

Developments in Research of Reactive Sulfur Species

Guest Editor: Tomohiro Sawa

Cardiac robustness regulated by reactive sulfur species

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(Received 2 July, 2021; Accepted 17 August, 2021; Released online in J-STAGE as advance publication 5 November, 2021)

The human myocardium contains robust cells that constantly beat from birth to death without being replaced, even when exposed to various environmental stresses. Myocardial robustness is thought to depend primarily on the strength of the reducing power to protect the heart from oxidative stress. Myocardial antioxidant systems are controlled by redox reactions, primarily via the redox reaction of Cys sulfhydryl groups, such as found in thioredoxin and glutathione. However, the specific molecular entities that regulate myocardial reducing power have long been debated. Recently, reactive sulfide species, with excellent electron transfer ability, consisting of a series of multiple sulfur atoms, i.e., Cys persulfide and Cys polysulfides, have been found to play an essential role in maintaining mitochondrial quality and function, as well as myocardial robustness. This review presents the latest findings on the molecular mechanisms underlying mitochondrial energy metabolism and the maintenance of quality control by reactive sulfide species and provides a new insight for the prevention of chronic heart failure.

Key Words: persulfide, reactive sulfide species, electrophile, mitochondrial quality control, cardiac senescence

The recent increase in the number of patients with chronic cardiovascular diseases, such as heart failure, is becoming a major problem worldwide. Cellular senescence is observed in cardiovascular tissues as a result of the progression of pathological structural and morphological remodeling,⁽¹⁻³⁾ and its suppression may lead to preventive and therapeutic strategies for the treatment of chronic cardiovascular diseases.⁽⁴⁾

Hydrogen sulfide (H₂S), a colorless and highly toxic gas with a rotten egg odor, has been detected in low concentrations in the body,⁽⁵⁾ including in the cardiovascular system.^(6,7) It has been reported that treatment with H₂S can have various physiological effects, including vascular smooth muscle relaxation,⁽⁸⁾ insulin signal suppression,⁽⁹⁾ and inflammation.⁽¹⁰⁾ In particular, the anti-aging effect of H₂S has attracted attention because it has been reported that H₂S can extend the life expectancy of yeast, nematodes, and flies.⁽¹¹⁻¹³⁾ Furthermore, there is increasing evidence that H₂S has a protective effect against various diseases associated with cellular senescence, such as cardiac hypertrophy,⁽¹⁴⁾ heart failure,⁽¹⁵⁾ atherosclerosis,⁽¹⁶⁾ and ischemia/reperfusion injury.⁽¹⁷⁾ The biological effects of H₂S are thought to be due to the nucleophilicity (or reducing power) similar to the redox-active cysteine thiol groups (Cys-SH, Cys-S⁻).⁽⁵⁾ Actually,

we have previously collaborated with Dr. Takaaki Akaike (Tohoku University), and found that hydrogen sulfide anion improves cardiac senescence induced by electrophilic secondary metabolite, 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP).⁽¹⁵⁾ However, we also found that H₂S itself is insufficient to react directly with electrophilic chemical substances but requires heavy metals as a catalyst.⁽¹⁵⁾ This indicates that highly nucleophilic substance(s) using H₂S as a substrate is formed in cells and mediate the protective action of H₂S on organs. Dr. Akaike's group found that reactive sulfur species (RSS) produced from the H₂S biosynthesis pathway are bona fide nucleophilic substances.⁽¹⁸⁾ Cysteine persulfide (Cys-SSH), a RSS, reacts directly with hydrogen peroxide and electrophilic substances for its metabolism and elimination.^(19,20) As described below, a RSS is mostly deprotonated under physiological pH conditions and has higher nucleophilicity than H₂S (HS⁻).⁽²¹⁻²³⁾ Currently, the role of RSS in life expectancy and tissue homeostasis is being investigated.

In this review, we describe the intracellular mechanism for the production of RSS and the actions of RSS in cellular senescence and cardiac homeostasis.

Cardiac Redox Homeostasis and Antioxidant System

Increasing evidence suggests that disruption of redox homeostasis such as excess oxidative stress play a critical role in the progression of cardiac diseases.^(24,25) Redox homeostasis is controlled by the balance between pro-oxidative production and antioxidant defense system. Mitochondria is a major source of reactive oxygen species (ROS) production. To produce a lot of energy for continuous beating, cardiomyocytes possess numerous mitochondria which occupy more than 30% of total cell volume.⁽²⁶⁾ Therefore, cardiomyocytes continuously produce a lot of ROS as a byproduct of mitochondrial respiration. To adapt these oxidative stress, cardiomyocytes have evolved an elegant antioxidant defense system.⁽²⁷⁾ For instance, mitochondrial H₂O₂ is increased in skeletal muscle in mice with both exercise and high-fat high-sucrose diet, whereas it is decreased in heart by increasing expression of thioredoxin reductase (TrxR)-2.⁽²⁸⁾ Antioxidant system in the heart has been studied by assessing

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He received "SFRR Japan Award of Scientific Excellence" in 2018 in recognition of his outstanding work.

H₂O₂ removing activities.⁽²⁹⁾ The respiration-dependent mitochondrial H₂O₂ removal is believed as the predominant endogenous ROS-eliminating machinery and its rate becomes higher with pyruvate/malate than with succinate. Compared with the capacity of rat liver mitochondria, myocardial mitochondria has higher H₂O₂-eliminating activity. The heart also has common H₂O₂-scavenging systems, including Trx/TrxR, peroxiredoxins, glutathione (GSH)/GSH reductase/GSH peroxidase, and catalase.⁽³⁰⁾ The GSH content is >2-fold higher in liver than heart and skeletal muscle,⁽³¹⁾ suggesting that the contribution of each enzyme to antioxidation may differ between organs. The significance of scavenging system ranked by the H₂O₂-removing rate (%) is estimated: catalase > TrxR > GSH in unenergized heart and GSH > TrxR > catalase in energized heart, and catalase > GSH > TrxR in unenergized and energized liver.⁽³²⁾ However, Two Selenoprotein families, GSH peroxidases and Trx reductases, and Zn²⁺-containing metallothioneins also act as ROS-scavenging enzyme in the heart.^(33,34) These ROS-eliminating and scavenging systems commonly use sulfur (CysSH) to contribute to ROS removal, and Cys polysulfidation will positively regulate intracellular antioxidant activities.

Electrophile-Mediated Cardiac Premature Senescence

It is believed that there is a close relationship between the aging of individuals and cellular senescence.⁽³⁵⁾ Senescent cells, which are observed in various tissues according to the degree of aging, secrete high levels of inflammatory cytokines, growth factors, and proteases (known as the senescence-associated secretory phenotype), leading to dysfunction in tissues and individuals.⁽³⁶⁾ Cells under various types of stress, including the progression of pathological conditions, exhibit a phenotype similar to cellular senescence (premature senescence).⁽³⁷⁾

Cardiovascular cells are constantly exposed to hemodynamic load, such as blood flow, so they are more robust than other cell types. However, increased stress caused by diabetes, hypercholesterolemia, and ischemic conditions, triggers premature senescence.^(2,15,38,39) Such premature senescence of cardiomyocytes causes decreased cardiac function (heart failure) and arrhythmia, which in turn can cause sudden death.⁽⁴⁰⁾

Telomere shortening, accumulated nucleotide damage, increased oxidative stress, and activation of oncogene products cause cellular senescence.⁽⁴¹⁾ Reactive oxygen species (ROS) and reactive nitrogen species (RNS) undergo a chemical reaction with lipids and nucleic acids in cells, producing various secondary metabolites. These secondary metabolites, such as 8-nitro-cGMP, nitro fatty acids, 15-deoxy-Δ^{12,14}-prostaglandin J₂, and 4-hydroxynonenal, often have the ability to accept electrons (electrophiles).⁽⁴²⁾ In addition to endogenous electrophiles, exogenous environmental pollutants, such as methylmercury (MeHg) and acrolein, also act as electrophilic substances.⁽⁴³⁾ These electrophiles covalently bind to the cysteine residues in a protein and change the structure and function of the protein.^(42,44) We have previously found that 8-nitro-cGMP was generated in the hearts of myocardial infarction model mice and sustained activation of H-Ras through covalent modification of cysteine 184 (S-guanylation) caused myocardial premature senescence.⁽¹⁵⁾ The hydrogen sulfide anion (HS⁻) is a nucleophile that can react with electrophilic substrates. Therefore, it has been speculated that H₂S inhibits cellular senescence through the metabolism and elimination of electrophiles. In fact, we found that the administration of sodium hydrosulfide (NaHS) to myocardial infarction model mice inhibited the S-guanylation of H-Ras and improved cardiac function.⁽¹⁵⁾

Reactive Sulfur Species (RSS)

The treatment of cells with HS⁻ induces the metabolism of

electrophilic substrates.⁽¹⁵⁾ However, recent studies have identified that sulfur intermediates rather than HS⁻ are likely to react with these electrophiles. RSS, such as Cys-SSH and glutathione persulfide (GSSH), have been identified as bona fide nucleophilic intermediates.⁽¹⁸⁾ These persulfides have a sulfane sulfur atom bound to a thiol group. Because of the α-effect, persulfides are more nucleophilic than the corresponding thiols.⁽²¹⁾ It has been reported that the pKa value of glutathione persulfide (GSSH) is 6.9 or 5.5, whereas that of GSH is 8.9.^(21,22) The pKa value of Cys-SSH is computationally calculated to be 4.3, whereas that of cysteine is 8.3.⁽²³⁾ Therefore, most persulfide is deprotonated under physiological pH conditions and can easily react with electrophiles. GSSH directly reacts with 8-nitro-cGMP, generating non-electrophilic 8-SH-cGMP.⁽¹⁸⁾

H₂S is believed to be produced by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) in the trans-sulfuration pathway that mediates the metabolism of sulfur-containing amino acids.^(5,8) However, the use of recent advances in mass spectrometry to detect sulfane sulfur atoms has identified that Cys-SSH is produced by CBS and CSE.⁽¹⁸⁾ This sulfane sulfur generated from cystine can be transferred to other thiol groups in peptides, such as GSH to produce GSSH, (trans-sulfidation).^(18,43) Cys-SSH and GSSH are ubiquitously present in cells, from yeasts to humans, in micromolar concentrations. For instance, quantitative mass spectrometry analysis identified that GSSH concentration is about 150 μM in brain and 50 μM in heart and liver, which is equivalent to 2–5% of GSH.⁽¹⁸⁾ Recently, cysteinyl-tRNA synthetases (CARs) have been identified as novel enzymes for Cys-SSH synthesis.⁽⁴⁶⁾ CARs were originally identified as the enzymes that catalyze the ligation of cysteine to tRNA. In mammal, there are two CAR genes: a cytosolic CAR (CAR1) and a mitochondrial isoform of CAR (CAR2). Akaike *et al.*⁽⁴⁶⁾ have reported that CARs directly catalyze Cys-SSH synthesis from cysteine and produce Cys-SSH bound to cysteinyl-tRNA. This persulfidated cysteinyl-tRNA can be incorporated into nascent polypeptides during the translation step; thus, mediating protein persulfidation (protein-SSH). Protein persulfidation occurs not only via co-translation but also by the post-translation (trans-sulfidation) pathway. Protein persulfidation is important for regulation of the structure and function of the protein.⁽⁴⁷⁾

H₂S is released from Cys-SSH and protein-SSH by non-enzymatic reaction.^(48,49) Sulfide quinone reductase (SQR) catalyzes the oxidation of H₂S and generates GSSH through sulfur atom transfer.^(50,51) Ethylmalonic encephalopathy protein 1 (ETHE1) catalyzes the oxidation of GSSH, yielding sulfite (SO₃²⁻) and GSH. Sulfite is further oxidized to sulfate (SO₄²⁻) by sulfite oxidase (Fig. 1).⁽⁴⁹⁾

RSS and Senescence

Cys-SSH and protein-SSH may be factors that regulate aging and cellular senescence. As described above, Cys-SSH is produced by the enzymatic reactions of CBS and CSE.⁽¹⁸⁾ Several studies have reported that expression of these trans-sulfuration enzymes is closely related with life span.⁽⁵²⁾ Sulfur-containing amino acid restriction increased the expression of CSE and endogenous H₂S production, which is essential for beneficial effects, including longevity and stress resistance, in *Caenorhabditis elegans* (*C. elegans*) and mice.⁽¹²⁾ CSE expression has been shown to be decreased in aged *C. elegans*.^(53,54) Overexpression of CBS increased the life span,⁽⁵⁵⁾ and overexpression of CSE decreased oxidative stress and protected against neurodegenerative disease, in *Drosophila melanogaster*.⁽⁵⁶⁾ Mouse embryonic fibroblasts from CSE knockout mice showed an increase in oxidative stress and an increase in the expression of p53 and p21, resulting in a senescence phenotype.⁽⁵⁷⁾ The level

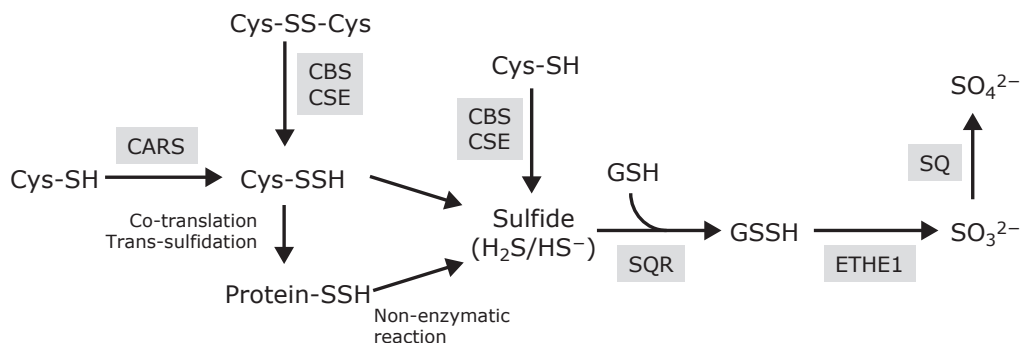


Fig. 1. The scheme of metabolic pathway of persulfides and sulfides. Enzymes are shown in gray background. Cys-SH, cysteine; Cys-SSH, cysteine persulfide; Cys-S-S-Cys, cystine; protein-SSH, protein persulfide; H₂S/HS⁻, hydrogen sulfide/hydrogen sulfide anion; GSH, glutathione; GSSH, glutathione persulfide; SO₃²⁻, sulfite; SO₄²⁻, sulfate; CARS, cysteinyl-tRNA synthetase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; SQR, sulfide quinone reductase; ETHE1, ethylmalonic encephalopathy protein 1; SQ, sulfite oxidase.

of protein persulfidation decreases during aging, whereas the level of irreversibly overoxidized cysteine is increased in aged animals.⁽⁵⁸⁾ Cysteine persulfidation protects the cysteine thiol from overoxidation induced by excess ROS, and this process is conserved in various species, including mice and humans.⁽⁵⁸⁾ Although these genetic studies speculate the potential relationship between RSS and senescence, it is still unclear why reduced levels of RSS trigger cellular senescence. Additionally, CBS and CSE are multifunctional enzyme that is related to biosynthesis of selenium-containing amino acids as well as sulfur-containing amino acids. Actually selenium deficiency is associated with normal lifespan through suppression of selenoprotein functions.^(59,60) However, it is still unclear the relationship between RSS and selenocysteine.

The Critical role of RSS in Mitochondrial Quality Control and Cardiac Homeostasis

Finally, we will describe our recent work regarding the role of RSS in myocardial senescence and cardiac vulnerability, through mitochondrial quality control.

Cardiomyocytes require a lot of energy for continuous contraction and relaxation. Therefore, the quality of the mitochondria in these cells is a determining factor for cardiac robustness.⁽²⁶⁾ The mitochondrial length and density are thought to be closely related to oxygen consumption.⁽⁶¹⁾ For instance, mitochondrial electron transport chains in skeletal muscle form a dense supercomplex and oxygen consumption is increased.⁽⁶²⁾ In contrast, in an anaerobic metabolic environment, such as found in hyperglycemia and hypoxia, promotes mitochondrial division.⁽⁶³⁾ Cells accurately regulate systemic energy metabolism by controlling the mitochondrial fission/fusion cycle according to the environment of the cells. Mitochondrial fission is regulated by dynamin-related protein 1 (Drp1), a large GTPase, whereas optic atrophy 1 (Opa1) and mitofusin 1/2 (Mfn1/2), which are also large GTPases, regulate mitochondrial fusion.⁽⁶⁴⁾ Drp1 is diffusely distributed in the cytosol and is translocated to the mitochondrial outer membrane when it is bound to GTP (the active form). Drp1 is oligomerized and forms a ring-like structure to divide the mitochondria. Opa1 and Mfn1/2 mediate the fusion of the mitochondrial inner and outer membranes, respectively.⁽⁶⁵⁾ Dysfunctional mitochondrial fragments are degraded by mitophagy.⁽⁶⁶⁾ Therefore, the proper balance of mitochondrial fission and fusion is indispensable for mitochondrial quality. Aberrant balance of mitochondrial fission and fusion is observed in various diseases, including ischemia-reperfusion injury,⁽⁶⁷⁾ cardiomyopathy,⁽⁶⁸⁾ amyotrophic lateral sclerosis,⁽⁶⁹⁾ Huntington's disease,⁽⁷⁰⁾ and Alzheimer's disease.⁽⁷¹⁾

The genetic mutation of mitochondrial regulatory proteins has

shown the important role of mitochondrial quality control on cardiac homeostasis. Cardiomyocyte-specific Drp1 deficient mice exhibit a dilated cardiomyopathy-like phenotype. Remarkable mitochondrial fusion and elongation and increased mitophagy and myocardial necrosis have been observed in the cardiomyocytes of these mice.⁽⁷²⁾ Cardiomyocyte-specific Mfn1/2 double knockout mice showed eccentric cardiac remodeling, including mitochondrial hyperfission, reduced mitophagy, and hypertrophy.⁽⁷²⁾ Cardiac-specific Drp1/Mfn1/2 triple deficient mice exhibited a concentric hypertrophy-like phenotype with heterogeneous mitochondrial morphology, massive mitochondrial accumulation, and sarcomeric distortion.⁽⁷³⁾ These studies indicated the importance of proper mitochondrial fission/fusion balance for maintaining cardiac homeostasis.

We analyzed the mitochondrial dynamics during cardiac remodeling and found that aberrant mitochondrial hyperfission and Drp1 hyperactivation occurred in cardiomyocytes in the early stage of myocardial infarction in a mice model. Hypoxic stress induced Drp1-mediated mitochondrial hyperfission, leading to myocardial senescence through mitochondrial ROS generation (Fig. 2).⁽⁷⁴⁾

The cellular features of stress-induced premature senescence are similar to those of CARS2-deficient cells. CARS2 knockout cells show severe mitochondrial fragmentation and a decrease in the mitochondrial membrane potential and oxygen consumption rate.⁽⁴⁶⁾ In collaboration with the Akaike group, we found that CARS2 mediated Drp1 Cys⁶²⁴ persulfidation. Drp1 activity was negatively regulated by persulfidation.⁽⁴⁶⁾ These findings suggested that Drp1 persulfidation by CARS2 affects mitochondrial quality control and energy homeostasis.

Because Drp1 persulfidation was found to negatively regulate mitochondrial fission, the pathophysiological role of Drp1 desulfidation in cardiomyocytes was investigated. In the environment, there are various electrophilic pollutants that adversely affect human health, including organic mercury, cadmium, aldehydes, and quinones.⁽⁴³⁾ MeHg is an environmental electrophile that accumulates in fish and marine mammals.⁽⁷⁵⁾ MeHg can covalently react with the sulfhydryl group of a protein to form a MeHg-S-protein complex (S-mercuration). This pathological modification disrupts the proper protein function causing neurotoxicity.⁽⁷⁶⁻⁷⁹⁾ Importantly, epidemiological studies have suggested that MeHg exposure can increase the risk of cardiac dysfunction at a lower concentration than that associated with neurotoxicity.⁽⁸⁰⁻⁸³⁾ Oral exposure of mice to a low dose of MeHg that does not induce neurotoxicity for 1 week did not alter the body weight, urine excretion/dietary intake, or activity of the mice but exacerbated cardiac dysfunction induced by pressure overload.⁽⁸⁴⁾ The level of Drp1 Cys⁶²⁴ persulfidation was

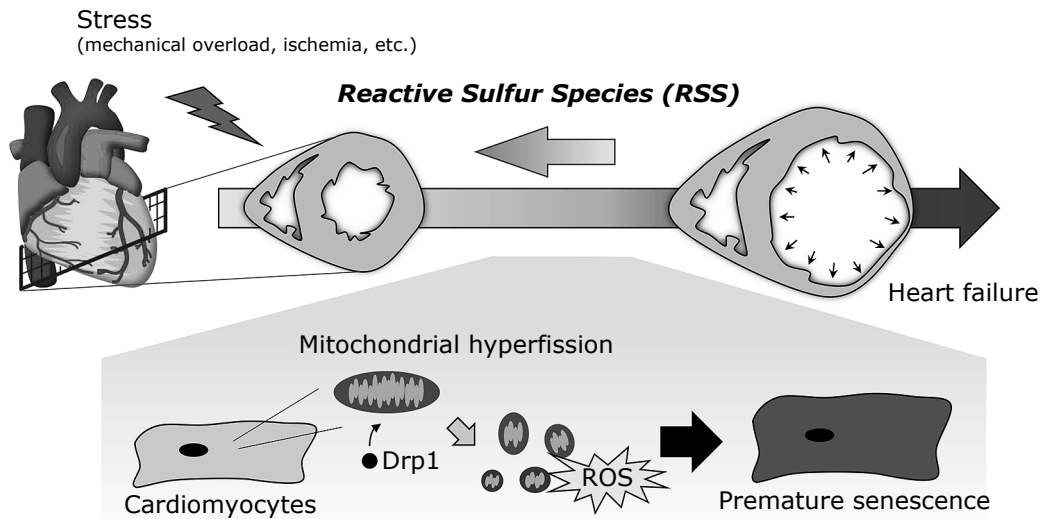


Fig. 2. Mitochondrial hyperfission-mediated myocardial senescence. When the heart is exposed to chronic stress, such as ischemia and mechanical overload, it gradually undergoes maladaptive remodeling, including premature senescence. These stresses induce Drp1-mediated mitochondrial hyperfission in cardiomyocytes, leading to myocardial premature senescence.

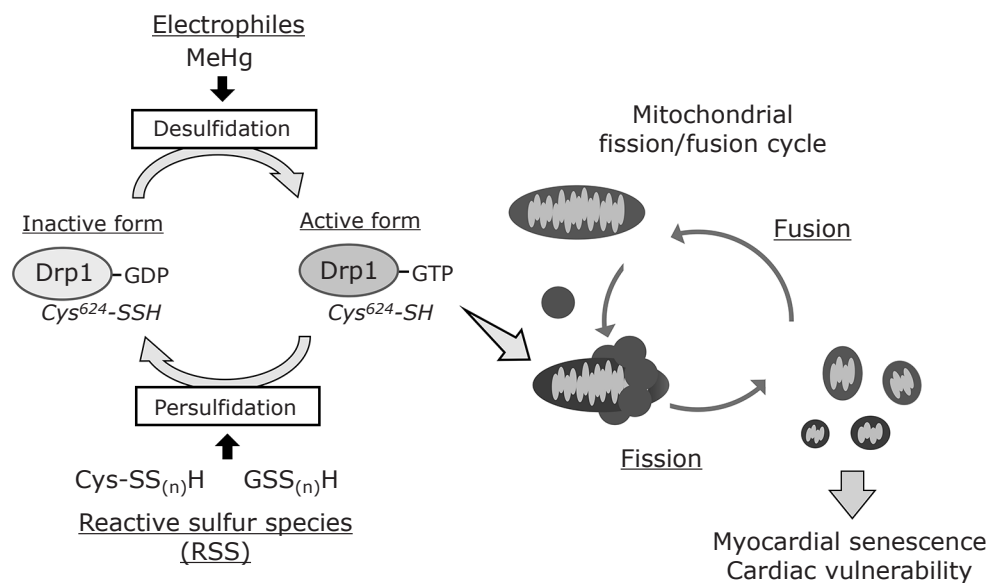


Fig. 3. Cross-talk between the mitochondrial fission/fusion cycle and reactive sulfur-dependent Drp1 activation cycle. Drp1 activity is negatively regulated by persulfidation at the redox-sensitive Cys624 in the basal state. Electrophiles, such as MeHg, induce sulfur deprivation of Drp1. The activation of desulfidated Drp1 is promoted through the interaction with filamin A, a guanine nucleotide exchange factor for Drp1, leading to mitochondrial fission.

decreased in MeHg-exposed cardiomyocytes, which was accompanied by an increase in Drp1 activation and mitochondrial hyperfission. Desulfidation of Drp1 and mitochondrial hyperfission because of MeHg exposure were almost completely abolished by the treatment of cardiomyocytes with NaHS, a sulfur substrate required to form RSS. MeHg-induced desulfidation of Drp1 promoted mitochondrial hyperfission by enhancing the interaction of Drp1 with filamin A, an actin-binding protein that acts as a guanine nucleotide exchange factor for Drp1.⁽⁷⁴⁾ In addition, administration of NaHS inhibited the hyperfission of myocardial mitochondria and rescued cardiac dysfunction after pressure overload in MeHg-exposed mice.⁽⁸⁴⁾ These results suggested that polysulfidation of Drp1 proteins negatively regulates cardiac vulnerability to mechanical stress through maintaining mitochondrial quality (Fig. 3).

Conclusion

For many years, H₂S has been believed to be a gaseous signaling mediator, such as nitric oxide (NO) and carbon monoxide (CO), and many papers have been published on this theme. However, with the development of advanced technology for detecting sulfur-containing molecules, it is clear that the active entity is not H₂S but RSS.⁽¹⁸⁾ The enzymes responsible for the production and metabolism of RSS have been identified and the biological significance of RSS has been revealed. The relationship between RSS and mitochondrial energy metabolism is important,⁽⁴⁶⁾ and our research has shown the influence of RSS in mitochondrial quality control and myocardial senescence-related cardiac diseases.⁽⁸⁴⁾ In the future, we would like to develop medical technology that utilizes these properties of RSS.

Author Contributions

AN and MN wrote and edited the manuscript. TT, YK, and KN edited the manuscript and prepared figures.

Acknowledgments

This work was supported by JST, CREST Grant Number JPMJCR2024 (20348438 to MN and AN) and Grants-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology (No. 19K07085 to AN, and 19K22443 and 19H03383 to MN), Innovative Areas (Research in a Proposed Research Area 'Integrated Bio-metal Science' (20H05512 to MN), and Mochida Memorial Foundation for Medical and Pharmaceutical Research (No. 6-11 to AN). Victoria Muir, PhD, from Edanz (<https://www.jp.edanz.com/ac>) edited a draft of this manuscript.

Abbreviations

CARS	cysteinyl-tRNA synthetase
CBS	cystathionine β -synthase
CO	carbon monoxide
CSE	cystathionine γ -lyase

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Cys-SSH	cysteine persulfide
Drp1	dynamamin-related protein 1
ETHE1	ethylmalonic encephalopathy protein 1
GSH	glutathione
GSSH	glutathione persulfide
H ₂ S/HS ⁻	hydrogen sulfide/hydrogen sulfide anion
MeHg	methylmercury
Mfn1/2	mitofusin 1/2
3-MST	3-mercaptopyruvate sulfurtransferase
8-nitro-cGMP	8-nitroguanosine 3',5'-cyclic monophosphate
NO	nitric oxide
Opa1	optic atrophy 1
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSS	reactive sulfur species
SO ₃ ²⁻	sulfite
SO ₄ ²⁻	sulfate
SQR	sulfide quinone reductase
TrxR	thioredoxin reductase

Conflict of Interest

No potential conflicts of interest were disclosed.

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