RAGE upregulation and nuclear factor-κB activation associated with ageing rat cardiomyocyte dysfunction

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Abstract. Evidence suggests that ageing is a major risk factor for cardiac dysfunction. Interactions between advanced glycation endproducts (AGEs) and the receptor for AGEs (RAGE) are known to cause chronic cellular activation, including activation of nuclear factor-κB (NF-κB), which has been implicated as a causal factor in the ageing process. To assess whether cardiomyocyte contractile function and the interaction of AGEs with RAGE in the heart are altered in ageing, 25- and 2-month-old male rats were compared. Mechanical properties were assessed in ventricular myocytes using an edge-detection system, including peak twitch amplitude (PTA), time-to-PTA (TPS), time-to-75% relengthening (TR75) and maximal velocity of shortening/relengthening (±dL/dt) in ventricular myocytes. AGEs were detected by using a fluorescence assay. The expression of RAGE and NF-κB was assessed through a Western blot analysis. Compared with young myocytes, aged myocytes displayed a prolonged TR75 at 1 Hz. With increasing stimulus frequency (from 2 to 4 Hz), aged myocytes' PTA was significantly reduced relative to young myocytes. Aged rat hearts displayed high level of AGEs, RAGE upregulation and NF-κB activation. These findings demonstrate impaired cardiomyocyte relaxation and reduced tolerance to increased stimulus frequency in aged rats, which might be associated with enhanced AGEs, RAGE expression, and NF-κB activation.

Key words: Cardiac myocytes — Ageing — Advanced glycation endproducts — Receptor for AGEs — Nuclear factor-κB

Introduction

Advanced glycation endproducts (AGEs) arise from a complex non-enzymatic multi-step reaction of reducing sugars with proteins, namely *via* their amino acid side chains and the terminal amino group. AGEs exacerbate disease progression through two general mechanisms – modifying molecules and forming nondegradable aggregates, thus impairing normal cellular/tissue functions, and altering cellular function directly through receptor-mediated activation (Baynes 2001). Although the other receptors such as AGE-R1, AGE-R2 and AGE-R3 are known to interact with AGEs, many of the cellular effects of AGEs have been attributed to their interaction with the receptor for AGEs (RAGE) (Thornalley 1998).

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RAGE is a member of the immunoglobulin superfamily of cell surface molecules and has been shown to be expressed in various organs including the heart (Petrova et al. 2002). AGEs bind to RAGE, inducing processes related to cellular activation and inflammation, such as the activation of nuclear factor-κB (NF-κB) (Bierhaus et al. 2001). NF-κB activation stimulates the expression of RAGE (Li and Schmidt 1997), thus initiating a self-amplifying cycle. The activation of NF-κB also promotes the expression of pro-inflammatory cytokines, such as IL-6 (Bierhaus et al. 2004) and TNF-α (Tanaka et al. 2000) which can ultimately impair normal cellular/tissue functions. Sustained RAGE-mediated cellular activation has been shown to contribute to disease progression in diabetes (Yan et al. 2003), Alzheimer's disease (Lue et al. 2001), arthritis (Sunahori et al. 2006) and neurodegeneration (Ramasamy et al. 2005).

Evidence suggests that AGEs form during natural ageing. Furthermore it was reported that RAGE over-expression leads impairments of Ca^{2+} handling in cultivated cardiac

myocytes (Petrova et al. 2002). Therefore, the mechanisms by which AGEs act in age-related cardiomyocyte dysfunction may involve RAGE upregulation and NF-κB activation. The aims of the present study were to examine the effect of ageing on cardiac contractile function and to assess the involvement of AGE-related pathways in the pathogenesis of cardiac dysfunction in aged rats.

Materials and Methods

Chemicals

Hydroxyethyl piperazine ethanesulfonic acid (HEPES), collagenase I, thiobarbituric acid, GSH, o-phthalaldehyde (OPT), collagenase VII, Nonidet P-40 (NP-40) and protease inhibitors cocktail were purchased from Sigma (St. Louis, MO, USA). The reagents obtained from other sources are detailed within the text. All other chemicals were of analytical grade.

Experimental animals

Animal housing and all experimental procedures followed the requirements of the Provisions and General Recommendations of the Chinese Experimental Animal Administration Legislation. Healthy male Sprague-Dawley rats used in this study were obtained from Laboratory Animal Center of the Fourth Military Medical University, Xi'an, China. Animals belonged to two different age groups: 2-months-old and 25-months-old. The rats were housed in large cages (five to six 2-month-old rats in one cage, two to three 25-month-old rats in one cage) and maintained on a 12 : 12 h/light : dark illumination cycle and provided food and water ad libitum.

Myocyte isolation

The rats were anesthetized with sodium pentobarbital (30 mg/kg) and heparinized with 200 U of heparin injected intraperitoneally. Hearts were quickly removed and perfused by intubation *via* the aorta with Krebs–Henseleit bicarbonate buffer (in mmol/l: 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 HEPES and 11.1 glucose, pH 7.4). Hearts were subsequently perfused with Ca^{2+}-free KHB containing 1 mg/ml collagenase I. After perfusion, the left ventricle was removed, minced, and the cells were filtered through a nylon mesh (200 mm). Myocytes were washed with Ca^{2+}-free KHB buffer to remove remnant enzymes, and extracellular Ca^{2+} was added incrementally for four times and slowly back to 1.25 mmol/l. Mechanical properties remained relatively stable in myocytes for 10 h. Cells that had any obvious sarcolemmal blebs or spontaneous contractions were not used; only rod-shaped myocytes with distinctly clear edges were selected for recording of mechanical properties.

Myocyte contractility

Mechanical properties of left ventricular myocytes were assessed by using a video-based edge-detection system (Crescent, USA). For this study, the initial stimulus frequency was 1 Hz and, following 10 min of stabilization, cell shortening was simultaneously recorded. The stimulus frequency was subsequently increased to 2, 3, and 4 Hz with 2 min of stabilization at each new frequency prior to recording cell shortening. Cell shortening and relengthening were assessed using the following indices: peak twitch amplitude (PTA), the amplitude myocytes shortened upon electrical stimulation, an indication of peak ventricular contractility; time-to-PTA (TPS), the duration of myocytes shortening, an indication of systolic duration; time-to-75% relengthening (TR75), the duration to reach 75% relengthening, an indication of diastolic duration; and maximal velocities of shortening (+dL/dt) and relengthening (–dL/dt), the maximal slope of shortening and relengthening phases, an indication of maximal velocities of ventricular pressure increase/decrease.

AGEs determination

AGEs content was estimated according to established procedures (Monnier et al. 1986). Briefly, the rats were anesthetized with sodium pentobarbital (30 mg/kg) and draw blood through abdominal aorta. The hearts were removed quickly, minced with 0.9% cold saline, and homogenized with cold phosphate-buffered saline. The samples were then centrifuged at 3000 × *g* at 4°C, and the supernatant was decanted. Lipids were extracted with chloroform-methanol for 24 h at 4°C. The solution was then rehydrated with 2 ml methanol and 0.5 ml distilled water. After two rinses with methanol and distilled water, the samples were transferred to HEPES buffer with 0.01 mol/l $CaCl_2$, and incubated for 12 h. The samples were then digested with 280 U/ml collagenase VII at 37°C during vigorous shaking. Finally, the supernatant was obtained and examined fluorometrically with a spectrophotometer at excitation/emission 370/440 nm. The values were corrected for the collagenase VII blank and then normalized to millimolar hydroxyproline content. Therefore, the level of AGEs was expressed by the intensity of fluorescence as arbitrary units per unit of millimolar hydroxyproline content in the collagenase-digested heart homogenate. Hydroxyproline levels in the supernatant were measured after hydrolysis with 6 mol/l HCl by using the method described by Inayama and colleagues (1978).

Preparation of membrane protein

Frozen heart tissues were homogenized on ice in buffer A (0.32 mol/l sucrose, 5 mmol/l Tris-HCl (pH 7.5), 20 mmol/l KCl, 1 mmol/l EDTA, 1 mmol/l EGTA) with a protease inhibitors cocktail. The homogenate was centrifuged at 4°C

at 100,000 × *g* for 1 h. The pellet was then re-suspended in buffer B (20 mmol/l HEPES (pH 7.5), 10% glycerin, 2% Triton X-100, 1 mmol/l EDTA, 1 mmol/l EGTA) with a protease inhibitors cocktail. The samples were stored at –80°C until use and the protein content of each sample was determined using the bicinchoninic acid protein assay (Sigma Chemical Co., St. Louis, MO, USA).

Preparation of nuclear and cytoplasmic protein

Frozen heart tissues were homogenized on ice in the hypotonic extraction buffer (in mmol/l: 10 HEPES, 10 KCl, 1 EGTA, 1 EDTA, 0.5 DTT, and 0.5% NP-40) with a protease inhibitors cocktail for 15 min on ice, and then centrifuged at 12,000 × *g* for 15 min. The supernatant corresponded to the cytoplasmic extract. The pelleted nuclei were re-suspended in the hypertonic extraction buffer (10 mmol/l HEPES, 0.4 mol/l KCl, 1 mmol/l EGTA, 1 mmol/l EDTA, 0.5 mmol/l DTT) with a protease inhibitors cocktail, constantly shaken at 4°C for 30 min, and then centrifuged at 20,000 × *g* for 20 min. The samples were stored at –80°C until use and the protein content of each sample was determined using the bicinchoninic acid protein assay (Sigma Chemical Co.).

Western blot analysis

The protein samples were boiled at 100°C for 5 min with a gel-loading buffer (0.125 mol/l Tris-HCl, 4% SDS, 10% β-mercaptoethanol, 0.2% bromphenol blue, pH 6.8) at a ratio of 1 : 1. Protein sample was separated by SDS-PAGE using 10% acrylamide gels (Laemmli 1970) and transferred to nitrocellulose membranes (BioRad, USA) using the Bio-Rad Mini Trans-Blot system. The membranes were blocked for 1.5 h at room temperature with 5% non-fat milk. The membranes were incubated with an anti-RAGE monoclonal IgG antibody (MAB1179; R&D Systems, USA) and an anti-NF-κB subunit p65 monoclonal IgG antibody (sc-8008; Santa Cruz, USA) in TBS (tris buffered saline) + 5% non-fat milk overnight at 4°C, followed by incubation with HRP (horseradish peroxidase)-conjugated goat anti-rabbit IgG (Santa Cruz, USA) or goat anti-mouse IgG antibody (Santa Cruz, USA) for 1 h at 37°C. Proteins bands were visualized with the enhanced chemiluminescence detection system (Pierce, USA). The X-ray films

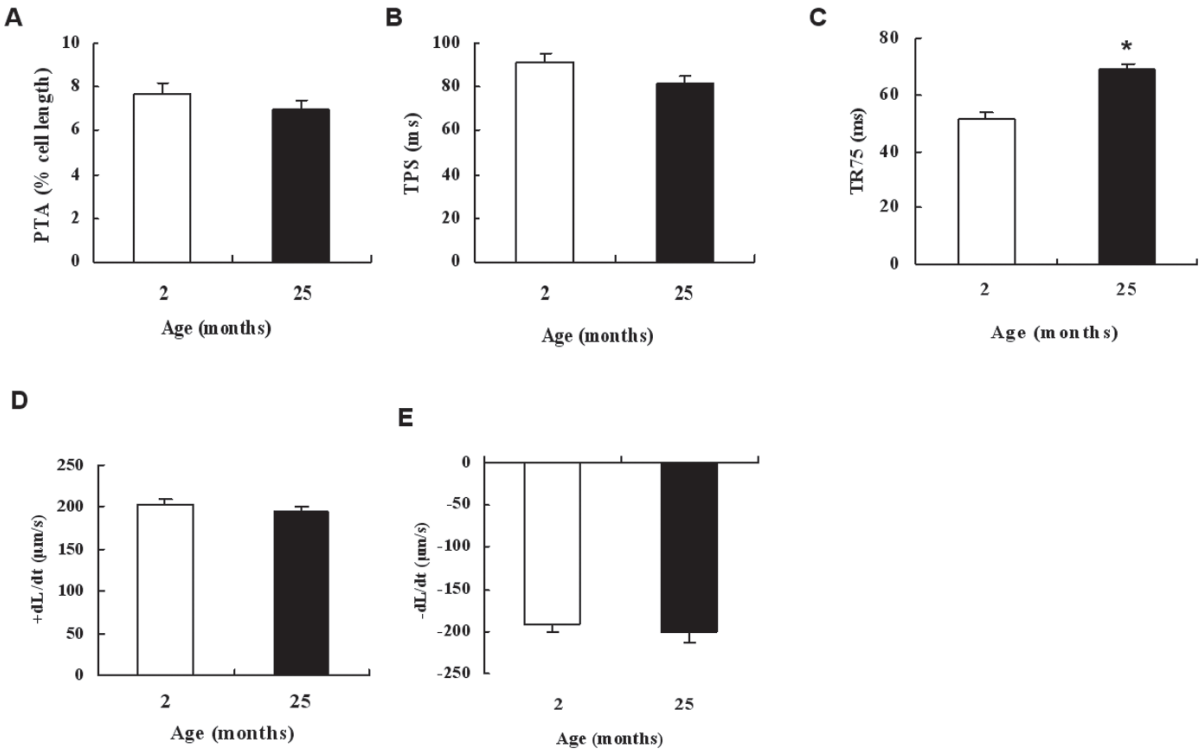

Figure 1. Contractile properties of left ventricular myocytes stimulated at 1 Hz from young ($n = 8$) and aged ($n = 8$) rat hearts. **A.** Peak twitch amplitude (PTA). **B.** Time-to-peak twitch amplitude (TPS). **C.** Time-to-75% relengthening (TR75). **D.** Maximal velocity of shortening (+dL/dt). **E.** Maximal velocity of relengthening (–dL/dt). Myocyte data from eight young and eight aged hearts were averaged and expressed as means ± SEM (5–7 myocytes were obtained from each heart; 48 cells/group). * $p < 0.01$ compared with young myocytes (Student's *t*-tests).

were scanned and band intensities were quantified by optical densitometry using image-analysis software.

Statistical analysis

Analysis was performed using the SPSS version 10.0 package. All data are presented as mean ± SEM. Differences between two groups (2-month-old versus 25-month-old) were assessed using Student's *t*-tests. Measurements made sequentially were compared by repeated measures ANOVA. Values of $p < 0.05$ were considered statistically significant.

Results

Mechanical properties of left ventricular myocytes stimulated at 1 Hz

As show in Figure 1, young and aged myocytes stimulated at a frequency of 1 Hz display a similar extent of contractile capacity as indicated by PTA and TPS. However, the duration of TR75 was significantly prolonged in aged myocytes compared with young myocytes ($p < 0.01$). There were no differences in +dL/dt and –dL/dt in aged myocytes compared with young myones.

To evaluate the impact of aging on cardiac contractile function at higher frequencies, the stimulus frequency was increased from 1 to 4 Hz. Figure 2 showed the result of PTA in aged myocytes at different stimulus frequency compared with that in young myocytes. At baseline (1 Hz), there was no difference in the PTA in aged myocytes (7.73 ± 0.48%) compared with young myocytes (6.96 ± 0.42%). At 2, 3, and 4 Hz, the PTA was significantly low in aged myocytes compared with young myones.

AGEs content

Cardiac AGEs content was significantly increased in aged rats (Figure 3; $p < 0.01$). Fluorescence of the AGEs increased from 2.54 ± 0.11 AU/mmol hydroxyproline in 2-month-old rats to 3.76 ± 0.23 AU/mmol hydroxyproline in 25-month-old rats.

RAGE expression

RAGE protein level analysis was performed by Western blotting (Figure 4). A significant increase in RAGE expression was observed in aged rat hearts compared with young ones.

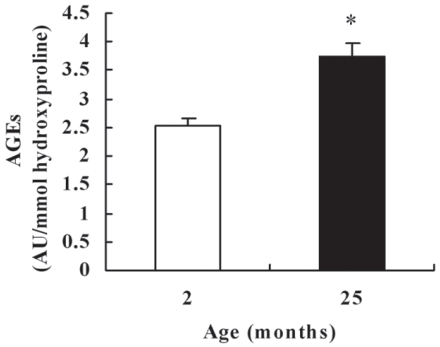

Figure 3. Fluorometric estimation of advanced glycation end products (AGEs) level in cardiac tissue. Values are arbitrary units (AU) per unit of millimolar hydroxyproline. Values are expressed as means ± SEM, $n = 8$. * $p < 0.01$ compared with 2-month-old rats (Student's *t*-tests).

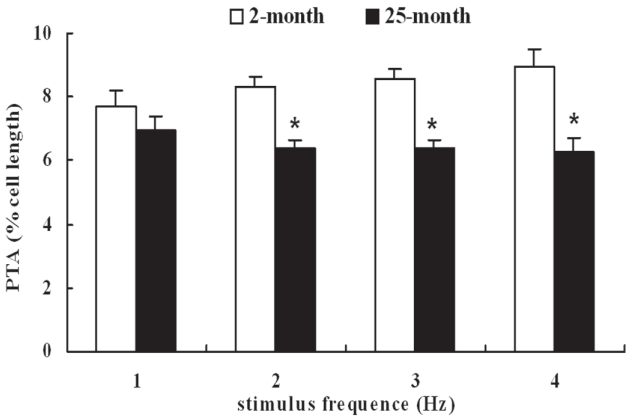

Figure 2. Peak twitch amplitude (PTA) of ventricular myocytes from young ($n = 8$) and aged ($n = 8$) rat hearts at different stimulation frequencies (1, 2, 3, and 4 Hz). Myocyte data from eight young and eight aged hearts were averaged and expressed as means ± SEM (5–7 myocytes were obtained from each heart, 48 cells/group). * $p < 0.01$ compared with young myocytes (repeated measures ANOVA).

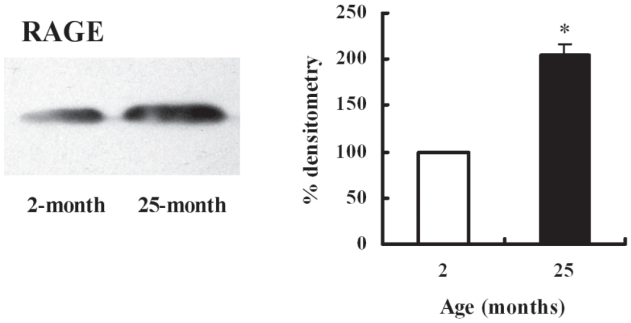

Figure 4. Effect of ageing on receptor for advanced glycation end-products (RAGE) expression in cardiac tissue examined by Western blot. The levels of each protein were quantified by densitometry. Values are means ± SEM, $n = 8$. The data shown are presented as the percentage relative to 2-month-old rats. * $p < 0.01$ compared with 2-month-old rats (Student's *t*-tests).

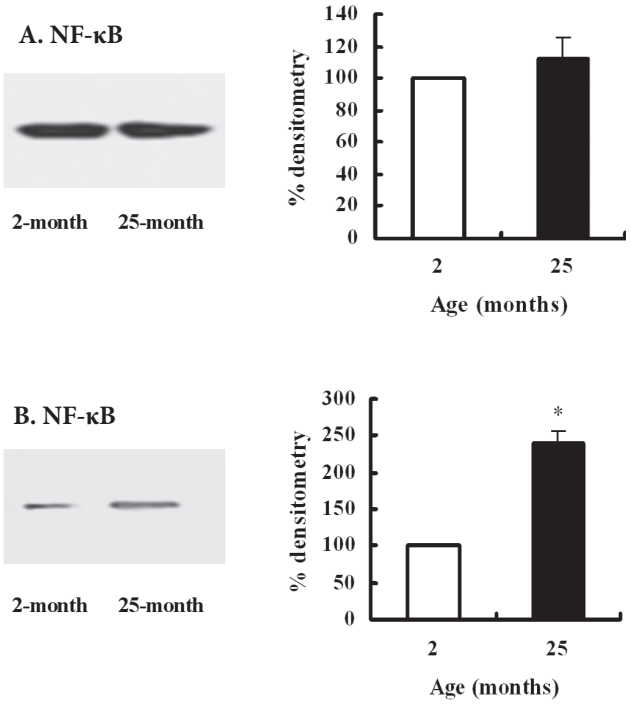

Figure 5. Effect of ageing on NF-κB expression in cytoplasmic (A) and nuclear (B) extracts from cardiac tissue. Cytoplasmic and nuclear extracts were analyzed by Western blot using an anti-NF-κB antibody specific for the p65 subunit. The levels of each protein were quantified by densitometry. Values are means ± SEM, $n = 8$. The data shown are presented as the percentage relative to 2-month-old rats. * $p < 0.01$ compared with 2-month-old rats (Student's *t*-tests).

NF-κB activation

It is known that NF-κB is first activated in the cytoplasm through phosphorylation and is subsequently translocated to the nucleus where it is thought to affect transcription. In the present study, NF-κB p65 protein was detected in the nuclear and cytoplasmic extracts of heart tissue. Higher expression levels were detected in the nuclear extracts and a similar level of expression was detected in the cytoplasmic extracts of the aged group compared with the young group (Figure 5).

Discussion

In our study, aged cardiocytes displayed prolonged relaxation duration and impaired tolerance to increased stimulus frequency which demonstrated ageing leading to cardiac contractile dysfunction. In addition, AGEs levels, RAGE expression, and activation of NF-κB were found in aged cardiomyocyte. We favor that AGEs accumulation and RAGE upregulation may be related to ageing-induced cardiomyocyte dysfunction.

The dysfunction of left ventricle can induce more diseases than that of right ventricle to old man. So, this study investigated the impact of aging on cardiac contractile function. We found a prolonged duration of relengthening (TR75) associated with normal PTA in aged myocytes stimulated at a frequency of 1 Hz. From data, there is a trend for PTA to decrease in aged myocytes, and an opposing trend for PTA to increase in young myocytes, with increasing stimulus frequency. At 2, 3, and 4 Hz, PTA was significantly low in aged myocytes compared with young myocytes. These results demonstrated ageing lead to cardiac contractile dysfunction. In the present study, ageing-induced cardiomyocyte dysfunction was associated with AGEs accumulation and RAGE upregulation, which implicated the involvement of AGE/RAGE interactions in the pathogenesis of ageing-associated cardiac mechanical dysfunction. It was reported that RAGE overexpression leads to impairments in Ca^{2+} handling in cultivated cardiac myocytes (Petrova et al. 2002). Altered myosin heavy chain isoform expression (Carnes et al. 2004) and impaired sarcoplasmic reticulum function (Janczewski et al. 2002; Kass et al. 2004; Bers et al. 2006) have been shown to be related to cardiac systolic and diastolic dysfunction, probably due to changes in cardiac intracellular calcium concentration. Thus, in the present study, whether RAGE upregulation led Ca^{2+} handling impairments and thus may account for cardiomyocyte dysfunction in aged rats requires further study.

Several AGE-binding proteins have been identified, including AGE-R1, AGE-R2, AGE-R3, the scavenger receptor II and RAGE (Thornalley 1998). Although other receptors are known to interact with AGEs, many effects of AGEs on cells are commonly attributed to the interaction of the modified protein with RAGE (Bucciarelli et al. 2002). AGEs through interactions with RAGE have been implicated in the pathophysiology of numerous age-related diseases such as diabetes (Goldin et al. 2006), neurodegeneration (Ramasamy et al. 2005), renal disease (Bohlender et al. 2005). However, there is a little study on RAGE expression in ageing heart tissue. Only Simm reported that in human subjects undergoing cardiac surgery, an age-dependent increase in RAGE protein in the atria was observed (Simm et al. 2004). In the present study, we compared the protein expression levels of RAGE in aged rat hearts with that in young rat hearts and found an upregulation in the protein expression of RAGE. The binding of AGEs to RAGE has been demonstrated to trigger signal transduction mechanisms, including NF-κB activation (Miyata et al. 1996; Alves et al. 2005). In thisstudy, a higher concentration of NF-κB was found in the nuclear extract from aged rat hearts. NF-κB is considered a critical contributing factor to the RAGE-dependent development of numerous age-related diseases, through promotion of several secondary messenger systems and increasing the production of pro-inflammatory cytokines (Ramasamy et al.

2005). More work is required to further characterize specific genes that contribute to ageing-associated cardiomyocyte dysfunction.

In summary, our study supports the idea that the 'ageing process' itself is a risk factor for cardiomyocyte dysfunction. This study provides evidence for the hypothesis that an increase in oxidative stress, AGEs, RAGE, and the activation of NF-κB, may be involved in the physiopathology of cardiomyocyte dysfunction during ageing. Ongoing studies will help to elucidate whether any of these steps could be a target for therapeutic intervention in ageing cardiomyocyte dysfunction.

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