Journal of Molecular and Cellular Cardiology 60 (2013) 72–83

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

# 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], Emily J. Tsai [a,c,f,*]

[a] Cardiovascular Research Center, Temple University School of Medicine, Philadelphia, PA, USA
[b] College of Veterinary Medicine, Auburn University, Auburn, AL, USA
[c] Department of Physiology, Temple University School of Medicine, Philadelphia, PA, USA
[d] Division of Cardiovascular Disease, Department of Medicine, University of Alabama Birmingham, Birmingham, AL, USA
[e] Department of Anatomy and Cell Biology, Temple University School of Medicine, Philadelphia, PA, USA
[f] Section in Cardiology, Department of Medicine, Temple University School of Medicine, Philadelphia, PA, USA

ARTICLE INFO

*Article history:*
Received 16 November 2012
Received in revised form 18 March 2013
Accepted 28 March 2013
Available online 6 April 2013

*Keywords:*
Eccentric hypertrophy
Cyclic guanosine monophosphate signaling
Caveolae
Soluble guanylyl cyclase

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; $sGC\beta_1$ at 4wkMR, followed by $sGC\alpha_1$ 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 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.



## 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].

*Abbreviations:* Cav3, caveolin-3; $Cav3^+LR$, caveolin-3 enriched lipid raft, caveolae; cGMP, cyclic guanosine monophosphate; DEA/NO, diethylammonium (Z)-1-(N, N-diethylamino)diazen-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; $NO_2$-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.

* 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.
*E-mail address:* emily.tsai@tuhs.temple.edu (E.J. Tsai).

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http://dx.doi.org/10.1016/j.yjmcc.2013.03.019

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 mechanotransduction [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 $MgCl_2$ 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 μL 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: $sGC\alpha_1$ (1:1000, Abcam); $sGC\beta_1$ (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 ($NO_2$-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-$sGC\alpha_1$ and -$\beta_1$ 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