteins and organelles. However, little is known about the mechanisms that govern this autophagic response. Cellular repressor of E1A genes (CREG1) is an evolutionarily conserved lysosomal protein, and an important new factor in regulating tissue homeostasis that has been shown to antagonize injury of tissues or cells. In the present study, we aimed to investigate the regulatory role of CREG1 in cardiac autophagy, and to clarify autophagy activation mechanisms. First, we generated a CREG1 haploinsufficiency (Creg1 +/-) mouse model, and identified that CREG1 deficiency aggravates myocardial fibrosis in response to aging or angiotensin II (Ang II). Conversely, exogenous infusion of recombinant CREG1 protein completely reversed cardiac damage. CREG1 deficiency in Creg1 +/- mouse heart showed a marked accumulation of autophagosome that acquired LC3II and beclin-1, and a decrease in autophagic flux clearance as indicated by upregulating the level of p62. Inversely, restoration of CREG1 activates cardiac autophagy. Furthermore, chloroquine, an inhibitor of lysosomal acidification, was used to confirm that CREG1 protected the heart tissue against Ang II-induced fibrosis by activating autophagy. Using adenoviral infection of primary cardiomyocytes, overexpression of CREG1 with concurrent resveratrol treatment significantly increased autophagy, while silencing CREG1 blocked the resveratrol-induced autophagy. These results suggest that CREG1-induced autophagy is required to maintain heart function in the face of stress-induced myocardial damage. Both in vitro and in vivo studies identified that CREG1 deficiency influenced the maturation of lysosomes and reduced the expression of Rab7, which might be involved in CREG1-induced cardiomyocyte autophagy. These findings suggest that autophagy activation via CREG1 may be a viable therapeutic strategy for improving cardiac performance under pathologic conditions. This article is part of a Special Issue entitled: Autophagy and protein quality control in cardiometabolic diseases.

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1. Introduction

Myocardial fibrosis is a critical aspect of cardiac remodeling following myocardial infarction, hypertension and other cardiovascular diseases [1,2]. This process is initially an adaptive response to maintain cardiac function by reducing stress on the walls of the heart and decreasing energy expenditure; impairing this adaptive mechanism leads to heart failure [3,4]. A more complete understanding of how the heart senses and responds to stress would allow for the development of more effective therapeutic strategies to treat myocardial fibrosis, with the ultimate goal of eradicating the problem entirely through prevention as well as treatment.

Autophagy is a lysosome-mediated catabolic process in which senescent or damaged proteins and organelles are sequestered by double membrane-limited vesicles called autophagosomes, and then degraded by lysosomes [5]. Accumulating evidence attests to a pro-survival role for autophagy when cells are under stress by facilitating the removal of damaged proteins and organelles, and by recycling basic building blocks that can be used for energy generation and targeted macromolecular synthesis to shore up cellular defenses [6,7]. Recently, autophagy was found to be upregulated during stress conditions such as heart failure, pressure overload, nutrient starvation, ischemia-reperfusion injury, and proteotoxic disease [8–10]. As a housekeeping process, autophagy is critical for the normal structure and function of the heart. Dysfunctional autophagy has been implicated in cardiac diseases, with particularly strong evidence in the case of cardiac impairment, which is thought to be a result of imbalance between autophagosome production and clearance [11]. As an important organelle involved in autophagosome clearance, lysosome function is an attractive target for therapeutic intervention to treat or prevent heart disease. However, up to now, our understanding of the molecular mechanisms contributing to the
lysosome–autophagosome balance in response to increased workload and stress remains rudimentary.

The cellular repressor of E1A-stimulated genes (CREG1) is a new, important glycoprotein that regulates tissue homeostasis. CREG1 has been shown to antagonize tissue or cellular injury [12,13]. Despite potentially significant roles of CREG1 remaining unclear, Xu et al. [14] reported that CREG1 expression is decreased during biomechanical stress in the heart, and function to attenuate cardiac hypertrophy. Recent studies [15–18] showed that CREG1 is a lysosomal protein that undergoes proteolytic maturation by lysosomal cysteine proteases in the course of its biosynthesis. CREG1 contains three mannose 6-phosphate (M6P) markers, and depends on interactions with M6P receptors for efficient delivery to lysosomes, which is implicated in the regulation of lysosomal functions. Therefore, in the present study, we aimed to investigate the role of CREG1 in cardiac stress mediated by angiotensin II (Ang II) and to clarify its molecular mechanisms.

2. Methods

2.1. Animals

Male heterozygous Creg1+/− mice were generated and bred at the Southern model animal center as described previously [19]. Age-matched male transgenic-negative wild-type (Creg1−/−) littermates were used as controls. All experiments were approved by the Animal Ethical Committee of Shenyang General Hospital and conducted in accordance with existing guidelines on the care and use of laboratory animals. Animal model surgery and echocardiography and hemodynamic analyses were performed as previously described [20].

2.2. Mouse experiments

Two-month-old Creg1+/− and Creg1+/+ mice were subjected to an infusion of Ang II (1.5 mg/kg · day) for 14 days. In another series of experiments (reversal experiment), 2-month-old male Creg1+/− mice were subjected to an infusion of Ang II for 14 days, while being treated with recombinant CREG1 protein (0.03/0.06/0.3 mg/kg · day) or with chloroquine (5 or 10 mg/kg · day) for 14 days (starting from day 1 and continuing until day 14). Chloroquine (N-(7-chloro-4-quinoliny1)-N1,N1-dimethyl-1,4-pentanediame diphasate salt) and resveratrol were purchased from Sigma-Aldrich (Poole, United Kingdom).

2.3. Immunohistochemistry

Hearts were isolated and fixed with buffered 3.7% formalin for 24 hours. Thereafter, tissue sections were dehydrated and embedded in paraffin. Another slice was embedded in tissue-tic, placed on a carton disk put on a Petri dish and immediately, but slowly, frozen above liquid nitrogen. For immunohistochemistry, 3-μm-thick sections were cut on a microtome and mounted on slides. Hearts were cut at the midpapillary level.

2.4. Masson’s trichrome staining

To measure fibrosis, Masson’s trichrome staining was performed on paraffin sections for all experimental animals. Whole stained sections were scanned (Nanozoomer 2.0-HT, Hamamatsu, Japan) and a fibrosis score for the entire section was calculated using 20 × magnification for mice (ScanScope, Aperio Technologies, Vista, CA, USA).

2.5. Transmission Electron Microscopy

Transmission electron microscopy (TEM) of perfusion-fixed myocardium was performed as previously described [9]. Autophagosomes or autolysosomes were identified by the characteristic structure of a double- or multi-lamellated smooth membrane completely surrounding compressed mitochondria, or membrane-bound electron-dense material.

2.6. Assessing autophagic flux in heart

Mice were delivered Ang II (Sigma), His-tagged human recombinant CREG1 protein (Abcam, CA, USA), or chloroquine (5 or 10 mg/kg · day) using osmotic pumps (Alzet Model 1004, PA, US), PBS as vehicle control. The mice were euthanized and ventricular myocardium was sampled for Western blot analyses of LC3II/I levels, Beclin 1 and p62 expression [8].

2.7. RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen, CA, USA). Reverse transcription was performed at 50 °C for 50 min, and then 85 °C for 5 min using the SuperScript III First-Strand Synthesis SuperMix for RT-PCR kit (Invitrogen, CA, USA). RT-PCR was performed on a Bio-Rad T100 PCR System (primer sequences are shown in Table 1).

2.8. Western blotting

Protein was extracted from cardiac tissue and cardiomyocytes. Equal amounts of protein (50 μg) were separated by SDS–PAGE (150 V, 1 h) and transferred into a polyvinylidene difluoride membrane. After blocking, blots were probed with anti-Beclin 1, anti-LC3, anti-p62 or anti-β-actin antibody (Cell Signaling, PA, USA) at 4 °C overnight. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated AffiniPure rabbit anti-goat IgG (H + L) (Thermo Scientific, PA, USA) for 1 h. After washing again, immunological complexes were detected using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, PA, USA).

2.9. Confocal microscopy

After removing the culture media, the samples were fixed with 3.7% formaldehyde at 37 °C. The samples were permeabilized with 1% Triton X-100 in PBS-T (Triton X-100: phosphate buffered saline 1:100, volume ratio) for 15 min. The samples were then incubated with Alexa Fluor 555 Phalloidin (1:40, Invitrogen) for 20 min. The cells were stained with the chromatin dye, DAPI (300 nmol/L, Invitrogen), for 5 min. Images were obtained using an Olympus confocal microscope (Olympus, Japan).

2.10. Primary culture of mouse cardiomyocytes

Primary cardiac myocytes (CMs) were isolated, purified and maintained in medium 199 (M199) supplemented with 10% fetal bovine serum at 37 °C in an atmosphere of 5% CO2 and 95% air. The phenotype of primary cardiomyocytes was confirmed morphologically and by positive immunofluorescence staining of anti-actinin. All experiments with cells were performed with subcultures 4–10 after cloning. For autophagic studies, early passage CMs (passage 2–3) were plated in

| Table 1 |
| RT-PCR primer sequences. |
| Raβ7-F | 5′-AGACCTGCAACTACTGGAGCCGAC-3′ |
| Raβ7-R | 5′-AGACCTGCAACTACTGGAGCCGAC-3′ |
| mCREG1-F | 5′-TGCCTGGAACTCGTCACACAG-3′ |
| mCREG1-R | 5′-TGCCTGGAACTCGTCACACAG-3′ |
| hCREG1-F | 5′-TTTCTGCGAGAACTTTGCTACAG-3′ |
| hCREG1-R | 5′-TTTCTGCGAGAACTTTGCTACAG-3′ |
| GAPDH-F | 5′-GGGGCTTCAGTCACAGAC-3′ |
| GAPDH-R | 5′-GGGGCTTCAGTCACAGAC-3′ |
Fig. 1. Creg1+/− mice are more susceptible to cardiac fibrosis with aging. A: Western blot of CREG1 expression in hearts from 2-month-old and 6-month-old Creg1+/− mice and age-matched Creg1+/+ controls; β-actin was probed for loading control. B: Densitometry of immunoblots showing quantification of changes in CREG1 expression. C: Real-time PCR for CREG1 expression in hearts from 2-month-old and 6-month-old Creg1+/− mice and age-matched Creg1+/+ controls; GAPDH was probed for loading control. D: Densitometry of bands showing the semi-quantification of changes in CREG1. E: Masson trichrome stains showed increased myocardial fibrosis in hearts from 2- or 6-month-old Creg1+/− mice compared with age-matched Creg1+/+ controls. F and G: Western blot of collagen I and III expression in hearts from 6-month-old Creg1+/− mice and age-matched Creg1+/+ controls; β-actin was probed for loading control. H: Sections were immunostained for CREG1 (green) and collagen I (red) in hearts from 6-month-old Creg1+/− mice and age-matched Creg1+/+ controls; bars = 100 μm. **p < 0.01 and ***p < 0.001.
24-well plates, grown to subconfluence, then resveratrol (10 μmol/L) treatment in 10% FCS for 24 hours.

2.11. Adenoviral infection

Adenoviral GFP and CREG1 vectors were created as described previously [17]. These replication-deficient vectors were propagated in AD293 cells using Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% (v/v) FBS. The CMs were infected with DMEM containing 1 × 10^8 AdGFP or AdCREG adenovirus particles/mL for 2 consecutive days. The expression of CREG1 was assessed by immunoblotting.

2.12. Generation of CREG1 and Rab7 knockdown cells

CREG1 and Rab7 RNAi vectors (Abcam, CA, USA) were transfected in primary cardiomyocytes using FuGene 6 (Roche, Mannheim, Germany) for immunofluorescence microscopy, or for Western blotting, according to instructions given by the manufacturer.

2.13. Statistical analysis

Data are expressed as mean ± SD. All data were analyzed using SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). Differences among two groups were compared using an unpaired Student’s t-test. Differences among three or more groups were compared using a one-way analysis of variance. Statistical significance was defined as p < 0.05 (two-tailed).

3. Results

3.1. Creg1^+/− mice are more susceptible to cardiac damage in response to aging or Ang II stimulation

Western blotting showed that CREG1 expression was significantly decreased in 2-month-old or 6-month-old Creg1^+/− mouse heart compared with age-matched Creg1^+/+ mice (Fig. 1A and B). RT-PCR analysis showed that there was no significant difference in mRNA levels in the 2-month-old Creg1^+/− mouse heart compared with aged Creg1^+/+ mouse heart; however, in 6-month-old Creg1^+/− mouse hearts, CREG1 expression in mRNA levels were slightly downregulated (Fig. 1C and D). Masson trichrome staining revealed the low level of interstitial fibrosis in 2-month-old Creg1^+/− mice hearts; however, with increasing age (from 2 to 6 months of age), fibrosis-positive labeling was strongly increased in 6-month-old Creg1^+/− hearts (Fig. 1E). Meanwhile, immunofluorescence staining and Western blot analysis showed that collagen I and III levels were significantly increased in 6-month-old Creg1^+/− mouse hearts compared with age-matched Creg1^+/+ controls (Fig. 1F to H). These data suggest that CREG1 deficiency plays a critical role in the emergence of myocardial fibrosis.

To further confirm these findings, we established an Ang II-induced myocardial fibrosis model in 2-month-old Creg1^+/− and Creg1^+/+ mice. Fourteen days after Ang II treatment, we observed cardiac damage results similar to those obtained with age increase using this model (Fig. 2A). More interestingly, three days after Ang II treatment, there was a rapid and dramatic downregulation of CREG1 both in protein (Fig. 2B and 2C) and mRNA levels (Fig. 2D and E) in Creg1^+/− mouse hearts and in Creg1^+/− mouse hearts. It is implied that deficiency of CREG1 expression was more susceptible to induce the myocardial fibrosis.

3.2. Deficiency of CREG1 impaired autophagy function in hearts of Creg1^+/− mice

Because CREG1 is a lysosome-related protein [18], we hypothesized that lack of CREG1 may be involved in the lysosome–autophagy function, which plays an important role in preventing heart disease. Western blot analysis identified that the ratio of LC3II/I and the level of Beclin-1, both indicators of macroautophagy and CMA autophagy, were increased in cardiomyocytes of Creg1^+/− mice compared with that in Creg1^+/+ mice (Fig. 3A and B). This was accompanied by increased accumulation of LC3-positive vesicles on immunofluorescence analysis (Fig. 3C). Meanwhile, p62, an adapter protein that indicates clearance of autophagosomes, was investigated to accumulate significantly in Creg1^+/− cardiomyocytes compared with that in Creg1^+/+ control (Fig. 3A and B). Moreover, Western blot analysis identified that the ratio of LC3II/I and the expression of Beclin-1 and p62 were

![Fig. 2. Creg1^+/− mice are more susceptible to myocardial fibrosis in response to Ang II stimulation. Two-month-old Creg1^+/− and Creg1^+/+ mice were subjected to an infusion of Ang II (1.5 mg/kg - day) for 14 days. A: Masson trichrome stain showed that myocardial fibrosis significantly increased in 2-month-old Creg1^+/− mouse heart compared with that in Creg1^+/+ mice 14 days after Ang II treatment. B and C: Western blot of CREG1 expression in 2-month-old Creg1^+/− mouse heart and age-matched Creg1^+/+ mice after Ang II treatment for 0, 3 and 7 days. β-Actin was probed for loading control. Densitometry of immunoblots showing quantification of changes in CREG1 expression. D and E: Real-time PCR and semi-quantification for CREG1 mRNA expression after 0, 3 and 7 days of treatment by Ang II stimulation in 2-month-old Creg1^+/− mouse heart and age-matched Creg1^+/+ mice, GAPDH was probed for loading control; bars = 100 μm. ***p < 0.001.](image-url)
markedly increased in Creg1+/- cardiomyocytes compared with Creg1+/+ controls after three days of Ang II treatment (Fig. 3D and E). Furthermore, immunofluorescent staining revealed that both the level of p62 and LC3 were markedly enhanced in Creg1+/- hearts compared to Creg1+/+ controls (Fig. 3F). These data suggest that autophagic flux clearance, but not the formation of early autophagosomes, was impaired because of the deficiency of CREG1, which might be related to the occurrence of myocardium impairment under chronic, severe stress.

3.3. Recombinant CREG1 protein infusion rescues Ang II-induced myocardial fibrosis via activation of autophagy in Creg1+/- mice

To further evaluate the effect of CREG1 on myocardial fibrosis and to determine the potential therapeutic value of increased CREG1 function, we used osmotic pumps (Alzet Model 1004, PA, US) to deliver His-tagged recombinant human CREG1 protein (0.03/0.06/0.3mg/kg · day; Abcam, CA, USA) and Ang II (1.5mg/kg · day) concurrent into the

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Fig. 3. Deficiency of CREG1 results in autophagosome accumulation and impairs autophagic flux clearance in Creg1+/- mice heart with and without Ang II treatment. A: sections were immunostained for LC3 (green) and actinin (red) in 6-month-old Creg1+/- mouse hearts and age-matched Creg1+/+ mice; yellow arrows show LC3 accumulation. Bars = 100 μm. B: Representative image of Western blot analyses of LC3II/I, Beclin 1 and p62 in 6-month-old Creg1+/- mouse hearts and age-matched Creg1+/+ mice. C: A summary of densitometry data for LC3II/I, Beclin1 and p62, β-actin was probed for loading control. D to F: Representative images of Western blot analyses of LC3, Beclin1 and p62, and a summary of densitometry data in 2-month-old Creg1+/- mouse hearts and age-matched Creg1+/- mouse with or without Ang II stimulation, β-actin was probed for loading control. G: Sections were immunostained for LC3 (green) and p62 (red) in 2-month-old Creg1+/- mouse hearts and age-matched Creg1+/+ mouse with or without Ang II stimulation; yellow arrows show LC3 accumulation. Bars = 100 μm. #p < 0.05, *p < 0.01 and **p < 0.001.
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HE Masson
Creg1−/− mice. An ELISA assay for anti-His antibody revealed that the recombinant CREG1 protein was detected in the serum of treated mice from 3 days up to 14 days. Additionally, Western blotting using anti-His antibody identified the recombinant CREG1 protein was delivered in treated myocardial cells (data not shown). Remarkably, HE and Masson staining showed that Ang II-induced myocardial fibrosis was rescued completely in a dose-dependent manner upon treatment with recombinant CREG1 (Fig. 4A). We further monitored autophagic flux after recombinant CREG1 treatment for 3 days by Western blot analysis. LC3II/I and Beclin-1 markedly increased, while the level of p62 markedly decreased in Creg1−/− hearts with recombinant CREG1 protein treatment compared with those without CREG1 protein treatment (Fig. 4B and C). To validate whether CREG1 antagonized myocardial fibrosis by modulation of autophagic activity, we blocked autophagic flux using different concentrations of chloroquine (5 or 10 mg/kg · day), a lysosome inhibitor, in Ang II-stressed Creg1+/- hearts with recombinant CREG1 protein treatment. LC3II/I, Beclin-1 and p62 levels were detected to increase in chloroquine-treated groups in dose-dependent manner (Fig. 4D and E), accompanied by further exaggeration of the fibrosis response, similar to what we saw with CREG1 deficiency (Fig. 4F). It is suggested that recombinant CREG1 protein treatment protected mice hearts against myocardial fibrosis accompanied by activation of autophagy.

3.4. Deficiency of CREG1 impairs the maturation of lysosome associated with reduction of Rab7

Excessive accumulation of autophagic vacuoles resulting from impairment of autophagosome–lysosome fusion or lysosome dysfunction [23]. Therefore, we further investigated the impact of CREG1 deficiency on the biogenesis of lysosome in the heart. In contrast to Creg1−/− hearts, lysosome-associated membrane protein-1 (LAMP-1) and LAMP-2 markedly decreased in Creg1−/− hearts, as did the mature form of cathepsin D (Fig. 5A and B). Meanwhile, immunofluorescence staining showed that co-localization of cathepsin D and LAMP-1 decreased in Creg1−/− hearts, which suggested that perinuclear lysosome aggregated impairment in Creg1−/− hearts (Fig. 5C). These results implied that CREG1 deficiency might be blocking the maturation of lysosome in Creg1−/− hearts. Because lysosome maturation played a pivotal role in autophagy, thereby, we deduced that CREG1 deficiency induced the reduction of LAMP-1 and LAMP-2, which might have mediated the dysfunction of autophagy in Creg1−/− hearts. Western blotting identified that LAMP-1, LAMP-2 and cathepsin D increased strikingly in Creg1−/− hearts compared with Creg1−/− mice hearts after 3 days with Ang II (Fig. 5D and E). Furthermore, transmission electron microscopy analysis also identified that lysosomal structure and autophagosome–lysosome fusion structure reduced significantly in the Creg1−/− hearts in contrast to Creg1+/+ hearts (Fig. 5F). It is implicated that a deficiency of CREG1 inhibits maturation of lysosome, but does not directly decrease the expression of its marker proteins.

To explore the mechanisms of CREG1 on lysosome formation, we monitored the process of lysosome biogenesis from early to late endosome by measuring Rab1a, Rab5a, Rab7 and Rab9 expression, and found a significant upregulation of Rab1a, Rab5a and Rab9, and a marked downregulation of Rab7 in Creg1−/− mouse hearts compared with Creg1+/+ controls (Fig. 5G and H). Meanwhile, immunofluorescence staining also identified that Rab7 expression was reduced in Creg1−/− hearts (Fig. 5I). More important, Rab7 expression was markedly downregulated in Creg1−/− hearts when it was stimulated by Ang II (Fig. 5D and E). It is suggested that Rab7 might be an important molecule downstream of CREG1, which is involved in CREG1-induced heart autophagy.

3.5. CREG1 activates autophagy in primary cardiomyocytes by modulating Rab7 expression

To explore the mechanism of CREG1 on myocardial autophagy, we overexpressed CREG1 using an adenovirus vector or silenced CREG1 by siRNA in primary cultured cardiomyocytes (CMs), respectively. CREG1 expression was monitored by immunoblotting and RT-PCR as shown in Fig. 6A to D. Resveratrol (10 μmol/L) markedly increased LC3II/I and Beclin-1 expression both in CREG1-overexpressed and CREG1-silenced CMs. However, increased p62 was only observed in CREG1-silenced CMs, but not in CREG1-overexpressed cells (Fig. 6E and F). Furthermore, transmission electron microscopy analysis also revealed excessive of autophagic vacuoles in CREG1-silenced CMs compared with that in CREG1-overexpressed cells (Fig. 6G). These results were consistent with in vivo data suggesting that CREG1 deficiency inhibits resveratrol-induced autophagy in CMs. In addition, Western blotting and immunofluorescence staining revealed that knockdown of CREG1 is sufficient to diminish the expression of Rab7, while overexpression of CREG1 increases Rab7 expression in CMs (Fig. 7A and B). However, RT-PCR revealed that CREG1 did not modulate the expression of Rab7 in the mRNA level (data not shown). Furthermore, mouse Rab7 siRNA duplexes (Ambion, CA, USA) were used to inhibit the endogenous Rab7 expression in CMs. As shown in Fig. 7C and D, 80% of Rab7 was abolished in siRab7-2 and siRab7-3 transfected CMs after 72 hours of transfection. As expected, resveratrol-induced autophagy in CREG1-overexpressed CMs was significantly inhibited in siRab7-7-transfected cells compared with that in scramble siRNA transfected cells (Fig. 7E and F). This result suggested that Rab7 might be involved in the autophagy of cardiomyocytes mediated by CREG1.

4. Discussion

An important finding in our study is that CREG1 is a positive contributor to myocardial autophagy, which prevents cardiomyocyte damage induced by aging or stress. This beneficial effect can be explained as follows: (a) CREG1 haploinsufficiency is capable of inducing a robust accumulation of autophagosomes in mice heart cells, and leads to myocardial fibrosis; (b) enhancing CREG1 function with infusion of recombinant CREG1 protein completely halts the progression of myocardial fibrogenesis by rapidly activating autophagy; (c) CREG1-induced increase of Rab7 expression might be a required molecular element that regulates the cardiac autophagic response to protect cardiomyocytes from damage.

Autophagy is a conserved catabolic process that is essential for maintaining cellular homeostasis, which is a critical survival mechanism in cells subjected to stress that preserves the cellular energy status and degrades damaged proteins and organelles [21–24]. A basal level of autophagy is particularly vital in cardiomyocytes, as these cells are normally terminally differentiated and cannot decrease toxic concentrations of waste by cell division [9]. Recently, cardiac autophagy was reported in models of short-term nutrient deprivation [25], chronic ischemia [26,27] and ischemia-reperfusion injury [28,29]. However, the mechanisms promoting autophagy in cardiomyocytes are not fully understood.

Fig. 4. Recombinant CREG1 protein infusion reverses Ang II-induced myocardial fibrosis via activating autophagy in Creg1−/− hearts. A: HE and Masson staining showed Ang II-induced cardiac fibrosis decrease in 2-month-old Creg1−/− mice hearts with recombinant CREG1 (0.03, 0.06, 0.3 mg/kg · day) infusion compared with Creg1−/− mice or Creg1+/+ mice heart. Bars = 50 μm. B: Representative images of Western blot analysis of CREG1. LC3II/I, Beclin-1 and P62 in 2-month-old Creg1−/− mice hearts and age-matched Creg1+/+ mice with Ang II and reCRGE1 protein infusion. β-actin was probed for loading control. C: A summary of densitometry data of CREG1, LC3II/I, Beclin-1 and P62 in 2-month-old Creg1−/− mice hearts stimulated by Ang II with or without reCREG1 protein infusion. D and E: Using different concentrations of chloroquine (5 or 10 mg/kg · day) blockade for 24 hours, Western blot of p62, Beclin1 and LC3II/I expression. β-actin was probed for loading control. F: Masson staining showed cardiac fibrosis in 2-month-old Creg1−/− mice hearts with Ang II, reCREG1 protein (0.3 mg/kg · day) and chloroquine (5 or 10 mg/kg · day) infusion for 14 days. Bars = 50 μm. #p < 0.05, **p < 0.01 and ***p < 0.001.
In the present study, we elucidate for the first time that a novel molecular, CREG1, promotes cardiomyocyte autophagy during aging or Ang II-induced damage. First, in a mouse model of CREG1 haploinsufficiency, we identified that CREG1 deficiency aggravates myocardial damage in response to aging or Ang II. Strikingly, overexpression of CREG1 by infusion of recombinant exogenous CREG1 completely reversed cardiac damage and resulted in normal cardiac morphology and function. This was consistent with previous studies [14,15], which implied that CREG1 plays an important role in modulating homeostasis in cardiomyocytes. Second, our data showed that both CREG1

FIG. 5. Deficiency of CREG1 impairs the maturity of lysosome and reduces the expression of Rab7 in Creg1+/− mice heart. A and B: Representative image of Western blot analyses and a summary of densitometry data for LAMP-1, LAMP-2 and cathepsin D, in 2-month-old Creg1+/− mice hearts and age-matched Creg1+/+ mice, β-actin was probed for loading control. C: Immunofluorescence staining showed co-localization of cathepsin D and LAMP-1 in 2-month-old Creg1+/− mice hearts and age-matched Creg1+/+ mice. D and E: Representative image of Western blot analyses and a summary of densitometry data for LAMP-1, LAMP-2, and cathepsin D in 2-month-old Creg1+/− mice heart and age-matched Creg1+/+ mice with Ang II treatment, β-actin was probed for loading control. F: Electron micrographs of myocardium from Creg1+/+ and Creg1+/− hearts with or without Ang II treatment (bars = 2 μm). Abundant autophagolysosome (white arrow) containing degenerating mitochondria and other cytoplasmic contents are evident in Creg1+/+ hearts compared with accumulation of autophagosomes (red arrow) in Creg1+/− hearts. G and H: Representative image of Western blot analyses and a summary of densitometry data for Rab7, Rab9, Rab5α and Rab1α in 2-month-old Creg1+/− mice hearts and age-matched Creg1+/− hearts with Ang II treatment, β-actin was probed for loading control; bars = 100 μm. #p < 0.05, **p < 0.01 and ***p < 0.001.
deficiency and CREG1 overexpression markedly enhanced accumulation of autophagosomes that acquired LC3II/I and beclin-1 in hearts with Ang II stimulation. Autophagy is a dynamic process, which begins with the formation of the double-membrane autophagosome that engulfs a part of the cytoplasm. Subsequently, the outer membrane of the autophagosome fuses with late endosomes and lysosomes to become an autolysosome, and the contents are finally degraded for the synthesis of new molecules and organelles[23,24]. Therefore, it is established that the initial step of autophagic maturation took place in the hearts of both CREG1-deficient and overexpressed mice. Dramatically, CREG1 deficiency-induced vacuolation did not reflect reduced but rather enhanced expression of p62, an adaptor protein that gets consumed during autophagy[30,31], which suggested that the aberrant accumulation of enlarged hybrid compartments was due to delayed/ arrested autolysosomal maturation in Creg1+/− mice. However, overexpression of CREG1 resulted promptly in the degradation of p62, implying that autophagy occurred rapidly. Therefore, we deduced that the large capacity of autophagosomes induced by CREG1 allows autophagy to remove damaged organelles and protein aggregates to maintain organelle function and protein quality, while suppression of CREG1 below physiological levels might induce heart damage with enhanced protein aggregation. To confirm this, we blocked autophagy flux using chloroquine, which is known to inhibit lysosomal acidification[32], and investigated the levels of Beclin-1, LC3II/I and p62 increased in chloroquine-treated cardiomyocytes, accompanied by further exaggeration of the fibrosis response, ignoring the effects of CREG1 on myocardial protection. The result suggested that CREG1-induced autophagy is required to withstand Ang II-induced myocardial fibrosis, and CREG1 is a critical mediator of autophagy activation and modulator of physiological or pathological cardiomyocyte stress. This novel protein

Fig. 6. CREG1 overexpression activates autophagy in primary cardiomyocytes (CMs). A and B: Representative images of Western blot analyses of CREG1 and a summary of densitometry data in CREG1-overexpressed cardiomyocytes (AdCreg1) or in CREG1-silent CMs (siCreg1-1, siCreg1-2 and siCreg1-3). C and D: Representative RT-PCR analyses of CREG1 and a summary of densitometry data in CREG1-overexpressed cardiomyocytes (AdCreg1) or in CREG1-silent CMs (siCreg1-1, siCreg1-2 and siCreg1-3). E and F: Representative images of Western blot analyses of LC3II/I, Beclin 1 and p62, and a summary of densitometry data in CREG1-overexpressed cardiomyocytes or in CREG1-silent CMs treated by resveratrol (10 μmol/L) for 24 hours. G: Transmission electron micrographs showed autophagosomes (red arrow) and autophagolysosome (white arrow) containing degenerating mitochondria and other cytoplasmic contents are evident in CREG1 overexpressed CMs compared with accumulation of autophagosomes in CREG1-silent CMs stimulated by resveratrol (10 μmol/L) for 24 hours. Bars = 0.5 μm. #p < 0.05, **p < 0.01 and ***p < 0.001.
is expressed broadly in mature tissue and organelle, which allows it to be used to study the effect of rapid autophagy in a stressed organ system.

Another contribution of this study is the confirmation of an important role of CREG1 in activating Rab7 expression in the cardiomyocytes. Here, we found the coexistence of increased autophagosomes with decreased autophagic flux both in adult Creg1+/− mouse hearts and with CREG1-ablated primary cultured cardiomyocytes, confirming that CREG1 is required for autophagosome–lysosome formation. Impaired autophagosome–lysosomes formation can result from defective fusion between autophagosome with lysosome, or lysosome malfunction [33,34]. Therefore, we further investigated the impact of CREG1 deficiency on the function of lysosome. Both in vitro and in vivo studies identified that CREG1 deficiency reduced the lysosome maturation and the formation of autophagolysosomes accompanied by a reduction of Rab7.

Rab proteins are a family of small molecular weight guanine nucleotide-binding proteins (G-proteins) that regulate vesicular budding and fusion reactions by oscillating between active (GTP-bound) and inactive (GDP-bound) forms [35,36]. Over 60 different Rab proteins have been identified, and each is unique in cellular and subcellular localization, as well as the vesicles that it fuses [37]. There are studies indicating that Rab5 and Rab1 have a role in mediating the fusion of the early endosome [38], Rab11 in facilitating recycling endosomes [39] and Rab7 and Rab9 in regulating late endosome fusion with lysosomes [40]. Rab7 is a 23-kDa protein that is localized to late endosome, which in

![Image of Rab7 expression](image_url)
particular has been suggested to regulate the transport from early to late endosomes that is essential for the maintenance of a functional lysosome compartment but not for the function of earlier endocytic compartments [41–44]. In the present study, CREG1 deficiency resulted in the absence of functional Rab7 protein, which might be directly involved in the aggregation and fusion of late endocytic structures/lysosomes and accumulation of autophagosomes. Although the mechanism by which CREG1 modulating Rab7 expression is not clear, the present study confirmed that downregulation of Rab7 had a negative effect on CREG1-induced autophagic vacuole maturation, implying that Rab7 is a nexus through which CREG1 regulates autophagolysosome maturation to activate autophagy. Certainly, more detailed studies will be done in Rab7 knockout mouse to confirm the function of Rab7 mediated by CREG1.

Taken together, CREG1 is indispensable to cardiomyocyte survival via keeping its autophagy homeostasis, not only in physiologic heart stress but also in pathological heart stress. This may provide a therapeutic target to reduce myocardial damage, particularly in patients with heart remodeling and heart failure.

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Disclosures
None.

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