Doctoral thesis (abridged version)

Role of Reactive Sulfur Species in Mitochondrial Quality Control and Ischemic Stress Resistance in Rodent Hearts

Shimoda, Kakeru

The Graduate University for Advanced Studies, SOKENDAI

School of Life Science

Department of Physiological Sciences

Introduction

Mitochondria are the organelles that produce adenosine 5'-triphosphate, the major energy molecule for cardiomyocytes, utilizing highly redox (reduction-oxidation)-dependent oxidative phosphorylation system. Mitochondria constantly undergo fission and fusion to maintain their integrity, which is critical for cardiac energy homeostasis and physiological function. In failing hearts, mitochondria in the cardiomyocytes are often found to be fragmented, and accumulating evidence suggests that mitigating mitochondrial hyperfission during heart failure ameliorates cardiac function. However, its molecular mechanism and practical therapeutic approach remain to be fully elucidated.

Reactive sulfur species (RSS), which have highly reactive sulfur atoms, are redox-active molecules that produced *in vivo*. Due to its high reactivity, it has been suggested that RSS can contribute to functional regulation of proteins by its polysufidation and energy metabolism in mitochondria. However, pathophysiological roles of RSS in the hearts are still unknown.

In this study, I revealed three factors for maintaining cardiac robustness via regulation of mitochondrial homeostasis; 1) approved anti-hypertensive drug cilnidipine, 2) control of polysulfidation of dynamin-related protein 1 (Drp1), and 3) RSS metabolism, using *in vivo* heart failure models.

Materials and Methods

Animal experiments

All animal experiments were reviewed and approved by the ethics committees at the National Institutes of Natural Sciences. All animals were maintained under air-conditioned and 12 hours light/dark cycle.

To develop heart failure model, male mice were anesthetized by isoflurane and intubated and ventilated, and chest cavity was open at the intercostal space. For myocardial infarction (MI) model, left anterior descending artery was ligated. For pressure overload-induced heart failure model by transverse aortic constriction (TAC), transverse aorta was constricted between the brachiocephalic artery and the left carotid artery by the width of 27-gauge needle. For myocardial ischemia reperfusion (I/R) model, left anterior descending artery was ligated with PE10 polyethylene tube for 15 min to stop the blood flow (ischemia) and then, ligation was removed to restart the blood flow (reperfusion). After the surgery, chest cavity was closed and buprenorphine was intraperitoneally injected as an analgesic.

Cilnidipine (20 or 30 mg/kg/day) or sodium hydrogen sulfide (NaHS, 50 μmol/kg/day) were administered intraperitoneally using a mini-osmotic pump (ALZET). For methylmercury (MeHg) exposure, MeHgCl was dissolved in drinking water (10 ppm) and mice were freely accessible. Sodium tetrasulfide (Na_2S_4, 3 nmol) was injected intracardially.

Echocardiography was performed by using Nemio-XG echocardiography (Toshiba) with a 14 MHz transducer or VEVO3100 with MX400 20-46 MHz transducer (Fujifilm VisualSonics).

Analysis of mitochondrial morphology

Mouse hearts were harvested and washed in ice cold phosphate-buffered saline. Then, hearts were fixed and processed for transmission electron microscopy imaging. Mitochondria in mouse hearts were imaged using JEOL1010 microscope (JEOL) with a Veleta CCD camera (Olympus). Mitochondrial circularity was calculated using ImageJ software (National Institutes of Health).

Analysis of mRNA expression

Total RNA were isolated from mouse hearts using RNeasy Fibrous Tissue Mini Kit (Qiagen) or from neonatal rat cardiomyocytes (NRCMs) using RNeasy Mini Kit (Qiagen) following manufacture's instruction. mRNA expression was analyzed by quantitative real-time PCR (qPCR) using Lightcycler 96 (Roche) with KAPA SYBR FAST qPCR kit (KAPA BIOSYSTEMS).

RSS imaging in cardiac tissue

The mouse hearts were harvested and embedded in O.C.T. compound (Sakura Finetek), and frozen in cold isopentane to make fresh-frozen section. The section was sliced in 12 μm thickness. RSS titer levels in cardiac tissue were visualized using fluorescent probe SSip-1 DA and QS10. For SSip-1 DA imaging, 10 μM of SSip-1 DA (Goryo Chemical) with 1 mg/mL bovine serum albumin (Nacalai Tesque) and 0.02% Cremophor (Sigma) was loaded at room temperature for 45 min. For QS10 imaging, 1 μM QS10 [provided from Dr. K. Umezawa (Tokyo Metropolitan Institute of Gerontology)] with 0.02% Pluronic F-127 (Invitrogen) was loaded at room temperature

for 15 min. Then, section was mounted with ProLong Diamond Antifade Mountant with DAPI (Invitrogen). Imaging was performed using BZ-X700 microscope (KEYENCE).

Isolation of NRCMs and gene knockdown by siRNA transfection

NRCMs were isolated from SD rats pups (1 to 2 days old). Isolated NRCMs were cultured in Dulbecco's Modified Eagle's Medium with low glucose (1 mg/mL) (D-MEM, Fujifilm Wako) containing 10% fetal bovine serum under 5% CO_2. To avoid proliferation of non-cardiomyocyte cells, 200 μM bromodeoxyuridine (Sigma) was used. For knockdown, Stealth siRNAs for cysteinyl aminoacyl tRNA synthetase 2 (CARS2) (Invitrogen) and Stealth RNAi^{TM} siRNA Negative Control Med GC Duplex #3 (Invitrogen) for siControl were used. NRCMs were transfected with 20 nM siRNA using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) following manufacture's instruction.

RSS, hydrogen sulfide, and mitochondrial membrane potential imagings in NRCMs

Isolated NRCMs were plated on φ8 triple well glass base dish (Iwaki) coated with Matrigel (Corning). For RSS imaging, 1 μM QS10 was loaded with 0.02% pluronic F-127 at 37℃ for 15 min. For hydrogen sulfide (H_2S) imaging, 2.5 μM SF7-AM (Cayman Chemical) with 0.02% Cremophor was loaded at 37℃ for 30 min. For mitochondrial membrane potential imaging, 1 μg/mL JC1 (Abcam) was loaded at 37℃ for 30 min. Imaging was performed using BZ-X700 microscope and analysis was performed using ImageJ software.

Results

Chapter 1. Cilnidipine improves heart failure after MI by suppressing mitochondrial hyperfission.

To investigate whether maintaining mitochondrial quality are beneficial for chronic heart failure, the effect of cilnidipine, which could suppress mitochondrial hyperfission during hypoxia *in vitro*, on mouse MI model were tested. MI surgery was performed 1 week before administration of cilnidipine and cardiac function was measured using echocardiography. Just before the drug administration, I confirmed that cardiac function was completely declined in MI mice. Surprisingly, 3 weeks continuous administration of cilnidipine recovered cardiac function. I also revealed that treatment of cilnidipine improved mitochondrial fragmentation induced by MI. These data suggest that mitochondrial quality control by cilnidipine is also effective *in vivo*.

Chapter 2. Restoration of Drp1 polysulfidation rescues MeHg-induced cardiac vulnerability to pressure overload.

In chapter 1, I found that mitochondrial quality control could be critical for cardiac robustness. In this section, therefore, I focused on mitochondrial fission-inducing protein Drp1, whose activity is regulated by depolysulfidation and polysulfidation. MeHg and sulfide donor NaHS was used to induce Drp1 depolysulfidation and polysulfidation, respectively. Prolonged exposure of MeHg along with TAC surgery caused significant decrease of cardiac contractility, indicating MeHg-induced cardiac vulnerability. In contrast, when NaHS was administered intraperitoneally, the decrease in cardiac

contractility was significantly attenuated. These results indicate that the administration of sulfide donor is cardioprotective against MeHg-induced cardiac vulnerability.

Chapter 3. The role of RSS in cardiac robustness.

In chapter 2, the physiological significance of Drp1 polysulfidation for cardiac robustness was demonstrated. The protein polysulfidation are mediated by RSS. However, the role of RSS in the heart is unknown.

First, I revealed that I/R injury induced decrease of RSS titer levels in mouse hearts. Moreover, ischemic stress-induced RSS decrease was also confirmed in NRCMs along with increase of H_2S titer levels. In addition, supplementation of RSS donor Na_2S_4 to I/R model mice improved I/R-induced heart failure. These data suggest that intracellular RSS are reduced to H_2S under hypoxic condition and RSS have protective effect against ischemic stress.

Next, I focused on endogenous RSS-producing enzyme CARS2. The mRNA expression of CARS2 was decreased in I/R-injured hearts. Moreover, in CARS2 heterozygous deficient (*Cars2*$^{(+/-)}$) mice, cardiac function after I/R was significantly decreased compared to WT (*Cars2*$^{(+/+)}$) mice. In *Cars2*$^{(+/-)}$ I/R hearts, RSS titer levels were dramatically decreased compared to *Cars2*$^{(+/+)}$ I/R hearts. Furthermore, CARS2 knockdown in NRCMs caused impairment of mitochondrial membrane potential after hypoxic stress. These data suggest that CARS2 has critical role for protecting against ischemic stress via maintaining mitochondrial membrane potential, and that RSS synthesized by CARS2 are important for cardiac robustness.

Finally, the role of RSS metabolism in I/R hearts was examined. The mRNA expression levels of RSS-catabolizing enzymes including sulfide:quinone oxidoreductase

(SQOR), persulfide dioxygenase (ETHE1), sulfite oxidase (SUOX), and thiosulfate sulfurtransferase (TST) were decreased in I/R hearts. In addition, another RSS-producing enzyme CARS1 expression level was increased in I/R hearts. These results suggest the compensation against reduction of RSS production through CARS2, to maintain endogenous RSS amounts in the heart. In summary, I revealed that RSS metabolism could be important for ischemic stress resistance via maintaining mitochondrial integrity.

Discussion

Collectively, these results suggest that RSS synthesis and catabolism contribute to cardiac homeostasis through protein polysulfidation and the maintenance of mitochondrial quality control, thereby inducing cardioprotective effect. The present study proposes that RSS are the key factors, along with reactive oxygen species and reactive nitrogen species, for understanding the molecular mechanism of cardiac pathophysiology. Moreover, this study suggests that the contribution of RSS to mitochondrial respiration (sulfur respiration) in the heart, proposing new insights for cardiac redox biology.