Published in final edited form as:

J Mol Cell Cardiol. 2013 July ; 60: 72–83. doi:10.1016/j.yjmcc.2013.03.019.

Volume overload induces differential spatiotemporal regulation of myocardial soluble guanylyl cyclase in eccentric hypertrophy and heart failure

Yuchuan Liu^a, A. Ray Dillon^b, Michael Tillson^b, Catherine Makarewich^{a,c}, Vincent Nguyen^a, Louis Dell'Italia^d, Abdel Karim Sabri^{a,c}, Victor Rizzo^{a,e}, and Emily J. Tsai^{a,c,f,*}

^aCardiovascular Research Center, Temple University School of Medicine, Philadelphia, PA, USA

^bCollege of Veterinary Medicine, Auburn University, Auburn, AL, USA

^cDepartment of Physiology, Temple University School of Medicine, Philadelphia, PA, USA

^dDivision of Cardiovascular Disease, Department of Medicine, University of Alabama Birmingham, Birmingham, AL, USA

^eDepartment of Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, PA, USA

^fSection in Cardiology, Department of Medicine, Temple University School of Medicine, Philadelphia, PA, USA

Abstract

Nitric oxide activation of soluble guanylyl cyclase (sGC) blunts the cardiac stress response, including cardiomyocyte hypertrophy. In the concentric hypertrophied heart, oxidation and re-localization of myocardial sGC diminish cyclase activity, thus aggravating depressed nitric oxide–cyclic guanosine monophosphate (NO–cGMP) signaling in the pressure-overloaded failing heart. Here, we hypothesized that volume-overload differentially disrupts myocardial sGC activity during early compensated and late decompensated stages of eccentric hypertrophy. To this end, we studied the expression, redox state, subcellular localization, and activity of sGC in the left ventricle of dogs subjected to chordal rupture-induced mitral regurgitation (MR). Unoperated dogs were used as Controls. Animals were studied at 4 weeks and 12 months post chordal rupture, corresponding with early (4wkMR) and late stages (12moMR) of eccentric hypertrophy. We found that the sGC heterodimer subunits relocalized away from caveolae-enriched lipid raft microdomains at different stages; sGC β_1 at 4wkMR, followed by sGC α_1 at 12moMR. Moreover, expression of both sGC subunits fell at 12moMR. Using the heme-dependent NO donor DEA/NO and NO-/heme-independent sGC activator BAY 60-2770, we determined the redox state and inducible activity of sGC in the myocardium, within caveolae and non-lipid raft microdomains. sGC was oxidized in non-lipid raft microdomains at 4wkMR and 12moMR. While overall

© 2013 Elsevier Ltd. All rights reserved.

*Corresponding author at: Cardiovascular Research Center, Temple University School of Medicine, 3500 N. Broad Street, MERB 1047, Philadelphia, PA 19140, USA. Tel.: +1 215 707 5512; fax: +1 215 707 5737. emily.tsai@tuhs.temple.edu (E.J. Tsai).

Disclosures

The authors have no conflicts of interests to disclose.

DEA/NO-responsiveness remained intact in MR hearts, DEA/NO responsiveness of sGC in non-lipid raft microdomains was depressed at 12moMR. Caveolae-localization protected sGC against oxidation. Further studies revealed that these modifications of sGC were also reflected in caveolae-localized cGMP-dependent protein kinase (PKG) and MAPK signaling. In MR hearts, PKG-mediated phosphorylation of vasodilator-stimulated phosphoprotein (VASP) disappeared from caveolae whereas caveolae-localization of phosphorylated ERK5 increased. These findings show that differential oxidation, re-localization, and expression of sGC subunits distinguish eccentric from concentric hypertrophy as well as compensated from decompensated heart failure.

Keywords

Eccentric hypertrophy; Cyclic guanosine monophosphate signaling; Caveolae; Soluble guanylyl cyclase

1. Introduction

Volume-overload cardiac stress, such as that associated with regurgitant valvular disease and dilated cardiomyopathy, triggers eccentric cardiac hypertrophy. Despite the predominance of volume-overload in heart failure, the molecular signaling of pathologic eccentric hypertrophy remains incompletely understood. Our understanding of cardiac hypertrophy has been largely derived from animal models of pressure-overload induced concentric hypertrophy and transgenic mice. Yet, animal and human studies of volume-overload induced eccentric cardiac remodeling point to a pathophysiology distinct from that induced by pure pressure-overload [1–4]. Volume-overload induces differential extracellular matrix remodeling, inflammation, metabolic dysfunction, and oxidative stress signaling [5–12].

NO–cGMP signaling protects the heart against various stressors, including pro-hypertrophic cardiac stress [13–18]. We previously reported oxidation and re-localization of the nitric oxide receptor soluble guanylyl cyclase (sGC) in pressure-overload induced concentric hypertrophy, revealing a novel regulatory mechanism of NO–cGMP signaling [19]. By assessing heme-dependent and heme-independent sGC production of cGMP in the myocardium, we found that oxidation of sGC greatly diminished cyclase activity in the concentric hypertrophied heart. We also identified caveolae as plasma membrane microdomains wherein relative protection from oxidation partially preserved NO-inducible cyclase activity. Small (50–100 nm), lipid- and protein-rich, flask-like invaginations of the plasma membrane, caveolae function in the compartmentalization of signal transduction, receptor-independent endocytosis, and mechano-transduction [20]. In concentric hypertrophied hearts, sGC heterodimer subunits re-localized away from caveolae, thus altering the spatial regulation of NO–cGMP signaling.

How volume-overload cardiac stress alters myocardial NO–cGMP signaling is unknown and unexplored. We hypothesized that volume-overload cardiac stress also disrupts myocardial NO–cGMP signaling but diverges from pressure-overload cardiac stress with regard to its impact on cyclase activity within caveolae. Several signaling molecules involved in eccentric and concentric hypertrophic signaling, including calcium channels and mitogen

activated protein kinases (MAPKs), reside within caveolae, suggesting this functional microdomain as a potential differential node in these hypertrophic signaling pathways [21].

In this study, we examined the submyocardial distribution, redox state, and inducible cyclase activity of the sGC heterodimer in a canine chronic mitral regurgitation model of volume-overload induced eccentric hypertrophy and heart failure. We exploited the variable redox state dependent responses of sGC to the heme-dependent NO donor DEA/NO (diethylamine NONOate) and heme-independent sGC activator BAY 60-2770. We also sought to relate changes in caveolae-localized NO–cGMP signaling with differential MAPK signaling. Whereas diuretics are used to manage volume-overload in heart failure patients, none of the current heart failure pharmacotherapies address the resultant eccentric hypertrophy [22]. By determining myocardial signaling abnormalities specific to volume-overload cardiac stress and eccentric hypertrophy, we aim to identify novel therapeutic targets that can fundamentally change the approach to heart failure therapy and complement current neurohormonal blockade strategies.

2. Materials and methods

2.1. Animal experiments

Mitral regurgitation (MR) was induced in conditioned mongrel dogs of either sex (19 to 26 kg) by chordal rupture with the use of a fluoroscopically guided catheterization method previously described [23,24]. Animals were maintained at a deep plane of general anesthesia using isoflurane (0.75–1.5%) and oxygen (2 L/min) and were mechanically ventilated during the catheterization procedure. Ten dogs underwent chordal rupture (n = 5 for 4wkMR, n = 5 for 12moMR); eight unoperated dogs served as Controls. Transthoracic echocardiography (2.25-MHz transducer, ATL Ultramark VI) and cardiac magnetic resonance imaging (cMRI, 1.5 T, GE Signa Horizon) were performed at baseline and at 4 weeks or 12 months after MR induction, as previously described (Appendix A) [25]. At time of euthanasia, the heart was arrested with intracardiac injection of KCl and quickly extirpated and placed in phosphate-buffered ice slush. The coronaries were flushed with oxygenated Krebs solution. A portion of the LV was cut and snap-frozen in liquid nitrogen for subsequent biochemical studies. These animal studies were approved by the Animal Services Committees at the University of Alabama at Birmingham and College of Veterinary Medicine, Auburn University.

2.2. Isolation of caveolin-enriched lipid raft fraction

Caveolin-enriched lipid raft fractions (Cav3⁺LR) were prepared from snap-frozen LV tissue, using a discontinuous 35–5% sucrose density gradient ultracentrifugation method as previously described [19]. LV tissue homogenization was carried out on ice, in detergent free buffer (50 mmol/L Tris–HCl, pH 7.6, 1 mmol/L EDTA, 1 mmol/L DTT, 2 mmol/L PMSF, 50 mmol/L NaF, 1 mmol/L Na Vanadate) with protease inhibitors (Mammalian Cocktail, Sigma-Aldrich). Following 1 hour incubation on ice with intermittent vortex, 0.6 mL of tissue homogenate was mixed with 1.4 mL of 60% (w/w) sucrose in 20 mmol/L KCl and 0.5 mmol/L MgCl₂ and placed at the bottom of an ultracentrifuge tube. A discontinuous 35%–5% sucrose gradient was formed by overlaying each sample with 1.3 mL of 35% sucrose and then with 1.3 mL of 5% sucrose. The sucrose density gradient was topped off

with 0.5 mL of 200 mmol/L KCl. Each sample was then centrifuged at $>180,000\ g$ for 18 h at 4 °C in a swinging bucket rotor (Beckman Instruments, Palo Alto, CA) without any brake. The top KCl layer was discarded and fractions were collected every 400 μ L from the top sucrose layer corresponding to F1 (top, most buoyant) to F11 (bottom, least buoyant/heaviest).

A light-scattering band confined to the 35–5% sucrose interface, typically F4–F6, corresponds to Cav3⁺LR fractions. Ponceau staining and protein concentrations determined by BCA assay confirmed that total protein distribution was weighted towards heavier sucrose density gradient fractions (F7 through F11) lacking Cav3 in both Control and MR hearts. Proteins were precipitated using 0.1% w/v deoxycholic acid in 100% w/v trichloroacetic acid. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce). Non-lipid raft (NLR, F11) and Cav3⁺LR fractions (F4–F5) without TCA precipitation were also collected for BCA and subsequent cGMP assays.

2.3. Reagents and antibodies

Primary antibodies used for western blot analysis included: sGC α_1 (1:1000, Abcam); sGC β_1 (1:4000, Cayman Chemicals); Cav-3 (1:10,000, BD Transduction); PDE2A (1:500, Fagennix); PDE3A (1:500, Santa Cruz); PDE5A (1:1000, Cell Signaling); PKG (1:250, Santa Cruz); VASP (1:250, BD Transduction); phospho-VASP (phospho-Ser239, 1:4000, Santa Cruz); nitro-tyrosine (NO₂-Tyr, 1:20,000, Millipore); p38 (1:500, Cell Signaling); phospho-p38 (1:500, Santa Cruz Biotech); ERK5 (1:1000, Cell Signaling); phospho-ERK5 (1:1000, Invitrogen); and GAPDH (1:10,000, Cell Signaling). Specificity of anti-sGC α_1 and - β_1 antibodies was confirmed using protein extracts from sGC $\alpha_1^{-/-}$ and sGC $\beta_1^{-/-}$ mouse hearts as previously published [19]. Primary antibody binding was visualized by horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

2.4. Western blot analysis

Protein extracts from LV tissue homogenate and the above mentioned subfractions were run on SDS-PAGE gels and transferred to nitrocellulose membranes. Total LV protein extracts were run in equal protein amount on SDS-PAGE electrophoresis, whereas each sucrose density gradient fraction was run in equal volume, as is convention for immunoblots of sucrose density gradient fractions. Immunoblot analysis was performed using primary antibody probes as detailed above. Total protein westerns were normalized to respective GAPDH signals. Sucrose density gradient fraction westerns were normalized to the sum of the target signal across all fractions for each heart. Densitometry analysis of immunoblots was performed using Image J Software (NIH).

2.5. sGC activity assay and determination of redox state

Baseline and agonist-stimulated cGMP levels of total LV, Cav3⁺LR, and NLR from Control, 4wkMR, and 12moMR hearts were measured by direct cGMP EIA kit from New East Biosciences (Malvern, PA). Homogenates were pre-incubated at room temperature for 15 min in a solution for final concentrations of Tris 50 mM, pH 7.6, IBMX (3-isobutyl-1-

methylxanthine) 0.75 mmol/L, creatine phosphate 3.5 mmol/L, creatinine phosphokinase 1 unit, GTP 1 mmol/L, and MgCl₂ 3 mmol/L. Samples were then incubated with or without DEA/NO (1 μ mol/L) or BAY 60-2770 (0.01 μ mol/L) at 37 °C for 10 min and subjected to diethyl ether extraction. cGMP levels of ether-extracted samples were measured by EIA according to kit manufacturer protocol. BAY 60-2770 compound was provided by J-P Stasch (Bayer AG, Wuppertal, Germany). A BAY 60-2770 response greater than the respective DEA/NO response indicated oxidation of sGC [19,26,27].

2.6. Statistics

All values are expressed as mean $\pm$ SEM. Statistical analyses were performed using: 2-way ANOVA when determining interaction of conditions or the source of variance, followed by Holm-Šídák multiple comparison testing, as appropriate; 1-way ANOVA followed by Tukey's multiple comparison test; and paired or unpaired Student's *t* test, as appropriate, and with Welch's correction, if variances were unequal. Statistical significance was defined as *P* < 0.05. GraphPad Prism 6.0 was used for statistical and graphical analysis.

3. Results

3.1. Chronic mitral regurgitation volume-overload induces eccentric hypertrophy and heart failure

Eccentric hypertrophy is characterized by increased LV mass accompanied by normal to reduced relative wall thickness. Assessment of cardiac structure and function by cardiac imaging revealed compensated LV eccentric hypertrophy at 4 weeks and decompensated LV eccentric hypertrophy at 12 months, as described previously (Table 1) [24,25]. By 12moMR, LV fractional shortening (FS) was markedly reduced.

3.2. Volume-overload cardiac stress compromises NO–cGMP signaling via oxidation of sGC and decreased sGC β_1 expression

Using the heme-dependent NO-donor DEA/NO and heme-/NO-independent sGC activator BAY 60-2770, we assessed the inducible cyclase activity and the redox state of sGC in control and eccentric hypertrophied hearts. By conducting these assays in the presence of the universal phosphodiesterase inhibitor IBMX, we ensured that agonist-induced cGMP levels reflected purely sGC-derived cGMP production. Of note, basal cGMP levels were similar in all hearts. While DEA/NO stimulation of total protein extract induced high levels of cGMP across all hearts, only those of Control and 4wkMR hearts were significantly greater than their respective baseline cGMP levels (Fig. 1A).

To determine whether myocardial sGC expression and/or redox state change with volume-overload cardiac stress, we also compared the DEA/NO-induced cGMP response of total LV protein extracts to that induced by BAY 60-2770 (Fig. 1B). A heme- and NO-independent sGC activator, BAY 60-2770 stimulates sGC cyclase activity irrespective of the redox state of sGC. That is, whereas oxidation of its ferrous heme moiety renders sGC unresponsive to NO, oxidation of sGC potentiates its response to BAY 60-2770. BAY 60-2770 can also activate sGC lacking the heme-moiety that is otherwise vital to NO activation.

The BAY 60-2770 response of 4wkMR total protein extract was markedly potentiated relative to its DEA/NO response and relative to the BAY 60-2770 response of Control and 12moMR hearts (Fig. 1B). While BAY 60-2770 also elicited marked cGMP production in 12moMR hearts (Fig. 1A), DEA/NO-induced cGMP levels in 12moMR hearts were not significantly greater than respective baseline cGMP (paired Student's *t*-test $P = 0.087$ for 12moMR DEA/NO vs Basal). Furthermore, the decreased BAY 60-2770 response of 12moMR hearts, relative to 4wkMR hearts, suggests that sGC β_1 expression had fallen in these decompensated, late-stage eccentric hypertrophied hearts.

Next we performed western analysis of the LV total protein extracts to determine if the differential responsiveness to BAY 60-2770 and DEA/NO of 4wkMR hearts reflected oxidation or, alternatively, increased expression of sGC. While sGC α_1 expression did not vary with volume-overload, sGC β_1 in 12moMR hearts fell to about 50% of Control levels (Fig. 2). Even in 4wkMR hearts, overall sGC β_1 expression trended towards a decline from Control levels. Thus the potentiated BAY 60-2770 response of 4wkMR hearts indeed reflected oxidation of sGC. Western analysis also confirmed that the diminished BAY 60-2770 response of 12moMR hearts, relative to 4wkMR hearts, could be partly attributable to overall decreased sGC β_1 expression.

3.3. Differential cardiac phosphodiesterase (PDE) isoform expression varies in transition from early-stage to decompensated, late-stage eccentric hypertrophied hearts, suggesting stress-induced variations in local cGMP pools within cardiomyocytes

Despite the observed differences in total sGC β_1 expression, basal cGMP levels were similar in Control and eccentric hypertrophied hearts, be it early or late stage. To better understand how baseline cGMP levels could remain relatively unchanged over this period of prolonged volume-overload cardiac stress, we also examined the expression of cardiac cGMP PDE isoforms in these hearts (Fig. 3). The expression of the cardiac PDE isoforms varied between the 4wkMR and 12moMR hearts, with decreased PDE2 levels in 4wkMR hearts and, conversely, decreased PDE3 and PDE5 levels in 12moMR hearts. As PDE2 is a dual substrate phosphodiesterase which selectively hydrolyzes cAMP upon cGMP activation, the decline in overall PDE2 expression in 4wkMR hearts theoretically should not impact basal cGMP levels. In contrast, the expression of PDE5, the only cGMP-activated and cGMP-selective PDE isoform, remained unchanged in 4wkMR hearts, compared to control, but ultimately fell in 12moMR hearts. With less PDE5 to hydrolyze sGC-derived cGMP in 12moMR hearts, the fall in sGC could effectively be counterbalanced, thereby maintaining similar overall basal cGMP levels across all groups. Interestingly, expression of PDE3, a dual substrate PDE inhibited by cGMP, also fell in 12moMR but not 4wkMR hearts. Given its selectivity for cAMP, changes in PDE3 expression would not be expected to impact global basal cGMP levels. Thus, these temporal changes in cardiac PDE isoform expression may aid to preserve basal cGMP levels in the presence of volume-overload cardiac stress.

3.4. Global changes in myocardial expression of sGC β_1 and PDE isoforms do not impact overall baseline NO–cGMP signaling but influences reactive nitrogen species (RNS) signaling in eccentric hypertrophied hearts

To study the effect of these global changes induced by volume-overload on downstream NO–cGMP signaling, we measured PKG I-mediated phosphorylation of vasodilator-stimulated phosphoprotein (VASP). Since NO can also alternatively signal independently of sGC activation and cGMP, via reactive nitrogen species (RNS), we also measured global tyrosine-nitration (NO₂-Tyr) via immunoblot detection. Overall baseline ratio of PKG-phosphorylated VASP-to-total VASP was similar across all three groups, whereas the ratio of total tyrosine-nitration to GAPDH in these hearts increased at 4wkMR and returned to Control level at 12moMR (Fig. 4).

3.5. Volume-overload impacts spatial regulation of myocardial NO–cGMP and RNS signaling

Given the differential expression of cardiac PDE isoforms and global changes in tyrosine-nitration detected in eccentric hypertrophied hearts, we sought to examine the intracellular distribution of myocardial sGC, as well as its cyclase activity and redox state within membrane microdomains. We hypothesized that, while global changes in myocardial NO–cGMP and RNS signaling may appear subtle with volume-overload cardiac stress, more profound changes occur in the submyocardial spatial regulation of NO signaling.

We first examined the localization of the sGC α_1 and sGC β_1 to caveolae by separating Cav3 enriched lipid rafts from non-lipid raft microdomains. In Controls, sGC α_1 and sGC β_1 were detected in both caveolae-enriched and nonenriched microdomains. Moreover, in Controls, the distribution of sGC α_1 , sGC β_1 , and Cav3 across SDG fractions revealed sGC α_1 and sGC β_1 to be relatively enriched in Cav3⁺LR (Fig. 5). However, in the MR hearts, the distribution of sGC β_1 and then sGC α_1 shifts away from the caveolae microdomain (Fig. A. 1).

To our surprise, sGC α_1 was undetectable in fractions F1–F11 in three of the five 12moMR hearts, at least at the expected molecular weight of approximately 75 kDa. Instead, a robust signal of about 40 kDa was detected, possibly corresponding to the C- α_1 splice variant previously described in humans (Fig. A.2) [28]. The distribution of this 40 kDa sGC α_1 splice variant was diffuse and remained similarly diffuse in Control and MR hearts.

3.6. Volume overload cardiac stress alters the redox state of sGC in a microdomain specific manner

Caveolae (Cav3⁺LR) and non-lipid-raft (NLR) microdomains were stimulated with DEA/NO and BAY 60-2770 to determine inducible cyclase activity as well as redox state of sGC (Fig. 6). DEA/NO responsiveness of caveolae-localized sGC was similar in Control and 4wkMR hearts but moderately blunted in 12moMR hearts ($P = 0.07$ vs Control). BAY 60-2770 responsiveness of sGC within Cav3⁺LR remained similar across all groups and, notably, less than or equal to DEA/NO responsiveness, indicating that caveolae-localized sGC is predominantly in the reduced state.

In contrast to Cav3⁺LR, NLR demonstrated differential responsiveness to DEA/NO and BAY 60-2770 in Control and MR hearts. The volume-overload induced changes in DEA/NO and BAY 60-2770 responses within the NLR of the hearts: 1) corroborate the re-localization of sGC away from Cav3⁺LR and towards NLR; and 2) indicate oxidation of NLR-localized sGC in the volume-overloaded hearts. Increased DEA/NO and BAY 60-2770 responses are expected in the 4wkMR NLR given the re-localization of sGC away from Cav3⁺LR in 4wkMR. However, the markedly potentiated BAY 60-2770 response in 4wkMR NLR is particularly interesting given that in the 4wkMR Cav3⁺LR, BAY 60-2770 responsiveness was much less than DEA/NO-responsiveness. This inversion in the relative responsiveness to BAY 60-2770 and DEA/NO suggests oxidation of sGC within the NLR microdomain of 4wkMR hearts. The modest decline in BAY 60-2770 response of 12moMR NLR, compared to 4wkMR NLR and concordant with the diminished DEA/NO response of 12moMR NLR, is consistent with the overall decline of sGC in 12moMR. The potentiated BAY 60-2770 response relative to DEA/NO response of 12moMR NLR indicated a predominance of oxidized or heme-free sGC in the NLR.

3.7. Re-localization and microdomain-specific oxidation of sGC appear to impact upon local NO–cGMP and RNS signaling

We hypothesized that these changes in localization and cyclase activity of sGC in the volume-overloaded hearts would also be reflected in downstream NO–cGMP and RNS signaling within the caveolae microdomain. To further investigate NO signaling within caveolae, we determined the distribution of phosphorylated VASP (pVASP) and nitrated-tyrosine (NO₂-Tyr) across sucrose density gradient fractions by western analysis (Figs. 7A and B). While detectable within Cav3⁺LR (F3–F6) of Control hearts, pVASP disappeared from Cav3⁺LR of 4wkMR and 12moMR hearts. Instead, pVASP was detected almost entirely in the heavy density fractions (F7–F11) of 4wkMR and 12moMR hearts (Fig. A.3). Distribution of pVASP was altered in MR hearts despite similar distribution of PKG and VASP in Control and MR hearts (Fig. A.4). Interestingly, nitrated-tyrosines were similarly detectable within the Cav3⁺LR (F3–F6) of Control but not 4wkMR or 12moMR hearts. The distribution of tyrosine-nitrated proteins was weighted heavily towards the heavy density fractions in the volume overloaded hearts (Figs. 7B and A.3.).

3.8. Volume-overload cardiac stress activated p38 and ERK5 overall and selectively activated caveolae-localized ERK5 signaling

We hypothesized that altered spatial localization and cyclase regulation of sGC might lead to regional changes in MAPK signaling associated with eccentric hypertrophy—stress-activated protein kinase p38 and extracellular-signal-regulated kinase 5 (ERK5) [1,4,29].

Assessment of overall p38 and ERK5 signaling via immunoblot detection of phosphorylated p38 (p-p38) and phosphorylated ERK5 (p-ERK5) relative to total p38 and ERK5 in LV total protein extracts, revealed an overall decrease in p-ERK5-to-ERK5 ratio at 12moMR (Figs. 8A and B). However, western analysis of sucrose density gradient fractions of Control, 4wkMR, and 12moMR hearts revealed that p-p38 and p-ERK5 were present in both Cav3⁺LR and NLR microdomains, with predominance in heavy density fractions F7–F11 (Figs. 8C and A.5). Both p-p38 and p-ERK5 were found in Cav3⁺LR microdomains of all

experimental groups. However, in volume overloaded hearts (4wkMR and 12moMR), caveolae-localization of p-p38 and p-ERK5 was greater than that of p38 and ERK5 (Fig. 8C). Cav3⁺LR localization of the phosphorylated MAPK exceeded that of total MAPK for both p38 and ERK5 in 4wkMR hearts. By 12moMR, only pERK was relatively enriched in the Cav3⁺LR microdomain.

4. Discussion

This study is the first to demonstrate that volume-overload cardiac stress alters myocardial localization, expression, and activity of soluble guanylyl cyclase (sGC). We discovered that altered microdomain NO–cGMP signaling is associated with the pattern of cardiac hypertrophic response and the transition to decompensation (Fig. 9). First, sGC is modified in the eccentric hypertrophied heart with respect to its expression, spatial localization, and inducible cyclase activity, with the most profound changes occurring in the late stage, decompensated eccentric hypertrophied heart (12moMR). Secondly, caveolae-localization protects myocardial sGC from oxidation. Thirdly, stress-induced modifications of sGC were also reflected in downstream NO–cGMP and MAPK signaling in a microdomain specific manner. Thus, chronic-volume overload induced differences in the redox regulation and DEA/NO-responsiveness of sGC may partially determine the pattern of cardiac hypertrophy.

4.1. Volume-overload differentially affects sGC subunit expression and localization

The ubiquitously expressed sGC $\alpha_1\beta_1$ is the predominant isoform in the myocardium and, as we have previously shown, localizes to plasmalemmal caveolae as well as the cytosol [19]. With volume-overload cardiac stress, overall expression of sGC α_1 and sGC β_1 declined, though with differential temporal patterns and statistical significance. More strikingly, both subunits relocated away from caveolae in volume-overloaded hearts, again with differential temporal patterns. Whereas the shift of sGC β_1 away from caveolae was detectable as early as 4wkMR and persisted through 12moMR, that of sGC α_1 was most notable at 12moMR. Dynamic caveolae-localization has been reported for a number of signaling proteins, including the angiotensin II type 1 receptor [30], endothelial nitric oxide synthase (eNOS) [31], endothelin receptor subtype A [32], β_2 adrenergic receptor (β_2 AR) [33], and muscarinic cholinergic receptor [34]. While the mechanistic details remain uncertain for many of these proteins, the reversible, post-translational modification s-palmitoylation is the most commonly imputed mechanism, as is the case for eNOS [31,35] and β_2 AR [33].

Relocalization of sGC α_1 was not appreciated in our prior studies of pressure-overload induced concentric hypertrophy and may reflect changes that depend on the chronicity, severity, and/or nature of the hemodynamic stress. More intriguing was the detection of a smaller ~40-kDa sGC α_1 diffusely throughout the canine LV myocardium, both within and without the caveolae microdomain. This ~40-kDa sGC α_1 variant may well correlate with the 54-kDa sGC α_1 -c splice variant described in humans to be a fully functional NO-sensitive enzyme that is susceptible to ODQ-inhibition but resistant to oxidation mediated protein degradation [28]. In our studies, the ~40-kDa sGC α_1 variant remained relatively unchanged in expression throughout chronic volume-overload but shifted away from caveolae as early as 4wkMR.

The spatiotemporal regulation of NO–cGMP signaling was also suggested by the differential overall expression of the PDE isoforms at early and late stage eccentric hypertrophy (Fig. 3). The PDE isoforms and their splice variants have subcellular regional specificity; PDE2A is confined to the membrane with the sarcomeric Z-band [36], PDE3A isoforms are either cytosolic or membrane-associated [37], and PDE5 localizes to the cardiomyocyte sarcomeric Z-band but becomes diffusely distributed in pathologically hypertrophied hearts [38,39]. Local cGMP pools within cardiomyocytes likely change over time with chronic volume-overload, as the relative expression and subcellular localization of these cardiac PDE isoforms vary along with the relocalization of the sGC subunits, and may mark the transition from compensated eccentric hypertrophy to clinical decompensation.

4.2. Redox regulation of sGC cyclase activity varies with caveolae localization

Consistent with our prior mouse studies, we again found that caveolae-localized sGC was relatively protected from oxidation. Assays of MR heart microdomains repeatedly revealed the presence of oxidized sGC in NLR but not Cav3⁺LR. Despite the consistent association between redox state and caveolae localization of sGC, DEA/NO responsiveness varied between eccentric and concentric hypertrophied hearts. Instead of a blunted DEA/NO response, as previously seen in the concentric hypertrophied mouse hearts, eccentric hypertrophied canine hearts had relatively preserved DEA/NO responses. This divergence in DEA/NO responsiveness may reflect fundamental differences in the hemodynamic stress (i.e., volume- versus pressure-overload) and pattern of hypertrophy (i.e., eccentric versus concentric). Most strikingly, the 40 kDa sGC α_1 variant was detected in the hearts of dogs but not mice. If analogous to the 54-kDa sGC α_1 -c splice variant in humans, the 40 kDa sGC α_1 variant in dogs may similarly resist oxidation-induced protein degradation and thus could partially account for the relatively preserved DEA/NO responsiveness of the volume-overloaded canine heart.

4.3. Alterations in subcellular localization and cyclase activity of sGC impact upon local pools of NO–cGMP and MAPK signaling

We examined the impact of the observed sGC modifications on caveolae-localized cGMP-PKG and MAPK signaling. That intracellular pools of cGMP are disrupted in cardiovascular disease has been demonstrated by several investigators, many of whom focus on the roles of PKG and PDE isoforms in the spatiotemporal regulation of cGMP signaling [38–41]. In this study, we provide evidence that relocalization of sGC in itself impacts downstream cGMP signaling. We demonstrated for the first time that PKG-mediated phosphorylation of VASP is detectable within caveolae of Control but not volume-overloaded canine hearts, despite similar global cGMP levels and similar distributions of PKG and VASP.

As mechanotransducers, caveolae may differentiate the mechanical load of volume-overload from that of pressure-overload. With volume-overload, increased diastolic stress causes a mechanical stretch parallel to the cardiomyocyte sarcomeric axis. In contrast, increased systolic stress with pressure-overload exerts mechanical stress perpendicular to the cardiomyocyte short axis. Positing that differential mechanical stress may induce differential MAPK signaling, Yamamoto et al. showed that p44/42 MAPK signaling is more pronounced in systolic (i.e., pressure-overload) rather than diastolic strain-induced protein

synthesis [42]. Such findings suggest that differential mechano-transduction may underlie differences between pressure- and volume-overload cardiac hypertrophy. Given that rapid and chronic activation of p38 [1,4] and ERK5 [29] is associated with eccentric hypertrophy, we sought to relate these MAPK signaling pathways to the volume-overload induced changes in caveolae-localized NO–cGMP signaling. cGMP has been shown to differentially regulate p38 activity in various models of cardiac stress [14,43–45]. Disparate findings of detrimental versus cardioprotective actions of p38 MAPK, either of which can be modulated by NO–cGMP, may reflect nuances in spatiotemporal regulation of cardiomyocyte signaling and the local integration of multiple stress signals. This may be particularly relevant to eccentric hypertrophy signaling given that p38 MAPK is activated by not only G-protein coupled receptors [46] but also reactive oxygen species [47,48].

By assessing the distribution of phosphorylated and total p38 and ERK5 across caveolae enriched lipid raft and non-lipid raft domains, we found an augmentation of p-p38 and p-ERK5, relative to total p38 and ERK5, within Cav3⁺LR in the volume-overloaded hearts. Moreover, the proportion of p-ERK5 within caveolae became markedly increased at 4wkMR, while caveolae localization of p-p38 remained relatively similar across all groups. These changes in spatial distribution of p-p38 and p-ERK5 suggest that caveolae-localized MAPK signaling may contribute pathophysiologically to the early stages of eccentric hypertrophy but not the transition to late stage, decompensated eccentric hypertrophy and heart failure. Such analysis however is only an indirect measure of caveolae localized MAPK signaling.

To define the mechanism by which caveolae-localized NO–cGMP signaling determines the pattern of cardiac hypertrophy, we would need to selectively manipulate myocardial caveolae-localized NO–cGMP signaling in face of both hemodynamic stress conditions (i.e., volume overload versus pressure overload) and systematically interrogate downstream signaling by either genetic or pharmacologic means. Hence this study represents an initial step at elucidating the mechanism and regulatory role of caveolae-localized NO–cGMP signaling. We are currently developing the tools to better study the functional significance of caveolae-localized NO–cGMP signaling.

Taken as a whole, our findings demonstrate differences in the regulation of myocardial NO–cGMP signaling that are unique to volume-overload cardiac stress and eccentric hypertrophy. The novelty of these results suggests therapeutic potential of caveolae-localized signaling in addressing eccentric remodeling. Cardiac therapeutic potential of caveolae-localized signaling was first proposed upon the discovery that caveolae and caveolin are essential to cardioprotection from myocardial ischemia/reperfusion injury [49]. Proof-of-concept was more recently provided by the selective blockade of hypertrophic signaling with gene transfer of a Cav-3 targeted calcium channel blocking protein into feline cardiac myocytes [50]. Further studies are underway to more fully explore the therapeutic potential of manipulating caveolae-localized NO–cGMP signaling.

5. Conclusions

Volume-overload cardiac stress induced relocalization and oxidation of myocardial sGC without compromising its responsiveness to DEA/NO, highlighting that dysregulation of NO–cGMP signaling differs in eccentric versus concentric hypertrophied hearts. Furthermore, early compensated and late decompensated stages of eccentric hypertrophy were differentiated by the expression, redox state, and subcellular localization of sGC subunits. Both PKG and MAPK signaling were altered in a microdomain-specific fashion, suggesting that myocardial caveolae signaling helps regulate the pattern of hypertrophic remodeling in response to the hemodynamic stress of volume-overload.

Acknowledgments

This work was supported in part by the American Heart Association Pre-Doctoral Research Fellowship to C. Makarewich; the NHLBI 5P50HL077100 to L. Dell'Italia; the Temple University School of Medicine Faculty Research Development Award, American Heart Association Scientist Development Grant, and NHLBI 1K08HL109159 to E.J. Tsai.

We thank Thomas Denney for providing technical support; Johanne-Peter Stasch for providing the BAY compound; and Steven R. Houser for careful reading of the manuscript.

Abbreviations

Cav3	caveolin-3
Cav3⁺LR	caveolin-3 enriched lipid raft, caveolae
cGMP	cyclic guanosine monophosphate
DEA/NO	diethylammonium (Z)-1-(N, N-diethylamino)diazene-1-ium-1,2-diolate, diethylamine NONOate
ERK5	extracellular signal regulated kinase 5
LR	lipid raft
LV	left ventricle
MAPK	mitogen activated protein kinase
MR	mitral regurgitation
NLR	non-lipid raft
NO	nitric oxide
NO₂-Tyr	nitrated tyrosine
PDE	phosphodiesterase
PKA	protein kinase A, cAMP-dependent protein kinase
PKC	protein kinase C
PKG	protein kinase G, cGMP-dependent protein kinase
RNS	reactive nitrogen species

sGC	soluble guanylyl cyclase
VASP	vasodilator-stimulated phosphoprotein

References

1. Miyamoto T, Takeishi Y, Takahashi H, Shishido T, Arimoto T, Tomoike H, et al. Activation of distinct signal transduction pathways in hypertrophied hearts by pressure and volume overload. *Basic Res Cardiol*. 2004; 99:328–37. [PubMed: 15309410]
2. Nakaoka Y, Nishida K, Fujio Y, Izumi M, Terai K, Oshima Y, et al. Activation of gp130 transduces hypertrophic signal through interaction of scaffolding/docking protein Gab1 with tyrosine phosphatase SHP2 in cardiomyocytes. *Circ Res*. 2003; 93:221–9. [PubMed: 12855672]
3. Nakaoka Y, Shioyama W, Kunimoto S, Arita Y, Higuchi K, Yamamoto K, et al. SHP2 mediates gp130-dependent cardiomyocyte hypertrophy via negative regulation of skeletal alpha-actin gene. *J Mol Cell Cardiol*. 2010; 49:157–64. [PubMed: 20226789]
4. Sopontammarak S, Aliharooob A, Ocampo C, Arcilla RA, Gupta MP, Gupta M. Mitogen-activated protein kinases (p38 and c-Jun NH2-terminal kinase) are differentially regulated during cardiac volume and pressure overload hypertrophy. *Cell Biochem Biophys*. 2005; 43:61–76. [PubMed: 16043884]
5. Lorell BH, Carabello BA. Left ventricular hypertrophy: pathogenesis, detection, and prognosis. *Circulation*. 2000; 102:470–9. [PubMed: 10908222]
6. Melenovsky V, Benes J, Skaroupkova P, Sedmera D, Strnad H, Kolar M, et al. Metabolic characterization of volume overload heart failure due to aorto-caval fistula in rats. *Mol Cell Biochem*. 2011; 354:83–96. [PubMed: 21465236]
7. Ulasova E, Gladden JD, Chen Y, Zheng J, Pat B, Bradley W, et al. Loss of interstitial collagen causes structural and functional alterations of cardiomyocyte subsarcolemmal mitochondria in acute volume overload. *J Mol Cell Cardiol*. 2011; 50:147–56. [PubMed: 21059354]
8. Hutchinson KR, Stewart JA Jr, Lucchesi PA. Extracellular matrix remodeling during the progression of volume overload-induced heart failure. *J Mol Cell Cardiol*. 2011; 48:564–9. [PubMed: 19524591]
9. Chen YW, Pat B, Gladden JD, Zheng J, Powell P, Wei CC, et al. Dynamic molecular and histopathological changes in the extracellular matrix and inflammation in the transition to heart failure in isolated volume overload. *Am J Physiol*. 2011; 300:H2251–60.
10. Ahmed MI, Gladden JD, Litovsky SH, Lloyd SG, Gupta H, Inusah S, et al. Increased oxidative stress and cardiomyocyte myofibrillar degeneration in patients with chronic isolated mitral regurgitation and ejection fraction >60%. *J Am Coll Cardiol*. 2010; 55:671–9. [PubMed: 20170794]
11. Marcil M, Ascah A, Matas J, Belanger S, Deschepper CF, Burelle Y. Compensated volume overload increases the vulnerability of heart mitochondria without affecting their functions in the absence of stress. *J Mol Cell Cardiol*. 2006; 41:998–1009. [PubMed: 17064727]
12. Ryan TD, Rothstein EC, Aban I, Tallaj JA, Husain A, Lucchesi PA, et al. Left ventricular eccentric remodeling and matrix loss are mediated by bradykinin and precede cardiomyocyte elongation in rats with volume overload. *J Am Coll Cardiol*. 2007; 49:811–21. [PubMed: 17306712]
13. Das A, Xi L, Kukreja RC. Protein kinase G dependent cardioprotective mechanism of phosphodiesterase-5 inhibition involves phosphorylation of ERK and GSK3beta. *J Biol Chem*. 2008; 283(43):29572–85. [PubMed: 18723505]
14. Fiedler B, Feil R, Hofmann F, Willenbockel C, Drexler H, Smolenski A, et al. cGMP-dependent protein kinase type I inhibits TAB1-p38 mitogen-activated protein kinase apoptosis signaling in cardiac myocytes. *J Biol Chem*. 2006; 281:32831–40. [PubMed: 16943189]
15. Takimoto E, Champion HC, Li M, Belardi D, Ren S, Rodriguez ER, et al. Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat Med*. 2005; 11:214–22. [PubMed: 15665834]

16. Gomes ER, Lara AA, Almeida PW, Guimaraes D, Resende RR, Campagnole-Santos MJ, et al. Angiotensin-(1-7) prevents cardiomyocyte pathological remodeling through a nitric oxide/guanosine 3',5'-cyclic monophosphate-dependent pathway. *Hypertension*. 2010; 55:153–60. [PubMed: 19996065]
17. Fiedler B, Lohmann SM, Smolenski A, Linnemuller S, Pieske B, Schroder F, et al. Inhibition of calcineurin-NFAT hypertrophy signaling by cGMP-dependent protein kinase type I in cardiac myocytes. *Proc Natl Acad Sci U S A*. 2002; 99:11363–8. [PubMed: 12177418]
18. Hsu S, Nagayama T, Koitabashi N, Zhang M, Zhou L, Bedja D, et al. Phosphodiesterase 5 inhibition blocks pressure overload-induced cardiac hypertrophy independent of the calcineurin pathway. *Cardiovasc Res*. 2009; 81(2):301–9. [PubMed: 19029137]
19. Tsai EJ, Liu Y, Koitabashi N, Bedja D, Danner T, Jasmin JF, et al. Pressure-overload-induced subcellular relocation/oxidation of soluble guanylyl cyclase in the heart modulates enzyme stimulation. *Circ Res*. 2012; 110:295–303. [PubMed: 22095726]
20. Gratton JP, Bernatchez P, Sessa WC. Caveolae and caveolins in the cardiovascular system. *Circ Res*. 2004; 94:1408–17. [PubMed: 15192036]
21. Patel HH, Murray F, Insel PA. Caveolae as organizers of pharmacologically relevant signal transduction molecules. *Annu Rev Pharmacol Toxicol*. 2008; 48:359–91. [PubMed: 17914930]
22. Borer JS, Bonow RO. Contemporary approach to aortic and mitral regurgitation. *Circulation*. 2003; 108:2432–8. [PubMed: 14623790]
23. Kleaveland JP, Kussmaul WG, Vinciguerra T, Ditters R, Carabello BA. Volume overload hypertrophy in a closed-chest model of mitral regurgitation. *Am J Physiol*. 1988; 254:H1034–41. [PubMed: 3381891]
24. Sabri A, Rafiq K, Seqqat R, Kolpakov MA, Dillon R, Dell'italia LJ. Sympathetic activation causes focal adhesion signaling alteration in early compensated volume overload attributable to isolated mitral regurgitation in the dog. *Circ Res*. 2008; 102:1127–36. [PubMed: 18356543]
25. Pat B, Killingsworth C, Denney T, Zheng J, Powell P, Tillson M, et al. Dissociation between cardiomyocyte function and remodeling with beta-adrenergic receptor blockade in isolated canine mitral regurgitation. *Am J Physiol Heart Circ Physiol*. 2008; 295:H2321–7. [PubMed: 18849331]
26. Knorr A, Hirth-Dietrich C, Alonso-Alija C, Harter M, Hahn M, Keim Y, et al. Nitric oxide-independent activation of soluble guanylate cyclase by BAY 60-2770 in experimental liver fibrosis. *Arzneimittelforschung*. 2008; 58:71–80. [PubMed: 18412020]
27. Mendes-Silverio CB, Leiria LO, Morganti RP, Anhe GF, Marcondes S, Monica FZ, et al. Activation of haem-oxidized soluble guanylyl cyclase with BAY 60-2770 in human platelets lead to overstimulation of the cyclic GMP signaling pathway. *PLoS One*. 2012; 7:e47223. [PubMed: 23144808]
28. Sharina IG, Jelen F, Bogatenkova EP, Thomas A, Martin E, Murad F. Alpha1 soluble guanylyl cyclase (sGC) splice forms as potential regulators of human sGC activity. *J Biol Chem*. 2008; 283:15104–13. [PubMed: 18381288]
29. Nicol RL, Frey N, Pearson G, Cobb M, Richardson J, Olson EN. Activated MEK5 induces serial assembly of sarcomeres and eccentric cardiac hypertrophy. *EMBO J*. 2001; 20:2757–67. [PubMed: 11387209]
30. Ishizaka N, Griendling KK, Lassegue B, Alexander RW. Angiotensin II type 1 receptor: relationship with caveolae and caveolin after initial agonist stimulation. *Hypertension*. 1998; 32:459–66. [PubMed: 9740611]
31. Yeh DC, Duncan JA, Yamashita S, Michel T. Depalmitoylation of endothelial nitric-oxide synthase by acyl-protein thioesterase 1 is potentiated by Ca(2+)-calmodulin. *J Biol Chem*. 1999; 274:33148–54. [PubMed: 10551886]
32. Chun M, Liyanage UK, Lisanti MP, Lodish HF. Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc Natl Acad Sci U S A*. 1994; 91:11728–32. [PubMed: 7972131]
33. Liu R, Wang D, Shi Q, Fu Q, Hizon S, Xiang YK. Palmitoylation regulates intracellular trafficking of beta2 adrenergic receptor/arrestin/phosphodiesterase 4D complexes in cardiomyocytes. *PLoS One*. 2012; 7:e42658. [PubMed: 22912718]

34. Feron O, Han X, Kelly RA. Muscarinic cholinergic signaling in cardiac myocytes: dynamic targeting of M2AChR to sarcolemmal caveolae and eNOS activation. *Life Sci.* 1999; 64:471–7. [PubMed: 10069512]
35. Feron O, Michel JB, Sase K, Michel T. Dynamic regulation of endothelial nitric oxide synthase: complementary roles of dual acylation and caveolin interactions. *Biochemistry.* 1998; 37:193–200. [PubMed: 9425039]
36. Mongillo M, Tocchetti CG, Terrin A, Lissandron V, Cheung YF, Dostmann WR, et al. Compartmentalized phosphodiesterase-2 activity blunts beta-adrenergic cardiac inotropy via an NO/cGMP-dependent pathway. *Circ Res.* 2006; 98:226–34. [PubMed: 16357307]
37. Wechsler J, Choi YH, Krall J, Ahmad F, Manganiello VC, Movsesian MA. Isoforms of cyclic nucleotide phosphodiesterase PDE3A in cardiac myocytes. *J Biol Chem.* 2002; 277:38072–8. [PubMed: 12154085]
38. Zhang M, Koitabashi N, Nagayama T, Rambaran R, Feng N, Takimoto E, et al. Expression, activity, and pro-hypertrophic effects of PDE5A in cardiac myocytes. *Cell Signal.* 2008; 20:2231–6. [PubMed: 18790048]
39. Zhang M, Takimoto E, Lee DI, Santos CX, Nakamura T, Hsu S, et al. Pathological cardiac hypertrophy alters intracellular targeting of phosphodiesterase type 5 from nitric oxide synthase-3 to natriuretic peptide signaling. *Circulation.* 2012; 126:942–51. [PubMed: 22829024]
40. Castro LR, Schittl J, Fischmeister R. Feedback control through cGMP-dependent protein kinase contributes to differential regulation and compartmentation of cGMP in rat cardiac myocytes. *Circ Res.* 2010; 107:1232–40. [PubMed: 20847310]
41. Castro LR, Verde I, Cooper DM, Fischmeister R. Cyclic guanosine monophosphate compartmentation in rat cardiac myocytes. *Circulation.* 2006; 113:2221–8. [PubMed: 16651469]
42. Yamamoto K, Dang QN, Maeda Y, Huang H, Kelly RA, Lee RT. Regulation of cardiomyocyte mechanotransduction by the cardiac cycle. *Circulation.* 2001; 103:1459–64. [PubMed: 11245653]
43. Kim SO, Xu Y, Katz S, Pelech S. Cyclic GMP-dependent and -independent regulation of MAP kinases by sodium nitroprusside in isolated cardiomyocytes. *Biochim Biophys Acta.* 2000; 1496:277–84. [PubMed: 10771096]
44. Braz JC, Bueno OF, Liang Q, Wilkins BJ, Dai YS, Parsons S, et al. Targeted inhibition of p38 MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling. *J Clin Invest.* 2003; 111:1475–86. [PubMed: 12750397]
45. Nishida K, Yamaguchi O, Hirotani S, Hikoso S, Higuchi Y, Watanabe T, et al. p38alpha mitogen-activated protein kinase plays a critical role in cardiomyocyte survival but not in cardiac hypertrophic growth in response to pressure overload. *Mol Cell Biol.* 2004; 24:10611–20. [PubMed: 15572667]
46. Marinissen MJ, Chiariello M, Pallante M, Gutkind JS. A network of mitogen-activated protein kinases links G protein-coupled receptors to the c-jun promoter: a role for c-Jun NH2-terminal kinase, p38s, and extracellular signal-regulated kinase 5. *Mol Cell Biol.* 1999; 19:4289–301. [PubMed: 10330170]
47. Clerk A, Fuller SJ, Michael A, Sugden PH. Stimulation of “stress-regulated” mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. *J Biol Chem.* 1998; 273:7228–34. [PubMed: 9516415]
48. Kulisz A, Chen N, Chandel NS, Shao Z, Schumacker PT. Mitochondrial ROS initiate phosphorylation of p38 MAP kinase during hypoxia in cardiomyocytes. *Am J Physiol Lung Cell Mol Physiol.* 2002; 282:L1324–9. [PubMed: 12003789]
49. Patel HH, Tsutsumi YM, Head BP, Niesman IR, Jennings M, Horikawa Y, et al. Mechanisms of cardiac protection from ischemia/reperfusion injury: a role for caveolae and caveolin-1. *FASEB J.* 2007; 21:1565–74. [PubMed: 17272740]
50. Makarewich CA, Correll RN, Gao H, Zhang H, Yang B, Berretta RM, et al. A caveolae-targeted L-type Ca(2)+ channel antagonist inhibits hypertrophic signaling without reducing cardiac contractility. *Circ Res.* 2012; 110:669–74. [PubMed: 22302787]

Appendix A. Supplementary Data

Cardiac Imaging

Two-dimensional and M-mode echocardiography (2.25-MHz transducer, ATL Ultramark VI) was performed in the conscious state at baseline and at 4 weeks after MR induction. Parasternal short axis view was used to obtain the following measurements: left ventricular end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and LV posterior wall thickness at end-diastole (LVPWt). Fractional shortening (%) was calculated as $[(LVEDD-LVESD)/LVEDD \times 100]$. Cardiac magnetic resonance imaging (cMRI) was performed in the anesthetized state at baseline and 12moMR at UAB on a 1.5 Tesla GE Signa Horizon (Milwaukee, WI) instrument optimized for cardiac application, as previously described [25]. The animals were allowed to recover and were sacrificed within 5 days of cMRI.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yjmcc.2013.03.019>.

Fig. A.1.

Western analysis of sGC α_1 and sGC β_1 across sucrose density gradient fractions of Control and MR hearts. sGC subunits shift away from caveolae enriched lipid rafts (F4–F6) towards heavy-density non-lipid raft fractions in chronically volume-overload hearts (4wkMR and 12moMR). One-way ANOVA $P < 0.05$ for sucrose density gradient fractions indicated. Student's *t*-test * $P < 0.10$, † $P < 0.05$, § $P < 0.01$ for 12moMR vs Control; ‡ $P < 0.10$, ** $P < 0.05$, # $P < 0.01$ for 4wkMR vs Control.

Fig. A.2.

Western analysis of total sGC α_1 -c (~40 kDa variant) expression and its distribution across sucrose density gradient fractions. (A) Representative western blot. (B) Summary densitometry analysis of sGC α_1 -c standardized to respective GAPDH signal and normalized to Control. One-way ANOVA $P = 0.66$. Control $n = 4$; 4wkMR $n = 5$; 12moMR $n = 5$. (C) Summary histogram of distribution across sucrose density gradients as determined by western analysis. Control $n = 7$; 4wkMR $n = 4$; 12moMR $n = 4$.

Fig. A.3.

Summary histogram of distribution of Ser239-pVASP and NO₂-Tyr across sucrose density gradients of Control and MR hearts. pVASP is detectable in F4 (within Cav3 + LR) of Control but not 4wkMR or 12moMR hearts. NO₂-Tyr histograms are shown for the total signal within each fraction as well as specific molecular weight NO₂-Tyr signals. Control $n = 2$; 4wkMR $n = 3$; 12moMR $n = 3$.

Fig. A.4.

Distribution of PKG and VASP across sucrose density gradients. (A) Representative western immunoblots and (B) summary densitometry histograms of PKG distribution. A greater proportion of PKG was localized within caveolae-enriched lipid raft fractions in early stage volume-overloaded hearts (4wkMR). (C) Representative western immunoblots and (D) summary densitometry histograms of VASP distribution. VASP distribution did not vary significantly amongst the heart groups. For PKG westerns, Control N = 5, 4wkMR N = 5, 12moMR N = 4. $P < 0.001$ on 2-way ANOVA. Multiple Student's *t*-test, * $P < 0.01$ 4wkMR vs Control; † $P < 0.05$ 4wkMR vs Control; ‡ $P < 0.001$ 4wkMR vs 12moMR; § $P < 0.01$ 4wkMR vs 12moMR; ** $P < 0.05$ 4wkMR vs 12moMR. For VASP westerns, Control N = 6, 4wkMR N = 5, 12moMR N = 5.

Fig. A.5.

Volume-overload cardiac stress selectively activates caveolae-localized p38 and ERK5 MAPK signaling. Western analysis of sucrose density gradient fractions of Control, 4wkMR, and 12moMR hearts was undertaken to assess the distribution of p-p38, p38, pERK5, and ERK5. Histograms show the summary densitometry data across all the sucrose density gradient fractions for Control (n = 4), 4wkMR (n = 3), and 12moMR (n = 5).

Fig. 1.

DEA/NO- and BAY 60-2770- induced cGMP production by sGC in control and volume-overloaded MR hearts. (A) cGMP levels were measured by ELISA in total LV protein extracts at baseline and following stimulation with NO-donor DEA/NO and NO-/heme-independent sGC activator BAY 60-2770. Measurements were made in the presence of non-selective PDE inhibitor IBMX. (B) Inducible cyclase activity represented as incremental change over baseline levels, expressed in percent of baseline. One-way ANOVA $P = 0.095$ for DEA/NO; $P = 0.0028$ for BAY 60-2770. A BAY 60-2770 response exceedingly greater than the DEA/NO response indicates oxidation of sGC. * $P < 0.0001$; † $P < 0.01$; ‡ $P < 0.05$; § $P < 0.10$ vs basal unless otherwise indicated on paired Student's *t*-test.

Fig. 2.

Overall expression of sGC heterodimer subunits in total LV tissue. (A) Summary bar graph shows densitometry analysis of sGC α ₁, sGC β ₁, Cav3 relative to Control levels for each signaling protein. Absolute densitometry values were standardized by the respective GAPDH signal before normalization to control levels. (B) Representative western blot is shown. Multiple measures were averaged for each heart. Total hearts analyzed: Control n = 6; 4wkMR n = 5; 12moMR n = 5. One-way ANOVA $P = 0.21$ for sGC α ₁, $P = 0.02$ for sGC β ₁. * $P < 0.05$; † $P = 0.10$ vs Control by unpaired Student's t -test.

Fig. 3.

Differential expression of cGMP PDE isoforms in eccentric hypertrophied hearts. (A) Bar graph summarizing densitometry analysis of PDE2A, PDE3A, and PDE5A, all standardized to respective GAPDH signals, and shown relative to Control levels. Replicate measurements were averaged for each heart. Total number of hearts analyzed: Control $n = 6$; 4wkMR $n = 5$; 12moMR $n = 5$. (B) Representative western blot is shown. One-way ANOVA $P < 0.01$ for PDE2 and PDE3; $P < 0.05$ for PDE5. Tukey's multiple comparison test $*P < 0.01$, $†P < 0.05$ vs Control unless otherwise indicated.

Fig. 4.

Global basal cGMP-PKG signaling remains unchanged by volume overload whereas reactive nitrogen species (RNS) signaling is increased at early stage eccentric hypertrophy. (A) Representative western blot of PKG-mediated phosphorylation of VASP and (B) tyrosine-nitration (NO₂-Tyr) in LV total protein extracts. (C) Summary densitometry analysis of results normalized to Control. For pVASP:VASP ratio, each signal was first standardized to a respective GAPDH signal. Total hearts analyzed: Control n = 6; 4wkMR n = 5; 12moMR n = 5. One-way ANOVA *P* values shown above respective bar graphs. **P* < 0.05 vs Control on Tukey's multiple comparison test.

Fig. 5.

sGC subunit distribution within the myocardium. Western analysis of sGC α_1 , sGC β_1 , Cav3 across sucrose density gradient fractions reveal a progressive shift away from caveolae-enriched lipid raft fractions (F4–F6) in volume overloaded hearts. Enrichment of Cav3 in F4–F6 represent caveolae. Cav3 distribution is unchanged. sGC subunits co-localize to myocardial caveolae of control dogs but less so in that of 4wkMR and 12moMR. Two-way ANOVA $P < 0.05$ for sGC α_1 , $P < 0.01$ for sGC β_1 , with the duration of MR identified as the source of variation for each subunit. Student's t -test * $P < 0.10$, † $P < 0.05$, ‡ $P < 0.01$ vs 12moMR; § $P < 0.05$ vs 4wkMR. Total hearts analyzed: Control $n = 7$; 4wkMR $n = 5$; 12moMR $n = 5$.

Fig. 6.

Volume-overload cardiac stress induces oxidation of sGC localized outside of caveolae microdomains. Sucrose density gradient fractions corresponding to caveolae (Cav3⁺LR, F4–F5) and non-lipid raft (NLR, F11) microdomains were stimulated with DEA/NO and BAY 60-2770 to determine inducible sGC cyclase activity as well as redox state of sGC. cGMP production is expressed as the incremental change in cGMP as a percentage of basal cGMP level. * $P < 0.10$; † $P < 0.05$; ‡ $P = 0.10$ vs Control unless otherwise indicated.

Fig. 7.

Western analysis of the distribution downstream NO signaling within Control and MR hearts. (A) Representative western blot of Ser239 phosphorylated-VASP across sucrose density gradient fractions F1–F11. (B) Representative western blot of NO₂-Tyr across sucrose density gradient fractions F1–F11. Summary densitometry analysis is included in Appendix A.

Fig. 8.

p38 and ERK5 MAPK activation appears to occur predominantly in the caveolae microdomain. (A) Western analysis of LV total protein extracts was performed to assess the relative expression of phosphorylated p38 (p-p38), phosphorylated ERK5 (p-ERK5), total p38, and total ERK5 in Control, 4wkMR, and 12moMR hearts. GAPDH was used as an internal control, to which the other proteins were respectively normalized. (B) Summary densitometry analysis shows phosphorylated-to-total MAPK ratios represented relative to Control. One-way ANOVA analysis $P = 0.51$ for p-p38:p38, $P = 0.01$ for p-ERK5:ERK5. Tukey's multiple comparison test $*P < 0.01$ vs Control; $\dagger P < 0.10$ vs 4wkMR. Total hearts analyzed: Control $n = 4$, 4wkMR $n = 5$, 12moMR $n = 5$. (C) Summary densitometry histogram of western immunoblots of p-p38, p38, p-ERK5, and ERK5 across sucrose density gradient fractions. Histograms are zoomed in to fractions F3–F6, with F4–F6 correlating with Cav3⁺LR, for Control $n = 4$, 4wkMR $n = 3$, and 12moMR $n = 5$. Complete histogram for fractions F1–F11 is provided in Appendix A. Repeated measures two-way ANOVA followed by Holm–Šídák multiple comparison testing ($\alpha = 0.01$) revealed significant differences in the phosphorylated versus total MAPK histograms across sucrose density gradient fractions F4 through F6 (Cav3⁺LR) for p38 and ERK5 for 4wkMR but only ERK5 for 12moMR.

Fig. 9.
Schematic of volume-overload induced relocalization, oxidation, and expression of sGC subunits.

Table 1

Cardiac remodeling assessed by imaging.

	Control	4wkMR	12moMR
LVEDD (cm)	3.7 ± 0.1	4.5 ± 0.2 *	4.9 ± 0.4 *
LVEDS (cm)	2.1 ± 0.1	2.5 ± 0.1 *	4.2 ± 0.4 *
LVWT/LVEDD	0.28 ± 0.03	0.19 ± 0.02 *	0.17 ± 0.01 *
FS (%)	43 ± 3	44 ± 3	14.6 ± 9.3 *

* $P < 0.05$ vs Control.