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Sestrin2 prevents age-related intolerance to post myocardial infarction *via* AMPK/PGC-1 α pathway

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Abstract

We have revealed that a novel stress-inducible protein, Sestrin2, declines in the heart with aging. Moreover, there is an interaction between Sestrin2 and energy sensor AMPK in the heart in response to ischemic stress. The objective of this study is to determine whether Sestrin2-AMPK complex modulates PGC-1 α in the heart and protects the heart from ischemic insults. In order to characterize the role of cardiac Sestrin2-AMPK signaling cascade in aging, C57BL/6 wild type young mice (3–4 months), aged mice (24–26 months) and young Sestrin2 KO mice were subjected to left anterior descending coronary artery occlusion for *in vivo* regional ischemia. Intriguingly, ischemic AMPK activation was blunted in aged WT and young Sestrin2 KO hearts as compared with young WT hearts. In addition, the AMPK downstream PGC-1 α was down-regulated in the aged and Sestrin2 KO hearts during post myocardial infarction. To further determine the regulation of AMPK on mitochondrial functions in aging, the downstream of mitochondrial biogenesis PGC-1 α transcriptional factor were measured. The results demonstrated that the PGC-1 α downstream effectors TFAM and UCP2 were impaired in the aged and Sestrin2 KO post-MI hearts as compared to the young hearts. While the apoptotic flux markers such as AIF, Bax/Bcl-2 were up-regulated in both aged and Sestrin2 KO hearts *versus* young hearts. Furthermore, both Sestrin2 KO and aged hearts demonstrated more susceptible to ischemic insults as compared to young hearts. Additionally, the adeno-associated virus (AAV9)-Sestrin2 delivered to the aged hearts *via* a coronary delivery approach significantly rescued the ischemic tolerance of aged hearts. Taken together, the decreased Sestrin2 levels in aging lead to an impaired AMPK/PGC-1 α signaling cascade and an increased sensitivity to ischemic insults.

Keywords

Sestrin2; Aging; Mitochondria; PGC-1 α

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Disclosures

The authors declare that they have no conflict of interest.

1. Introduction

The clinical findings and animal models of myocardial infarction demonstrated that an ability of the myocardium to tolerate ischemia becomes significantly compromised with aging [1–3]. AMP-activated protein kinase (AMPK) acts as an energetic stress sensor that modulates crucial processes, such as substrate metabolism and autophagy during myocardial ischemia [4]. We have demonstrated that during myocardial ischemia, AMPK activation is significantly impaired in the aged heart and that this is directly associated with increased myocardial infarction and cardiomyocyte dysfunction [5,6]. A novel group of proteins that lack kinase activity, known as the Sestrins, have been shown to increase the activation of AMPK *in vitro* and *in vivo* [7]. We postulate that Sestrin2 (Sesn2) may represent a particularly important survival mechanism in the heart during myocardial ischemia, in part by amplifying AMPK activation during ischemia. The induction of Sesn2 has been shown to be primarily mediated by hypoxia-inducible factor 1 α (HIF-1 α) [8,9]; we have found that there is an impaired HIF-1 α response in the aged *versus* young hearts [5]. Thus, in the event of myocardial ischemia in the aged heart, Sesn2 induction and/or function is likely to be blunted, which may alter AMPK activation. Moreover, Sestrins are indeed important in the aging process, as Sestrin gene deletion in *Drosophila* results in hallmark age-related pathologies such as increased lipid accumulation, increased oxidative stress, poor cardiac performance, and defective autophagy [10].

The mitochondria are the major organelles that produce reactive oxygen species (ROS) [11]. The ROS-detoxifying system and mitochondrial biogenesis may play vital roles as endogenous protective mechanisms during myocardial ischemia [11]. There is evidence that the peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α) is a powerful stimulator of mitochondrial biogenesis and gene transcription in the liver, heart, and skeletal muscle [12]. We have reported that AMPK regulates PGC-1 α which is critical for modulating mitochondrial biogenesis [13]. Recently, we revealed that Sesn2 as a scaffold protein promotes cardiac AMPK activation in response to ischemic stress [14]. Therefore, we hypothesized that Sesn2-AMPK signaling enhanced mitochondrial biogenesis and alleviated myocardial ischemic injury by eliminating ROS through the Sesn2-AMPK-PGC-1 α pathway. The impaired Sesn2-AMPK signaling cascade in aging could lead to the intolerance of aged hearts to ischemic insults, and rescue of the decreased Sesn2 levels in the aged hearts could improve the resistance of aged hearts to ischemic stress. We anticipate that an age-related protein Sestrin2 mediates the cardioprotection of AMPK-PGC-1 α signaling cascade against ischemic insults *via* modulation of mitochondrial functions.

2. Materials and methods

2.1.1. Animals

Young (3–4 months) and aged (24–26 months) C57BL/6J mice were from Charles River. Transgenic AMPK kinase dead (KD) mice that express a KD α 2 isoform (K45R mutation), driven in heart and skeletal muscle by the muscle creatine kinase promoter, were gifted from Dr. Morris Birnbaum [15]. Sestrin2 KO mice (C57BL/6J) were generated as previously described [7,14] and Sesn2 KO mice (male and female) back-crossed for at least 9 generations with C57BL/6J mice. The numbers of animals used in each experimental

condition are presented in the Results section. All animal protocols in this study were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Centre and conform to the NIH Guide for the care and use of laboratory animals.

2.1.2. Adeno-associated viral delivery

Mice were anesthetized with isoflurane (2%) and placed on a ventilator. The chest was entered from the left side through the fourth intercostal space. After dissection of the aorta and pulmonary artery, the adeno-associated virus (AAV9)-Sesn2 (5×10^7 gc per mouse) (Applied Biological Materials Inc., AAVP0205933) was injected into the left ventricular cavity through a 27G catheter while the aorta and pulmonary artery were transiently cross-clamped for 50 s ($n = 4$) [16]. In sham-operated animals ($n = 4$), normal saline was injected into the left ventricular cavity while the aorta and pulmonary artery were cross-clamped for 50 s. This procedure allows the solution that contains the adeno-associated virus (AAV) to circulate down the coronary arteries and perfuse the heart without direct manipulation of the coronaries. After 50 s, the clamp on the aorta and pulmonary artery was released. After removal of air and blood, the chest was closed, and animals were extubated and transferred back to the cages.

2.1.3. In vivo regional ischemia and infarct size measurement

Mice were anesthetized with isoflurane (2%), placed on a ventilator (Harvard apparatus, Holliston, MA, USA), and core temperature was maintained at 37°C with a heating pad. After left lateral thoracotomy, the left anterior descending coronary artery was occluded for 10 min ($n = 4$), 6 h ($n = 5$) or 24 h ($n = 4-5$) for biochemical and functional analysis as shown in the below graphic illustration with an 8-0 nylon suture and polyethylene tubing to prevent arterial injury. An electrocardiogram (ECG) and blanching of LV confirmed ischemic repolarization changes (ST-segment elevation) during coronary occlusion (ADInstruments, Colorado Springs, CO, USA). The hearts were then excised and stained with 2, 3, 5-triphenyltetrazolium (TTC) to delineate the extent of myocardial necrosis. Hearts were then fixed, sectioned, photographed with a Leica microscope, and analyzed with the ImageJ Software (U.S. National Institutes of Health, Bethesda, MD, USA) [14,17].

2.1.4. Immunoblotting

Western Immunoblots were performed as previously described [14,18,19]. Rabbit antibodies p-AMPK α (Thr¹⁷²) (#2535), AMPK α (#5831), p-acetyl-CoA carboxylase (pACC) (Ser⁷⁹) (#11818), ACC (#3676), mitochondrial uncoupling protein 2 (UCP2) (#89326), apoptosis-inducing factor (AIF) (#5318), Bcl-2 (#3498), Bax (#14796), and β -Tubulin (#2146) were obtained from Cell Signaling Tech (Danvers, MA, USA). Rabbit Sestrin2 antibody (#10795) was obtained from Protein Tech (Chicago, IL, USA). Rabbit peroxisome proliferator-

activated receptor gamma coactivator 1-alpha (PGC-1 α) (sc13067) was obtained from Santa Cruz Technology (Santa Cruz, CA, USA). Mitochondrial transcription factor (TFAM) antibody (AB131607) was obtained from Abcam (Cambridge, MA, USA).

2.1.5. Transmission electron microscope

Heart tissues were rapidly immersed in tissue fixative buffer (10% formaldehyde, buffered, pH 7.4, Carson-Millonig formulation; RI31911; Ricca Chemicals, Arlington, TX, USA) at 4 °C for at least 8 h. Fixed tissues were trimmed to 1 mm³ in size, stained with OsO₄ for 1 h, dehydrated in a graded ethanol series for 10 min per step (once in 35, 50, 70, and 95% and twice in 100% ethanol), washed twice with acetone for 15 min per wash, washed in a solution of 1:1 acetone:Epon for 1 h, and finally embedded in 100% Epon (Ted Pella, Redding, CA, USA) for 30 min. After incubating at 60 °C overnight, the Epon block was semithin sectioned (10–12 1- μ m-thick sections) by using Sorvall MT-6000-XL (RMC Boeckeler, Tucson, AZ, USA) and a glass knife. Semithin sections were stained with 1% toluidine and observed under a light microscope to locate interested areas for ultrathin sections. After trim, the Epon block was thin sectioned (70 nm thick) by using a Leica Reichert Ultracut microtome and diamond knife. Thin sections were then applied on copper grids, air dried, stained with 2% uranyl acetate for 3 min and calcinated lead citrate for 30 s, and rinsed in distilled water by briskly dipping up and down for 20 s. The stained grid was loaded in a Jem1400 transmission electron microscopy (Jeol, Tokyo, Japan) with an ANT camera system located in the Department of Pathology, University of Mississippi Medical Center. At least 5 sections from each sample were examined under transmission electron microscope. Each entire section was thoroughly viewed at low magnifications to find areas of interest and to observe size, shape and arrangement of subcellular organelles, including mitochondria, lipid droplets, nuclei, chromatin, muscle fibers, and autophagosomes [20].

2.1.6. In vivo evaluation of heart function by echocardiography

The animals from each group (n = 4–5) were anesthetized (isoflurane), and transthoracic M-mode echocardiography (Vevo 770, Visual-Sonics, Toronto) was performed to measure cardiac function, wall thickness, and chamber volumes. Left ventricle (LV) wall thickness were measured using a modified version of the leading-edge method by the American Society for Echocardiography using three consecutive cycles of M-mode tracing [18]. Myocardial peak velocities (systolic and diastolic) were derived at the mitral valve level, and the ratio between early mitral inflow velocity and mitral annular early diastolic velocity (E/E') ratio was used as an indicator of LV filling pressure [21]. Other indicators of diastolic function included isovolumetric relaxation time (IVRT), mitral valve deceleration time (MVDT), ratio of early (E) and late (A) mitral inflow velocities (E/A), and Tei index (calculated as sum of isovolumetric relaxation and contraction times (IVRT + IVCT) divided by ejection time (ET) were also calculated. Simpson's measurements were performed to obtain an averaged ejection fraction (%EF) and fraction shortening (%FS) of all coronary artery ligation animals and 6 representative samples of Sham group. Only a representative sample of Sham hearts was required since wall motions were observed to have synchronous motion, whereas infarcted group had asynchronous wall motion. Since all coronary artery ligation mice were expected to have infarcts and asynchronous wall motion, the averaged %EF and %FS were calculated using Simpson's measurements [22].

2.1.7. Immunohistochemistry

Immunohistochemical staining was performed as previously described [23]. The histological sections were localized to the ischemic area. Heart sections were stained with primary antibodies against Caspase-3 (1:200, Cell signaling Technology, Beverly, MA, USA) or IgG control at 4°C overnight, and then with second antibody (Vectastain ABC Kit, VECTOR Laboratories, Inc., Burlingame, CA). Peroxidase activity was visualized with use of diaminobenzidine (Peroxidase Substrate Kit, VECTOR Laboratories, Inc., Burlingame, CA), and the sections were counterstained with hematoxylin. The numbers of Caspase-3 positive cells were counted blindly and expressed as a percentage of total number of cardiomyocytes in six sequentially cut 5 µm sections of the ischemic lesion for each heart. Digital photographs were taken at 200 × magnifications of over 20 random fields from each heart, and the positive areas were calculated by NIH Image J software.

2.1.8. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels

The SOD activity can be measured in terms of its ability to inhibit reduction of nitro blue tetrazolium, a superoxide radical-dependent reaction [24]. SOD activity and MDA levels were measured using commercially available assay kits (Cayman Chemical, Ann Arbor, MI). Cardiac SOD activities and MDA levels in young WT, aged WT and young *Sesn2* KO hearts under sham (n = 4 per group) or post myocardial infarction conditions (n = 5 per group) were measured following kit instructions.

2.1.9. RNA isolation and real-time qPCR

Total RNA was isolated from heart tissues using Trizol reagent (Invitrogen) and real-time qPCR was performed as previously described [18]. Q-PCR was performed in a 20 µL reaction mixture prepared with SYBR GREEN PCR Master Mix (Applied Biosystems, Warrington, UK) containing an appropriately diluted cDNA solution and 0.2 mM of each primer at 95 °C for 10 min, followed by 35 cycles at 95 °C for 10 s and 60 °C for 45 s. All reactions were conducted in triplicated and data were analyzed using the delta Ct (DDCt) method. These transcripts were normalized to β-actin. The primers for PGC-1α were as follows: forward, 5'-TGATAAACTGAGCTACCCTTG-3'; reverse, 5'-ACACTGAGTCTCGACACGG-3', and β-actin was used as a housekeeping gene.

2.1.10. Relative quantification of mtDNA copy number

Total DNA was isolated from hearts of young WT, aged WT, and young *Sesn2* KO mice (n = 5 per group) and treated with RNase A (Invitrogen). The mtDNA content relative to nuclear DNA was assessed by real-time qPCR (Bio-Rad) [25]. Relative mtDNA content was determined using C_T -method. Primers of the mtDNA encoded *COI* gene were: forward, 5'-TGCTAGCCGCAGGCATTAC-3'; reverse, 5'-GGGTGCCCAAAGAATCAGAAC-3'. Primers of the single-copy nuclear gene *Ndufv1* were: forward, 5'-CTTCCCCACTGGCCTCAAG-3'; reverse, 5'-CCAAAACCCAGTGATCCAGC-3'.

2.1.11. Statistical analysis

Data are reported as the mean ± SEM. The number of experiments in each group is presented in the text, figure, or figure legend. Differences between treated group and vehicle

control groups were assessed by Student two-tail *t*-test. Changes in LV contractile functions over time were determined by two-way ANOVA using Tukey's test for *post hoc* comparisons using GraphPad Prism 5.0. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Cardiac AMPK mediates PGC-1 α levels by post myocardial infarction

In order to characterize the role of Sesn2-AMPK signaling cascade in cardiac PGC-1 α levels under normal physiological and myocardial ischemic conditions, the C57BL/6J wild type (WT), Sesn2 KO, and AMPK kinase dead (AMPK KD) transgenic mice were subjected to ligation of left anterior descending coronary artery (LAD) by suture to induce an *in vivo* regional ischemia 10 min for AMPK signaling measurements and 6 h for post myocardial infarction (Post-MI) determination. The results demonstrated that the Sesn2 levels are the same between WT and AMPK KD hearts, indicating that cardiac AMPK activation does not affect Sesn2 protein levels in the myocardium (Fig. 1A). The immunoblotting results demonstrated that activation of cardioprotective AMPK signaling was impaired in the Sesn2 KO hearts and was abolished in AMPK KD hearts as compared to WT hearts in response to ischemic stress (Fig. 1B). Interestingly, the levels of PPAR- γ coactivator-1 α (PGC-1 α) were up-regulated in response to 6 h of post myocardial infarction in WT hearts but not in AMPK KD hearts (Fig. 1C). Moreover, the PGC-1 α levels of post-MI Sesn2 KO hearts were impaired *versus* WT hearts (Fig. 1C). The mRNA levels of PGC-1 α in WT, Sesn2 KO, and AMPK KD hearts demonstrated a similar pattern as the alterations in PGC-1 α protein levels between WT, Sesn2 KO, and AMPK KD hearts (Fig. 1D). In order to assess the contributions of Sesn2 to the impaired PGC-1 α levels in Sesn2 KO hearts during post-MI conditions, the adeno-associated virus (AAV9)-Sesn2 was injected into the left ventricular cavity through a 27G catheter while the aorta and pulmonary artery were crossed-clamped for 50 s. This procedure allows the solution that contains the adeno-associated virus (AAV9) to circulate down the coronary arteries and perfuse the heart without direct manipulation of the coronaries. After seven days, the immunoblotting data showed that AAV-Sesn2 rescued the levels of Sestrin2 in the Sesn2 KO hearts (Fig. 1E), and the impaired AMPK phosphorylation of Sesn2 KO hearts during myocardial ischemia was significantly improved with AAV-Sesn2 treatment (Fig. 1F). Importantly, AAV-Sesn2 treatment rescued PGC-1 α levels in Sesn2 KO hearts but not AMPK KD hearts during post-MI (Fig. 1G).

3.2. Impaired AMPK/PGC-1 α signaling in the aged and Sens2 KO hearts

Sestrin2 (Sesn2) is postulated to be an anti-aging protein, as Sestrin deficiency in *Drosophila* and Sesn2 deletion results in classic age-related pathologies such as increased lipid accumulation, defective autophagy, and reduced cardiac function [10]. The age-related Sens2 protein serves as a stress induced scaffold protein modulates ischemic AMPK activation [14,16]. In order to determine whether the decreased Sesn2 in aged hearts was a reason of intolerance of aged hearts to ischemic damage, we compared the cardiac functions between young WT, aged WT and young Sesn2 KO hearts under sham operation and post-MI conditions. The echocardiographic data showed that cardiac contractile functions were similar between young, aged and Sesn2 KO hearts under sham operations (Fig. 2A); moreover, after post-MI 24 h by LAD ligation, cardiac dysfunction was observed in all

groups, with the cardiac dysfunction becoming worse in aged and *Sesn2* KO mice as shown by ejection fraction and fractional shortening (Fig. 2A). The hearts from young, aged, and *Sesn2* KO groups were stained with Evan's blue and TTC to determine the extent of necrosis, and myocardial infarction size was significantly increased in aged and *Sesn2* KO hearts *versus* young hearts (Fig. 2B). The transmission electronic microscopy results demonstrated that there is no obvious alterations in mitochondrial morphology of young, aged, and *Sesn2* KO hearts under sham operations, but the mitochondria in aged and *Sesn2* KO hearts appear more susceptible to post-MI *versus* young hearts' mitochondria as shown by mtDNA amount (Fig. 2C). The mtDNA analysis data showed that the amount of mtDNA was decreased during post-MI, and greater loss of mtDNA occurred in aged and *Sesn2* KO hearts *versus* young WT hearts (Fig. 2C), all of which suggesting less tolerance of aged and *Sesn2* KO heart mitochondria *versus* young heart mitochondria to ischemic insults.

The immunoblotting data demonstrated that AMPK downstream PGC-1 α was significantly lower in aged and *Sesn2* KO hearts than that in young hearts (Fig. 2D). The mitochondria-related proteins mitochondrial transcription factor A (TFAM) and uncoupling protein 2 (UCP2) were also impaired in aged and *Sesn2* KO hearts *versus* young hearts after post-MI (Fig. 2D); however, the apoptotic pathways related protein, apoptosis-inducing factor (AIF) was significantly upregulated in aged and *Sesn2* KO hearts *versus* young heart post-MI (Fig. 2D). The oxidative stress levels of aged and *Sesn2* KO hearts post-MI were significantly higher than that of young hearts, as shown by lower anti-oxidative superoxide dismutase (SOD) activities and higher levels of lipid peroxidation product malondialdehyde (MDA) (Fig. 2E). These results indicate that the impaired *Sesn2*-AMPK signaling cascade under ischemic stress in aging contributed to age-related defects in mitochondria maintenance in response to ischemic insults that caused alterations in intracellular redox status with more damage occurring during post-MI.

3.3. Post-MI causes more apoptosis in the aged and *Sesn2* KO hearts

The caspase 3 immunohistochemistry and the Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay results showed that there was significantly more apoptosis occurring in the aged and *Sesn2* KO hearts *versus* young hearts after 6 h myocardial infarction (MI) (Fig. 3A and B). The immunoblotting data with apoptosis related proteins demonstrated that MI can trigger down-regulation of anti-apoptosis protein Bcl-2 and up-regulation pro-apoptosis protein of Bax in young, aged and *Sesn2* KO hearts (Fig. 3C); interestingly, aged and *Sesn2* KO hearts *versus* young hearts demonstrated a higher ratio of Bax/Bcl-2, indicating that the aged and *Sesn2* KO hearts were more susceptible to ischemic insults than young hearts.

3.4. Rescue of impaired *Sesn2* level in aged heart improves tolerance of aged heart

In order to assess the contributions of *Sesn2* to the impaired cardiac functions in aged hearts during post-MI conditions, the adeno-associated virus (AAV9) was injected into the left ventricular cavity through a 27G catheter while the aorta and pulmonary artery were crossed-clamped for 50 s. This procedure allows the solution that contains the adeno-associated virus (AAV9) to circulate down the coronary arteries and perfuse the heart without direct manipulation of the coronaries. After seven days, the AAV-*Sesn2* rescues the

levels of Sestrin2 in the aged hearts [16]. Importantly, AAV-Sesn2 treatment significantly improved cardiac functions in aged hearts under post-MI conditions as shown by ejection fraction and fractional shortening of echocardiography analysis (Fig. 4A). Moreover, AAV-Sesn2 delivery significantly reduced myocardial infarction size in aged hearts after 6 h post-MI as shown by Evan's blue and TTC staining (Fig. 4B). All the benefits of AAV-Sesn2 for the aged hearts could be due to the rescued PGC-1 α levels and downstream effectors in response to 6 h post-myocardial ischemia in the aged hearts such as up-regulation of mitochondria related proteins TFAM and UCP2 and, down-regulation of apoptosis proteins AIF and Bax/Bcl-2 as shown by the immunoblotting (Fig. 4C).

4. Discussion

The results elucidate the fact that Sestrin2 is an essential part of the age-related adaptive response to ischemic stress as aged and Sesn2 KO hearts display similarly exacerbated myocardial infarction and impaired post-MI cardiac function as indicated by echocardiography analysis. Sestrin2 is a member of a highly conserved group of individual proteins collectively known as the Sestrins [26,27] and is expressed in various tissues including the heart. The induction and regulation of Sestrin2 expression has previously been demonstrated to be stress inducible and initiated as a result of DNA damage and oxidative stress *via* a p53-dependent manner [7]. Previous studies have attributed Sestrin2 to many important cellular processes and functions such as autophagy [28], metabolism [29], and reactive oxygen species quenching [30].

Sestrin plays a significant role in maintaining basal cardiac integrity as Sestrin-deficient hearts display aging related phenotype such as increased arrhythmias, bradycardia, disrupted myofibrils, and defective autophagy [10]. In the current study, we did not detect any significant changes in the basal cardiac phenotype between young WT, aged WT, and young Sesn2 KO mice that may have influenced any sensitivity when the ischemic insults were conducted. Despite the absence of any basal cardiac phenotype abnormalities, subjecting aged WT and young Sesn2 KO hearts to myocardial ischemia demonstrated exacerbated myocardial infarction and impaired contractile function when compared to young hearts. Moreover, viral delivery of AAV9-Sesn2 rescued Sesn2 levels of aged hearts [16], significantly improving cardiac resistance of aged hearts to ischemic insults. Therefore, Sestrin2 is cardioprotective in nature, as the reason for impaired cardiac function after post-MI was due to increased cell necrosis and apoptosis as demonstrated by TTC staining and TUNEL staining. Sestrin2 is thus an age related stress inducible protein in the heart, the down-regulation of Sestrin2 with aging could be an important factor responsible for the impaired ischemic AMPK activation that causes PGC-1 α signaling response to be blunted in the aged heart and intolerance to ischemic insults in aged hearts.

The results presented here indicate that one of the mechanisms of increased sensitivity to post-MI injury in the aged and Sesn2 KO hearts is due to the significant impairment of ischemic AMPK activation and PGC-1 α signaling, as evidenced by impaired AMPK activation [14,16] and down-regulation of mitochondria proteins post-MI in the aged and Sesn2 KO hearts. This study is the first examination of mammalian age related stress inducible protein Sestrin2 in the context of ischemic heart disease. It provides an explanation

on how Sestrin2-AMPK signaling cascades control cardiac PGC-1 α signaling cascades under ischemic stress conditions. The beneficial effects of Sestrin2 against ischemic injury by post-MI are mediated through AMPK regulating PGC-1 α [13,31]. The impaired ischemic AMPK activation observed in aged and Sestrin2 KO hearts facilitated down-regulation of PGC-1 α during post myocardial infarction (post-MI) that could potentially augment generation of more reactive oxygen species (ROS) in the mitochondria to damage cardiomyocytes [11]. The impaired mitochondrial related proteins such as TFAM and UCP2 in aged WT and Sestrin2 KO hearts as compared to young hearts implicates that more mitochondria dysfunction/damage may occur in aged WT and Sestrin2 hearts *versus* young WT hearts. The basic knowledge gained from the study is that development of Sestrin2 inducers or mimetics can lead to prevention of impaired AMPK signaling and its associated pathologies occurring in the aging population. Currently available AMPK activators were shown to be inappropriate for treatment of diabetes and metabolic syndrome due to potential liabilities of systemic AMPK activation [32]. Thus, the activity of Sestrin2 will provide an alternative treatment strategy to target Sestrin2 for type II diabetes and ischemic heart disease in the elderly. The modulation of Sestrin2 activity or expression with small molecule or peptide Sestrin2 analogues may represent a handle to further understand and eventually prevent and treat some of the most common diseases now considered to be an inevitable part of aging.

In summary, we have demonstrated that Sestrin2 is an age-related stress inducible protein and a significant player in the adaptive response to ischemic injury by influencing AMPK activation and PGC-1 α signaling cascades. Further understanding of Sestrin2-AMPK-PGC-1 α signal transduction remains an important feat for the development of novel therapeutic strategies against aging related ischemic injury. Future studies are warranted to determine how to improve Sestrin2-mediated AMPK activation which directs PGC-1 α signaling in aged hearts to achieve a cardioprotective response to ischemic insults.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yjmcc.2018.01.005>.

Abbreviations

AAV Adeno-associated virus

ACC	acetyl-CoA carboxylase
AIF	Apoptosis-inducing factor
AMPK	AMP-activated protein kinase
EF	Ejection fraction
FS	Fractional shortening
INF	Infarct
LAD	Left anterior descending coronary artery
LV	Left ventricle
MDA	Malondialdehyde
mtDNA	Mitochondrial DNA
Post-MI	Post myocardial infarction
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1- α
Sesn2	Sestrin2
SOD	Superoxide dismutase
TEM	Transmission electronic microscopy
TFAM	Mitochondrial transcription factor A
TTC	2, 3, 5-triphenyltetrazolium
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UCP2	Mitochondrial uncoupling protein 2

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Fig. 1. Sestrin2-AMPK complex regulates cardiac PGC-1α levels post-MI. (A) Immunoblotting measured the level of Sestrin2 protein expression in wild type (WT), Sestrin2 (Sesn2) KO, and AMPK kinase dead (AMPK KD) hearts, n = 4. (B) Immunoblotting measured the phosphorylation levels of AMPK and AMPK downstream acetyl CoA carboxylase (ACC) in WT, Sesn2 KO, and AMPK KD hearts under sham or ischemia (10 min of ligation of left anterior coronary artery) conditions. Values are means ± SEM, n = 4–5, *p < 0.05 vs. sham respectively; †p < 0.05 vs. WT ischemia. (C) Immunoblotting measured PGC-1α protein

levels of ischemic region in WT, Sesn2 KO and AMPK KD hearts under sham or post-MI (6 h of ligation of left anterior coronary artery) conditions. Values are means $\pm$ SEM, n = 5, *p < 0.05 vs. sham respectively; † p < 0.05 vs. WT post-MI. (D) Real-time RT-PCR showed mRNA levels of PGC-1 α in ischemia region in WT, Sesn2 KO and AMPK KD hearts under sham or post-MI (6 h of LAD ligation) conditions. Values are means $\pm$ SEM, n = 5, *p < 0.05 vs. sham respectively; † p < 0.05 vs. WT post-MI. (E) Immunoblotting showed the recovery of Sesn2 expression levels in Sesn2 KO hearts by coronary delivery of AAV9-Sesn2, n = 4. (F) Immunoblotting measured the phosphorylation level of AMPK and AMPK-downstream acetyl CoA carboxylase (ACC) in WT, Sesn2 KO, and AMPK KD hearts with or without AAV9-Sesn2 delivery under sham or ischemia (10 min of LAD ligation). Values are means $\pm$ SEM, n = 4–6, *p < 0.05 vs. sham respectively; † p < 0.05 vs. WT ischemia; $^{\#}$ p < 0.05 vs. Sesn2 KO Scram. (G) Immunoblotting measured PGC-1 α protein levels of ischemia region in WT, Sesn2 KO, and AMPK KD hearts with or without AAV9-Sesn2 delivery under sham or post-MI (6 h of LAD ligation) conditions. Values are means $\pm$ SEM, n = 5, *p < 0.05 vs. sham respectively; † p < 0.05 vs. WT post-MI.

Fig. 2. Deficiency Sestrin2 protein in aging sensitizes hearts to ischemic insults. (A) Echocardiography showed that MI causes cardiac dysfunction as shown by the decreased ejection fraction (EF) and fractional shortening (FS) in young, aged, and Sestrin2 KO mice, and the impaired EF and impaired FS occurred in aged and Sestrin2 KO hearts *versus* young hearts post-MI (24 h of LAD ligation). Values are means ± SEM, $n = 4-5$, * $p < 0.05$ *vs.* sham respectively; † $p < 0.05$ *vs.* young post-MI. (B) Evan's blue and TTC staining showed the myocardial infarction size after post MI 24 h in young, aged and Sestrin2 KO hearts. Upper: representative sections of the extent of myocardial infarction. Lower: ratio of the area at risk (AAR) to myocardium; and ratio of the infarct area (INF) to AAR. Values are means ± SEM, $n = 5$, * $p < 0.05$ *vs.* young. (C) Upper: transmission electronic microscope showed mitochondria morphology in young, aged, and Sestrin2 KO hearts under sham operations or 24 h post-MI conditions. Lower: real-time PCR measured the relative mitochondrial DNA (mtDNA) content (normalized to the single-copy nuclear gene Ndufv1) in heart tissue of young WT, aged WT, and Sestrin2 KO mice. Values are means ± SEM, $n = 5$, * $p < 0.05$ *vs.* sham respectively; † $p < 0.05$ *vs.* young post-MI. (D) Immunoblotting measured protein levels of PGC-1α, TFAM, UCP2, AIF and β-tubulin in the ischemia region of young, aged hearts and Sestrin2 KO hearts under sham or post-MI (6 h of LAD ligation) conditions. Values are means ± SEM, $n = 4$, * $p < 0.05$ *vs.* sham respectively; † $p < 0.05$ *vs.* young post-MI. (E) Measurements of SOD activity and MDA levels in young, aged and Sestrin2 KO hearts under sham or post-MI 6 h conditions. Values are means ± SEM, $n = 4-5$, * $p < 0.05$ *vs.* sham respectively; † $p < 0.05$ *vs.* young post-MI.

Fig. 3.

More apoptosis were triggered by post myocardial infarction in aged hearts. TUNEL staining (A) and Caspase 3 immunohistochemistry (B) showed more apoptosis occurred in aged and Sesn2 KO hearts *versus* young hearts under post-MI conditions (6 h of LAD ligation). Values are means $\pm$ SEM, $n = 5$, * $p < 0.05$ vs. sham respectively; $^{\dagger}p < 0.05$ vs. young post-MI. (C) Immunoblotting measured protein levels of Bcl-2, Bax and β -tubulin in the ischemia region of young, aged, and Sesn2 KO hearts under sham or 6 h post-MI

conditions. Values are means $\pm$ SEM, n = 5, *p < 0.05 vs. sham respectively; †p < 0.05 vs. young post-MI.

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Fig. 4. Viral recovery Sesn2 levels in aged hearts rescue PGC-1α levels in response to post-MI. (A) Echocardiography showed that recovery of impaired Sesn2 levels in hearts by coronary delivery of AAV9-Sesn2 to the aged heart improved the resistance of aged hearts to MI as shown by ejection fraction (EF) and fractional shortening (FS). Values are means $\pm$ SEM, $n = 4$, * $p < 0.05$ vs. Sham respectively; $\dagger p < 0.05$ vs. young post-MI. (B) Young, aged, and Sesn2 KO mice were subjected to *in vivo* myocardial ischemia by ligation of left anterior coronary artery for 24 h (post-MI). Upper: representative sections of the extent of myocardial infarction. Lower: ratio of the area at risk (AAR) to myocardium and ratio of the infarct area (INF) to AAR. Values are means $\pm$ SEM, $n = 5$, * $p < 0.05$ vs. young; $\dagger p < 0.05$ vs. aged. (C) Immunoblotting showed that the coronary delivery of AAV9-Sesn2 but not AAV9-scram significantly rescues the protein levels of PGC-1α, TFAM, UCP2, and Bcl-2 while reducing levels of AIF and Bax in aged heart under post-MI 6 h conditions. Values are means $\pm$ SEM, $n = 5$, * $p < 0.05$ vs. Sham, respectively; $\dagger p < 0.05$ vs. young post-MI; $\# p < 0.05$ vs. aged Sesn2 Scram.