Role of 8-nitro-cGMP and its redox regulation in cardiovascular electrophilic signaling

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Abstract

Structural and morphological changes of the cardiovascular systems (cardiovascular remodeling) are a major clinical outcome of cardiovascular diseases. Many lines of evidences have implied that transfiguration of reduction/oxidation (redox) homeostasis due to excess production of reactive oxygen species (ROS) and/or ROS-derived electrophilic metabolites (electrophiles) is the main cause of cardiovascular remodeling. Gasotransmitters, such as nitric oxide (NO) and endogenous electrophiles, are considered major bioactive species and have been extensively studied in the context of physiological and pathological cardiovascular events. We have recently found that hydrogen sulfide-related reactive species function as potent nucleophiles to eliminate electrophilic modification of signaling proteins induced by NO-derived electrophilic byproducts (e.g., 8-nitroguanosine 3',5'-cyclic monophosphate and nitro-oleic acid). In this review, we discuss the current understanding of redox control of cardiovascular pathophysiology by electrophiles and nucleophiles. We propose that modulation of electrophile-mediated post-translational modification of protein cysteine thiols may be a new therapeutic strategy of cardiovascular diseases.

Keywords: reactive oxygen species, electrophile, nucleophile, G protein, cardiovascular remodeling

Contents

1. Introduction: Redox homeostasis in the cardiovascular system
  - 1.1. Role of electrophiles and nucleophiles in redox signaling
  - 1.2. Regulation of ROS signaling in the cardiovascular system
  - 1.3. Formation, function, and metabolism of 8-nitro-cGMP
  - 1.4. Redox control of vascular tone
2. Redox control of G protein signaling
  - 2.1. G proteins and cardiovascular remodeling
  - 2.2. Redox control of G protein cycling
  - 2.3. Regulation of G protein activity by PTMs
3. Redox control of cardiac remodeling
  - 3.1. Formation of 8-nitro-cGMP in cardiac cells
  - 3.2. Cardiac cell senescence via S-guanylation of H-Ras
  - 3.3. Prevention of cardiac senescence via elimination of electrophiles by reactive sulfur species
4. Conclusions

1. Introduction: Redox homeostasis in the cardiovascular system

1.1.Role of electrophiles and nucleophiles in redox signaling

Redox homeostasis, a redox-based cellular metabolism, is maintained in a precise balance between reactive oxygen species (ROS) and antioxidant systems, counteracting each other during diverse cellular metabolism and signaling [1]. Physical and chemical stimuli activate ROS-dependent signaling pathways via several ROS-producing enzymes. There are two major sources of these enzymes, NADPH oxidase (Nox) and the mitochondrial electron transport chain. Additionally, xanthine oxidase, aldehyde oxidase, cytochrome P450, urate oxidase, D-amino oxidases, and uncoupled NO synthases (NOSs) are involved [2-4]. Each enzyme forms an original protein signaling complex with specific sensor/effector proteins to activate specific signaling pathways. A decline in ROS signaling is mainly regulated by ROS-scavenging enzymes (e.g., superoxide dismutase [SOD], catalase, glutathione peroxidase [GPx], and peroxiredoxins) [5-7]. SOD is an enzyme that reacts with superoxide, and catalase and peroxiredoxins catabolize hydrogen peroxide (H_2O_2). GPx is a selenium-dependent enzyme that contains a selenium atom incorporated within the selenocysteine residue [7]. GPx scavenges H_2O_2 in the presence of reduced glutathione (GSH). Because the intracellular concentration of GPx-coupled GSH is 1–3 mM and patients with selenium deficiency have heart failure, most H_2O_2 in the cardiac system is thought to be eliminated through GPx [8]. Additionally, the thioredoxin (Trx) and glutaredoxin family of proteins play an essential role in maintaining redox homeostasis by NADPH-dependent reduction of disulfide bridges in various target proteins. Deletion of every ROS-scavenging enzyme results in several cardiovascular abnormalities or a shorter life span [9-12]. Therefore, maintenance of redox homeostasis is predominantly regulated not only by a formation of ROS via ROS-generating enzymes, but also by a complex web of antioxidant molecules.

In addition to ROS-producing/scavenging systems, the reversibility of redox-dependent post-translational modification (PTM) of proteins regulates a spatiotemporal diversity of ROS signaling in cells [13]. The ROS signaling system, as an unstable primary signal of ROS, transforms to a stabler secondary signal. During this process, intracellular nucleic acids, nucleotides, lipids, and cysteine (Cys) sulfhydryl-containing proteins function as chemical sensors to interact with ROS/NO. The secondary signal molecules formed via reaction of ROS/NO and intracellular sensor molecules are termed “electrophiles”. Electrophiles also target Cys sulfhydryls to form stable covalent modification of proteins, leading to continuous activation of redox-dependent signaling. We recently found that hydrogen sulfide-related reactive

sulfur molecules function as a potent nucleophile to quench electrophilic PTM of proteins [14]. The physiological functions of hydrogen sulfide-related reactive sulfur molecules have been well documented in the vascular system [15-17]. Balancing electrophile and nucleophile signaling may provide a new aspect of redox control of cardiovascular homeostasis.

1.2.Regulation of ROS signaling in the cardiovascular system

Cardiovascular diseases are the leading cause of morbidity and mortality in developed countries. Many lines of evidence have suggested that ROS mediate the development of cardiovascular remodeling induced by neurohumoral factors and hemodynamic load via oxidative PTM of intracellular signaling proteins [18,19]. The above-mentioned ROS-generating enzymes are found to mediate agonist-induced activation of signaling proteins via oxidation of sulfur-containing amino acids (methionine and Cys), which are found at active or allosteric sites of effector proteins [1,20]. Several studies using gene-targeting mice have shown that ROS and/or reactive nitrogen oxides (RNS) function as a cytotoxic factor, and also act as an essential signaling mediator for maintaining physiological functions. This concept is supported by the surprising discovery that its counterpart, reductive stress, which is caused by an increase in reduced GSH, contributes to cardiomyopathy triggered by protein aggregation [20,21]. Additionally, our recent study showed that irreversible Cys modification by electrophilic byproducts, such as nitrated cyclic nucleotide (e.g., 8-nitroguanosine 3',5'-cyclic monophosphate [8-nitro-cGMP]), generated via reactions of ROS, RNS, and intracellular guanine nucleotide, acts as an important mediator of chronic heart failure [14]. Therefore, reversibility of protein modification by ROS or ROS-derived electrophiles may determine the transition from adaptation to maladaptation of the heart against oxidative stress. These findings are of great value because they provide a new aspect for the management of cardiovascular diseases.

The spatiotemporal property of ROS signaling functions depends on the ROS production system and chemical reactivity, which defines the molecular reaction environment. To elucidate the mechanisms of ROS signal production and sensing, the structure and function of ROS-producing enzymes need to be analyzed. Among ROS-producing enzymes, the structure and function of Nox family proteins have been well studied [22]. The activation of Nox2 primarily depends on intracellular Ca^{2+} concentrations, where an increase in Ca^{2+} activates a small GTP-binding protein, Rac, and protein kinase C (PKC)-mediated phosphorylation of p47^{phox} [22,23]. The cytosolic regulatory subunits (p47^{phox} , p67^{phox} , and Rac1) of Nox2 are localized to the

T-tubule/sarcoplasmic reticulum junction. These subunits contribute to diastolic stretch-induced ROS production, followed by oxidative modification (activation) of ryanodine receptor 2, leading to an increase in diastolic Ca^{2+} spark rate in the heart [24,25]. Additionally, transient receptor potential canonical (TRPC) 3 channel contributes to Nox2-mediated ROS production in rodent cardiomyocytes [26]. TRPC channels have two functions: they act as a sensor for mechanical stresses and as a protein scaffold at the plasma membrane to control the amplification and co-ordination of receptor signaling [27]. TRPC3 functionally interacts with PKC β II [28], and Ca^{2+} -dependent activation of PKC β II may induce Nox2 activation through phosphorylation of the p47^{Nox} subunit [23]. Because PKC-mediated phosphorylation of Rho GDP dissociation inhibitor (GDI) α induces Ca^{2+} -dependent Rac activation [29], TRPC3-mediated Ca^{2+} influx might dissociate RhoGDI α from Rac through PKC β II-dependent phosphorylation of RhoGDI α . Therefore, functional interaction between TRPC3 and Nox2 may be involved in mechano-chemo transduction in the heart.

Topologically, Nox isozymes, except for Nox4, generate O_2^- in the extracellular area [22]. Conversion of O_2^- into H_2O_2 by SOD is suggested to be essential for initiating ROS signaling. H_2O_2 might simply diffuse into the cell across the plasma membrane. However, recent studies suggest that H_2O_2 preferentially enters the cell through aquaporin channels on the plasma membrane [30,31]. Several ROS target proteins have been identified, including Trx [22], Nrf-2 [33], TRP [34,35], protein tyrosine phosphatase [36], and GTP-binding proteins (G proteins) [37]. These proteins contain particular Cys residues at active or allosteric sites, which are susceptible to oxidative modification, leading to regulation of specific downstream signaling pathways. Methionine is also a target amino acid for ROS. Oxidation of methionine on Ca^{2+} /calmodulin-dependent kinase II contributes to angiotensin (Ang) II-induced cardiac dysfunction in mice [38,39]. The reversibility of these oxidative modifications was confirmed by the discovery of their specific reductases, Trx reductase, GSH reductase, and methionine sulfoxide reductase [1].

1.3. Formation, function and metabolism of 8-nitro-cGMP

One of the major endogenous electrophiles is 8-nitro-cGMP, originally identified by Akaike's research group [40,41]. Formation of 8-nitro-cGMP requires RNS, such as peroxynitrite (ONOO^-) generated from Nox2-derived ROS and inducible NOS (iNOS)-derived NO. More recently, mitochondria-derived superoxide production regulated by H_2O_2 generated from Nox2 was found to be essential for 8-nitro-cGMP

formation in cells [42]. Using a stable isotope dilution technique and liquid chromatography coupled with tandem mass spectrometry analysis, more than 40 nM of 8-nitro-cGMP was formed in cells, and these levels were much higher than cGMP levels formed in the same cells. This result indicates that 8-nitro-cGMP is one of the major cyclic nucleotides formed in cells. Additionally, 8-nitro-cGMP is not formed by direct nitration of cGMP, but is eventually generated after nitration of abundant GTP. This results in production of 8-nitro-GTP, which then reacts with guanylate cyclases to generate 8-nitro-cGMP. Among various guanine nucleotides, GTP is most susceptible to ONOO⁻-mediated nitration [43]. 8-Nitro-GTP formed by the reaction of GTP and ONOO⁻ appears to be an efficient substrate for soluble guanylate cyclases to form 8-nitro-cGMP [44]. In fact, the electrophilicity of 8-nitro-cGMP is much lower than that of other endogenous electrophiles. The second-order rate constant for the reaction of 8-nitro-cGMP with GSH sulfhydryl is 0.03 M⁻¹ s⁻¹ at pH 7.4 and 37°C [41,45]. Therefore, 8-nitro-cGMP is believed to be much less reactive compared with certain other electrophiles, such as α,β -unsaturated aldehydes, ω -6, and ω -3 unsaturated fatty acids, and nitroalkene fatty acids. These nitroalkene fatty acids include 4-hydroxy-2-nonenal (HNE), 15-deoxy-D^{12,14}-prostaglandin J₂ (15d-PGJ₂), and nitro-linoleic and nitro-oleic acids. The second-order rate constants for the reaction of those electrophiles with GSH are 1.3 M⁻¹ s⁻¹ (HNE), 0.7 M⁻¹ s⁻¹ (15d-PGJ₂), 355 M⁻¹ s⁻¹ (nitro-linoleic acid), and 183 M⁻¹ s⁻¹ (nitro-oleic acid) at pH 7.4 and 37°C [45-47]. These reactive electrophiles react readily with GSH and thus can be scavenged by GSH sulfhydryls more than 20–10,000 times more rapidly than by 8-nitro-cGMP.

Reactive electrophiles produced in cells during oxidative stress are detoxified by the actions of glutathione *S*-transferase (GST) [48]. However, 8-nitro-cGMP is not metabolized by GSH and is stable enough to remain at appreciable levels in cells, even with excessive amounts of GSH. Based on the fact that Cys sulfhydryls react with 8-nitro-cGMP, we found a considerable effect of endogenous hydrogen sulfide-related reactive molecules on 8-nitro-cGMP metabolism. Two important enzymes involved in reactive sulfur biosynthesis, cystathionine β -synthase (CBS) and cystathionine γ -lyase, mediate the metabolism of cellular 8-nitro-cGMP in various cultured mammalian cells. The electrophilic nitro moiety undergoes nucleophilic substitution, yielding the novel product 8-SH-cGMP (Figs. 1 and 2). This metabolism occurs concomitantly with the release of nitrite to generate 8-SH-cGMP. This suggests a mechanism that depends on the nucleophilic qualities of HS⁻-like molecular species that predominate in neutral biological solutions. Similar electrophile sulfhydration occurs with a broad array of biological electrophiles. The metabolic fate of these sulfhydrated derivatives allows the

sorting of electrophiles into three groups (Fig. 1). In the first group (including 8-nitro-cGMP and 15d-PGJ₂), the sulfhydrated derivative is so stable that no additional reaction occurs, except for oxidative degradation of SH by ROS and other reactive species. The second group (including nitro-oleic acid, 1,2-naphthoquinone (1,2-NQ), 1,4-naphthoquinone, *tert*-butylbenzoquinone, *N*-ethylmaleimide, diethylmaleate, and monobromobimane) involves relatively stable bis product formation. The third group may include other highly reactive electrophiles, such as HNE and acrolein, for which additional metabolism and secondary chemical reactions follow SH addition. The end product 8-SH-cGMP appears to be extremely stable among sulfhydrated derivatives of the above-mentioned electrophiles. 8-SH-cGMP contains sulfhydryls but the sole product observed by further reaction with H₂O₂ was cGMP, rather than oxidized derivatives of 8-SH-cGMP, such as 8-sulfenyl-, 8-sulfinyl-, and 8-sulfonate-cGMP, or 8-OH-cGMP [14]. This result provides the first demonstration of oxidant-induced desulfhydration of SH-containing compounds. All these findings suggest that reactive sulfur species produced in cells are a critical factor in 8-nitro-cGMP metabolism.

8-Nitro-cGMP undergoes nucleophilic substitution with a protein sulfhydryl, releases the nitro group, and forms a protein Cys-cGMP adduct in a process called protein *S*-guanylation. Several ROS sensor proteins, such as Keap1 and H-Ras, are modified (*S*-guanylated) by 8-nitro-cGMP [14,41]. More interestingly, *S*-guanylated bacteria are selectively marked with Lys63-linked polyubiquitin, a known molecular tag for selective transport to autophagosomes [49]. This autophagic protein degradation may be an alternative 8-nitro-cGMP metabolic pathway.

1.4.Redox control of vascular tone

Vascular endothelium is a major modulator of vascular tone, releasing vasoactive substances, such as endothelium-derived relaxing factor (EDRF), endothelium-derived hyperpolarizing factors (EDHFs), cyclooxygenase metabolites, and endothelium-derived constricting factors [50,51]. Nobel laureates Furchgott, Murad, and Ignarro discovered NO as a major EDRF. NO induces vasodilation through cGMP-dependent protein kinase (PKG)-mediated phosphorylation of myosin light chain kinase in vascular smooth muscle cells. Another major EDRF is prostacyclin, which induces vasodilation through cAMP-dependent protein kinase-mediated phosphorylation of myosin light chain kinase. Accumulating evidence has shown that the contribution of EDRF in large arteries is higher than that in small arteries, while EDHF rather than EDRF contributes to vasodilation in smaller arteries [52]. Since the first report on the existence of EDHF, several substances/mechanisms have been

proposed for the nature of EDHF, including epoxyeicosatrienoic acids, K^+ , and electrical communications through myoendothelial gap junctions. Additionally, endothelium-derived H_2O_2 via the endothelial NOS system, which is functionally coupled with Cu,Zn-SOD, has been reported to work as an EDHF in mammalian coronary arteries [52]. Although it is still unclear whether endothelial NOS-derived H_2O_2 actually functions as an EDHF, H_2O_2 has been reported to induce vasodilation via oxidative activation of protein kinase and PKG [53-55]. Likewise, it has pharmacologically been shown that hydrogen sulfide, exogenously introduced, can exhibit vasodilatory effects indirectly by delaying cGMP degeneration through phosphodiesterase 5 inhibition [56] or ATP-sensitive K^+ channel activation [57]. However, hydrogen sulfide may also trigger dose-dependent vasoconstriction or vasodilatory effects depending on the type of vessel and the animal species examined. This conditional response toward hydrogen sulfide is suggested to be mediated via covalent modification of K^+ channel protein thiols or due to nitrosothiol formed as a result of NO/hydrogen sulfide interaction [58]. 8-Nitro-cGMP also shows dose-dependent vasoconstriction or vasodilatory effects [59]. Considering the nucleophilic property of hydrogen sulfide and its related reactive sulfur compounds, formation of 8-SH-cGMP via its nucleophilic substitution with reactive sulfur species is suggested to contribute to the vasodilatory effect of hydrogen sulfide because 8-SH-cGMP also has a potent vasodilatory activity (Fig. 2).

2. Redox control of G protein signaling

2.1. G proteins and cardiovascular remodeling

Cardiovascular remodeling is a process induced by common cardiovascular risk factors, such as high blood pressure, hyperglycemia, overweight/obesity, and smoking, which combine to modify the functional and structural characteristics of the myocardium and blood vessels. Cardiovascular remodeling includes a preclinical stage, during which symptoms are not evident. Cardiovascular remodeling also includes a clinical disease stage, which corresponds to the progression of preclinical disease and to the action of additional triggering mechanisms representing obvious tissue damage. Several neurohumoral factors, such as Ang II and noradrenaline, and their corresponding G protein-coupled receptors (GPCRs) have been implicated in the development of cardiovascular remodeling [60]. In most cases, protein expression levels of small G proteins, Ras, Rho, and Rac, and vasoconstrictive GPCRs (e.g., Ang type 1 receptor [AT_1R]) are significantly increased in the heart with heart failure [61]. Interestingly, recent studies have shown that the activities of G proteins and their

regulators, especially guanine nucleotide exchange factors and GTPase-activating proteins (GAPs), are functionally controlled by ROS and/or ROS/RNS-derived electrophiles through PTM mechanisms.

2.2.Redox control of G protein cycling

G proteins function as binary molecular switches to regulate a wide range of cellular processes [62]. There are two classes of G proteins. The first class functions as monomeric small G proteins, while the second functions as heterotrimeric G protein complexes. The latter class of complexes is composed of α , β , and γ subunits. The β and γ subunits can form a stable dimeric complex referred to as the $\beta\gamma$ complex. The conformation switch between the GDP-bound state and GTP-bound state is called the "G (protein) cycle" (Fig. 3). The exchange of GTP for GDP on G proteins is predominantly regulated by guanine nucleotide exchange factors and GAPs, which are originally believed to regulate the activation state of G proteins without covalently modifying them. Several studies have shown that PTM (especially oxidative modification) directly or indirectly modulates G cycle balance [63-66]. An example of this situation is where the protein expression level of regulator of G protein signaling type 4 (RGS4), which is a major GAP for α subunits of heterotrimeric G_q and G_i proteins among more than 20 RGS proteins, is negatively regulated by NO. This mechanism is termed "Arg/N-end rule", where S-nitrosylation of the N-terminal Cys residue, which is targeted for ubiquitin-dependent degradation by arginylation, promotes arginylation-dependent protein degradation via Cys oxidation [65,67,68]. This mechanism also participates in cardiovascular development [67] and placental growth factor-induced myocardial angiogenesis and hypertrophic growth of cardiomyocytes through endothelial NOS-dependent NO release [68].

Heterotrimeric G proteins located within the cell are activated by GPCRs that span the cell membrane. Therefore, GPCR and G proteins working together transmit signals from many hormones, neurotransmitters, and other signaling factors. The AT_1R , one of the guanine nucleotide exchanging factors for heterotrimeric G_q , G_i , and G_{12} family proteins, contributes to various cardiovascular functions and diseases [60]. AT_1R activity is negatively regulated by oxidation or reduction of AT_1Rs [69,70]. Disulfide bridges between the N-terminal region and the third extracellular loop, and between the first and second extracellular loops, are formed to maintain the conformation of AT_1R protein. AT_2R is resistant to inactivation by reducing agents because of the absence of disulfide bridges between the N-terminal region and the third extracellular loop. Nitro-oleic acid binds to AT_1R , and reduces G_q protein-mediated coupling without

affecting Ang II binding to the receptor [71].

ROS and RNS also regulate AT₁R transcriptional activity via PTM of a redox-sensitive transcriptional factor, nuclear factor- κ B [72]. We found that endogenous ROS generated by *Pertusis* toxin stimulation on Toll-like receptor 4 increases AT₁R density in rat cardiac fibroblasts [73]. Toll-like receptor 4 stimulation by *Pertusis* toxin activates NADPH oxidase through Rac, a small G protein, and subsequently induces nuclear factor- κ B activation through ROS-dependent I κ B α phosphorylation and degradation. In contrast, NO and nitro-unsaturated fatty acids reportedly decrease Ang II signaling in the heart [71,74]. Cys-38 of nuclear factor- κ B wedged between acidic and basic amino acids is nucleophilic, acidic, and redox active, and numerous reactions may occur on this Cys thiol side chain. The reactive Cys located in the Rel homology domain is involved in DNA binding or dimer formation of p65. NO-mediated Cys38 modification (S-nitrosylation) of p65 is expected to decrease transcriptional activity of nuclear factor- κ B. This mechanism includes formation of a protein signaling complex among iNOS, p65, and b-arrestin2 around the perinuclear region. Therefore, the spatiotemporal regulation of Cys modification by ROS or RNS may underlie the resultant changes of AT₁R signaling.

2.3.Regulation of G protein activity by PTM

The first two decades of research on the redox control of G protein signaling were developed by the understanding of how oxidative PTM enhances the exchange of GTP for GDP in Ras family G proteins. Many G proteins have reactive Cys residues in the guanine nucleotide-binding domain of GTPase (Fig. 4). The monomeric Ras family G proteins commonly possess Cys residues in the center (NKCD) or in the phosphate-binding loop (P-loop) of the guanine nucleotide-binding region [64,66]. Additionally, α subunits of heterotrimeric G_{i/o} proteins have reactive Cys residues at the α G/ α 4 and b6/ α 5 loops located near the guanine nucleotide-binding region [75]. The modification of these Cys residues actually increases the dissociation rate of guanine nucleotide, while this modification does not necessarily increase GTP-binding activity [76]. In fact, thioredoxin-1-sensitive oxidative modification or S-glutathionylation of Ras mediates α -adrenergic receptor-stimulated or mechanical strain-induced hypertrophy in adult rat ventricular myocytes [77,78]. This mechanism is fundamentally explained by the modification of Cys-118, a critical reactive Cys located in the guanine nucleotide-binding site. In contrast, Raines *et al.* reported that nNOS-mediated S-nitrosylation of H-Ras at Cys-118 suppresses H-Ras activity induced by Ca²⁺ ionophores [76]. We have also found that modification of Cys-118 is not stable and

gradually decreases GTPase activity of recombinant H-Ras (unpublished observation). Therefore, it appears that modification of Cys-118 on Ras transiently, but not continuously, activates Ras-dependent signaling pathways.

Recent studies have shown that PTM of G proteins, especially monomeric small G proteins, direct them to various cellular membranes and modulate GTP-GDP exchange [79]. Important PTM of G proteins includes the constitutive and irreversible remodeling of its carboxy-terminal CAAX motif. In this remodeling in which C is Cys, A is usually an aliphatic amino acid, and X is any amino acid, by farnesylation (isoprenylation), proteolysis and methylation, reversible palmitoylation, and conditional modifications, including phosphorylation, peptididyl-prolyl isomerization, mono- and di-ubiquitination, ADP ribosylation, and glycosylation. One of two palmitoylation sites (Cys-184) of H-Ras has been shown as a redox-active Cys. Oxidative modification of Cys proximal to the CAAX motif suppresses or in some cases accelerates other PTMs, leading to modification of G protein activity [80,81].

3. Redox control of cardiac remodeling

3.1. Formation of 8-nitro-cGMP in cardiac cells.

Chronic heart failure is a leading cause of morbidity and mortality in developed countries. ROS and NO generated from Nox2 and iNOS have been implicated in the pathogenesis of heart failure. In fact, 3-nitrotyrosine, an endogenous nitrating product, is actually generated in mouse hearts after myocardial infarction (MI), accompanied by a dramatic increase in expression levels of Nox2 and iNOS proteins. Using liquid chromatography coupled with tandem mass spectrometry analysis and immunohistochemistry with an anti-8-nitro-cGMP specific antibody, we showed that 8-nitro-cGMP is produced in mouse hearts 4 weeks after MI and 6 weeks after pressure overload [14]. Inducible NOS-derived NO may be essential for 8-nitro-cGMP formation in mouse hearts because 8-nitro-cGMP formation after MI is completely diminished in iNOS-deficient hearts. Although protein expression levels of iNOS, but not Nox2, are promptly increased in mouse hearts after MI, 8-nitro-cGMP formation is not present in mouse hearts within 1 week after MI. These results indicate that mouse hearts produce 8-nitro-cGMP in response to ischemia or mechanical stress in an iNOS-dependent manner, while Nox2-derived ROS may be essential for the formation of 8-nitro-cGMP by iNOS-derived NO [14].

3.2. Cardiac cell senescence via S-guanylation of H-Ras

Cardiac aging is one of the major causes of sudden death or left ventricular

dysfunction after MI. Cellular senescence is reportedly induced by oxidative stress, nucleotide damage (e.g., UV irradiation), and abnormal activation of oncogenes, including Ras family G proteins [82,83]. Recent studies of oxidative inflammatory reactions suggest that an H-Ras oncogenic cellular response can be induced by ROS- or RNS-derived species and 15d-PGJ₂ [64,84], which in turn may activate p53-dependent cellular senescence. We found that 8-nitro-cGMP mediates cardiac cellular senescence via *S*-guanylation of H-Ras at Cys-184 in failing hearts in mice after MI. In contrast, another electrophile, 1,2-NQ, does not modify Cys-184 of H-Ras or induce H-Ras activation, although 1,2-NQ strongly increases ERK activity in cardiomyocytes [14]. Among several endogenous and exogenous electrophiles, 8-nitro-cGMP is suggested to function as a specific physiological ligand for H-Ras activation. H-Ras possesses two palmitoylation sites (Cys-181 and Cys-184) located at its carboxy-terminal domain, and palmitoylation of Ras proteins plays an important role in Ras protein localization and activity [80]. Monopalmitoylation of Cys-181 is required for efficient trafficking of H-Ras to the plasma membrane, and GDP-bound H-Ras is predominantly detected in lipid rafts [80]. GTP loading of H-Ras releases H-Ras from the rafts so that they become more diffusely distributed in the plasma membrane, an event necessary for efficient activation of Raf. Although Cys-184 is not essential for targeting of H-Ras to the plasma membrane, it is essential for control of GTP-regulated lateral segmentation of H-Ras between lipid rafts and non-rafts. Inhibition of Cys-184 by 8-nitro-cGMP palmitoylation efficiently delivers H-Ras to the plasma membrane. This indicates that *S*-guanylation of H-Ras at Cys-184 promotes localization of H-Ras to the plasma membrane and association with Raf by dissociating H-Ras from lipid rafts. Oxidative stress-related nucleotides that induce DNA and nucleotide damage, as well as activation of oncogenic Ras, can induce cellular senescence via p53 and Rb tumor suppressor pathways [82,83]. Our recent findings provide evidence that electrophilic modification causes precise structural alterations of H-Ras, making it more accessible to Raf [14]. This leads to transduction of electrophile-mediated signaling to downstream phosphorylation signaling, including extracellular signal-regulated kinase, p38 mitogen-activated protein kinase, p53, and Rb.

3.3.Prevention of cardiac senescence via elimination of electrophiles by reactive sulfur species

Although endogenous hydrogen sulfide is thought to be formed by CBS and cystathionine γ -lyase in mammalian cells and tissues [58], the exact chemical nature and physiological functions of the products of their enzymatic reaction are mostly undefined.

CBS knockdown significantly suppresses generation of reactive sulfur metabolites, which are responsible for electrophile sulfhydration as discussed above, in several tumor cell lines (A549, HepG2, and C6 cells) expressing abundant CBS proteins [14]. In contrast, lower levels of CBS and cystathionine γ -lyase expression as well as a much lower production of reactive sulfur metabolites are observed in mouse hearts, primary-cultured rat neonatal cardiomyocytes, and cardiac fibroblasts compared with all tumor cell lines. Therefore, reactive sulfur metabolites in the heart may be limiting in the context of the amount of endogenous electrophiles that can be generated during basal metabolism and inflammatory responses. Therefore, myocardial cells may be relatively sensitive to endogenous electrophiles, particularly when they are excessively generated during inflammatory processes.

We evaluated pharmacological activities of hydrogen sulfide derivatives in an *in vivo* model of chronic heart failure by exogenous treatment with NaHS [14]. Considerable 8-nitro-cGMP production, which depends on iNOS expression and activity, is strongly inhibited by low-dose NaHS (50 mmol/kg/day, i.p.) in mouse hearts after MI. NaHS treatment has no significant effects on tyrosine nitration reaction occurring in cardiac tissues, suggesting that hydrogen sulfide does not directly scavenge ROS and RNS, but other related sulfur species might eliminate ROS/RNS, and even their derived electrophiles. The metabolic pathways related to cGMP biosynthesis and degradation (e.g., sGC and PDEs) are not affected by the same treatment.

NaHS treatment fully inhibits H-Ras activation and concomitant H-Ras S-guanylation in post-MI mouse hearts. NaHS treatment *in vivo* after MI significantly improves left ventricular dilation and limits left ventricular dysfunction in mice, although ischemic scars are equally developed in hearts of NaHS- and vehicle-treated mice [14]. Furthermore, phosphorylation of p38 mitogen-activated protein kinase, extracellular signal-regulated kinase, p53, and Rb protein is increased in mouse hearts after MI, with NaHS suppressing their activation. These results confirm that any particular reactive sulfur metabolites attenuate left ventricular dilation and dysfunction after MI, in part by suppressing myocardial cell S-guanylation-dependent activation of H-Ras and its downstream signaling pathways.

4. Conclusions

Although 8-nitro-cGMP is a moderate electrophile compared with other electrophiles generated by ROS, 8-nitro-cGMP functions as a newly discovered important player in the development of heart failure. ROS signaling is precisely regulated by various sensor and effector molecules, and the unique property of 8-nitro-cGMP appears to contribute

to its specificity and selectivity for ROS signal transduction. The emergence of new evidence supporting the role of electrophilic modifications of Ras family proteins may presage a new era in cardiovascular biology. H-Ras knockout mice show hypotension via enhancing the NO-cGMP-PKG pathway [85] and R-Ras knockout mice show abnormal maturation of new blood vessels in tumors [86]. Therefore, further extensive *in vivo* studies focusing on S-guanylation induced by 8-nitro-cGMP may reveal new aspects of redox control of cardiovascular physiological functions, such as vascular tone and angiogenesis. Notably, our recent investigation revealed that ROS and electrophiles were primarily regulated by reactive sulfur species such as Cys hydropersulfide (CysSSH) and its derivatives, endogenously occurring in most tissues and cells, rather than other standard sulfur-containing metabolites like glutathione and hydrogen sulfide (unpublished observation). Control of electrophilic PTM using nucleophilic properties of reactive sulfur compounds could be a new therapeutic strategy for the prevention and treatment of chronic cardiovascular diseases.

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Figure legends

Figure 1. Sulphydration of electrophiles (E) by reactive sulfur speices and subsequent regulation of metabolic pathways. Reactive sulfur species that can behave like nucelophilic HS^- act as a potent nucleophile and are thought to suppress cardiac senescence and dysfunction by inhibiting electrophilic modification of sensor proteins, including H-Ras.

Figure 2. Putative redox control of vascular tone. In small arteries, both electrophiles (i.e., 8-nitro-cGMP) and reactive sulfur function as vasodilators. However, nucleophilic reaction of HS^- may quench 8-nitro-cGMP, leading to suppression of vasodilation mediated by 8-nitro-cGMP and reactive sulfurs.

Figure 3. Redox control of the G cycle. GAP; GTPase activating protein, GDI; GDP dissociation inhibitor, GEF; GTP/GDP exchange factor, PB; carboxy-terminal polybasic domain, SW; switch region.

Figure 4. Conserved reactive Cys residues in Ras family proteins.

Figure 1

Figure 2

Figure 3

Figure 4