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Pressure-overload induced subcellular re-localization/oxidation of soluble guanylate cyclase in the heart modulates enzyme stimulation

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Abstract

Rationale—Soluble guanylyl cyclase (sGC) generates cyclic guanosine monophophate (cGMP) upon activation by nitric oxide (NO). Cardiac NO-sGC-cGMP signaling blunts cardiac stress responses, including pressure-overload induced hypertrophy. The latter itself depresses signaling via this pathway by reducing NO generation and enhancing cGMP hydrolysis.

Objective—We tested the hypothesis that the sGC response to NO also declines with pressure-overload stress, and assessed the role of heme-oxidation and altered intracellular compartmentation of sGC as potential mechanisms.

Methods and Results—C57BL/6 mice subjected to transverse aortic constriction developed cardiac hypertrophy and dysfunction. NO-stimulated sGC activity was markedly depressed, while NO- and heme-independent sGC activation by BAY 60-2770 was preserved. Total sGCα_1 and β_1 expression were unchanged by TAC, however sGCβ_1 subunits shifted out of caveolin-enriched microdomains. NO-stimulated sGC activity was 2–3-fold greater in Cav3-containing lipid raft

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versus non-lipid raft domains in control, and 6-fold greater after TAC. In contrast, BAY 60-2770 responses were >10 fold higher in non-Cav3 domains with and without TAC, declining about 60% after TAC within each compartment. Mice genetically lacking Cav3 had reduced NO and BAY-stimulated sGC activity in microdomains containing Cav3 for controls, but no change within non-Cav3-enriched domains.

Conclusions—Pressure-overload depresses NO/heme-dependent sGC activation in the heart, consistent with enhanced oxidation. The data reveal a novel additional mechanism for reduced NO-coupled sGC activity related to dynamic shifts in membrane microdomain localization, with Cav3-microdomains protecting sGC from heme-oxidation and facilitating NO-responsiveness. Translocation of sGC out of this domain favors sGC oxidation and contributes to depressed NO-stimulated sGC activity.

Keywords

hypertrophy; soluble guanylyl cyclase; caveolae; signaling; cardiomyocyte

Heart failure affects over 6 million patients in the United States alone, and its prevalence continues to rise despite recent diagnostic and therapeutic advances. A leading risk factor for the disease is chronic pressure-overload due to arterial hypertension, which afflicts nearly a third of the world's population. Such sustained stress often induces pathological hypertrophy of the chamber wall, and can lead to depressed function and electrical instability. Current treatment reduces the pressure load and suppresses neurohormones that contribute to maladaptive remodeling. However, clinical outcome remains poor, and as a consequence, novel approaches to leverage intrinsic negative modulators of hypertrophy are garnering increasing attention.

One such pathway involves the second messenger cyclic guanosine monophosphate (cGMP).[1–3] Cyclic GMP is generated by either the particulate guanylyl cyclase (pGC) linked to the natriuretic peptide receptor, or "soluble" GC (sGC), activated by nitric oxide (NO). Once generated, cGMP can modulate cardiomyocyte function by interacting with phosphodiesterases (PDEs) that regulate cAMP and its associated pathways, or by activating its primary target kinase, protein kinase G (PKG, also called cGK-1). Cyclic GMP-PKG activation plays a key role in modulating vascular tone and confers anti-fibrotic effects. Newer studies support a potent role in suppressing cardiomyocyte pathobiology, including blunting *in vivo* pressure-overload induced hypertrophy and protecting against ischemia-reperfusion injury and myocyte apoptosis.[4–11] However, in chronic pressure-overload, myocardial NO synthesis and secondary signaling via cGMP is itself depressed[9, 12–16] likely contributing to the pathophysiology. The latter appears related in part to an increase in cGMP hydrolysis by targeting phosphodiesterases such as PDE1 and PDE5.[9, 15, 16]

Another key component of this signaling pathway is sGC itself, yet no prior studies have examined its activity or potential modulators of activity in cardiac hypertrophy. While sGC-derived cGMP is mostly thought to accumulate in the cytosol, some NO-stimulated cGMP production is detectable at the plasma membrane.[17–19] In endothelial cells, endothelial NO synthase (NOS3) and PKG co-localize with caveolin, an integral membrane scaffolding protein that compartmentalizes and concentrates signaling molecules within specialized

regions of the plasma membrane. This has suggested that caveolae, small (50–100nm) flask-like lipid- and protein-rich invaginations of the plasma membrane, may serve as microdomains for optimized NO-sGC-cGMP signaling.[20] In this regard, sGC has been found at the plasma membrane in rat neurons, rat vascular endothelial cells, human and rat skeletal myocytes, rat cardiac myocytes, and human platelets.[19–23] The importance of this localization and whether it is impacted by heart disease to contribute to altered sGC function is unknown.

The present study tested the hypothesis that sustained pressure-overload depresses NO-stimulated sGC activation, and explored mechanisms for such change including loss of NO- and heme-dependent activity, and shifts in micro-domain localization. We reveal novel evidence for both, revealing a shift of sGC out of caveolae-enriched membrane microdomains after sustained pressure-overload, with enhanced sGC oxidation, and net depression of NO-stimulated cGMP generation.

Methods

Full details can be found in the Methods section of the Online Data Supplement at http://circres.ahajournals.org.

Animal Model

Male C57BL/6 mice (age 9–12 wk, Jackson Labs, Bar Harbor, Maine) were used. Pressure overload was produced by transverse aortic constriction (TAC) as previously described.[9] Left ventricular cardiac tissue of caveolin-3 null[24] (Cav3 KO background strain C57BL/6 mice) and littermate controls (WT) mice were provided by JF Jasmin (Thomas Jefferson University).[25] All animals received humane care according to NIH guidelines, and all animal protocols were approved by the respective IACUCs of Johns Hopkins University and Temple University.

Physiological Study

Cardiac function was assessed in conscious and isoflurane-anesthetized mice by transthoracic, 2D, M-mode echocardiography (Acuson Sequoia C256, Siemens at Johns Hopkins and Vevo660, Visual Sonics at Temple) with a 15 MHz linear-array transducer. M-mode LV end-systolic and end-diastolic dimensions were averaged from 3–5 beats. LV percent fractional shortening (FS) and mass were calculated as described previously.[9]

Immunoflourescent and Confocal Microscopy

Immunohistochemical analysis was performed on paraffin-embedded, 10% formaldehyde gravity-perfused LV tissue. LV tissue slices were imaged with a Nikon C-1 PLUS confocal microscope system.

Cell fractionation

Protein was prepared from snap-frozen heart tissue, and membrane-cytosol fractionation was performed as previously described.[9, 26]

Isolation of caveolin-enriched lipid raft fraction

Caveolin-enriched lipid raft fractions ($Cav3^+LR$) were prepared from freshly harvested and snap frozen LV tissue using a discontinuous 35%–5% sucrose density gradient ultracentrifugation method as previously described,[27] with some modifications.

Western blot analysis

Protein extracts from LV tissue homogenate and above mentioned subfractions were run on SDS-PAGE gels and transferred to nitrocellulose membranes. Immunoblot analysis was performed using primary antibody probes as detailed in Online Data Supplement.

sGC activity and cGMP measurement

Baseline and agonist-stimulated cGMP levels of total LV, $Cav3^+LR$ (F4, F5), and non-enriched fractions (NLR) from Sham, TAC, WT, and Cav3 KO hearts were measured by direct cGMP ELISA kit from New East Biosciences (Malvern, Pennsylvania, USA).

Statistical Analysis

All values are expressed as mean±SEM. Statistical analyses were performed using Student's t-test, one-way ANOVA followed by Tukey's test when comparing multiple groups, or two-way ANOVA when determining interaction of conditions. For data sets with unequal variances, a Welch's t-test was used. Statistical significance was defined as $P< 0.05$.

Results

Cardiac sGC activity declines in hypertrophied myocardium despite preserved overall myocardial $sGC\alpha_1$ and β_1 expression

C57BL/6J mice subjected to 3-weeks of transverse aortic constriction (TAC)[9] developed left ventricular hypertrophy, systolic dysfunction, and dilatation (Figure 1*A*), as previously reported.[9, 28] The capacity to stimulate myocardial sGC was assessed by the change in cGMP generation before and after exposure to NO, using the NO-donor diethylamine NONOate (DEA/NO, 1μmol/L) in the presence of the broad PDE inhibitor IBMX (3-isobutyl-1-methylxanthine, 0.75mmol/L). Mean baseline cGMP was ~40% lower after TAC (Table 1), but this disparity was markedly amplified upon DEA/NO stimulation (Figure 1*B*), with a 10 fold higher NO-stimulated response in Sham versus TAC. As a control, tissue extracts were pre-treated with the selective, irreversible, heme-site sGC inhibitor 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin1-one (ODQ at 10, 30, and 90μM) which blocked NO-stimulated cGMP by 80–90% (Online Figure I).

Since TAC induces myocardial oxidative stress[13] and oxidation of the sGC heme moiety suppresses its NO-stimulated activity[29], we tested whether this may have contributed to the response in TAC hearts. sGC was activated in a NO- and heme-independent manner by BAY 60-2770 (0.1μmol/L)[30] in the presence of IBMX (Figure 1*B*). BAY 60-2770 induced similar cGMP production as with DEA/NO in Sham controls, but also achieved this response in TAC heart isolate. This indicates the depressed DEA/NO response in TAC is unlikely related to reduced sGC expression per se, but rather to sGC heme-oxidation.

To confirm whether sGC expression was in fact unaltered by TAC, immunoblots were performed using specific column-purified antibodies to the α_1-subunit[31] or β_1-subunit. Functional sGC exists as a heterodimer with an α (73–82 kDa) and β subunit (70 kDa), the latter reportedly expressed diffusely within cells and necessary for NO-responsiveness.[32, 33] The $\alpha_1\beta_1$ isoform is the most ubiquitously expressed across tissue types and the only isoform in myocardium.[34] Negative controls were provided using heart tissue from mice genetically lacking either sGCα_1 [35] or sGCβ_1 [31] (Figure 1*C*). We found expression of sGC α_1- and β_1- subunits, relative to GAPDH, was unaltered by TAC (Figure 1*D*).

Myocardial sGC localizes in cytosolic and membrane compartments

Given unchanged protein expression, we next questioned whether TAC alters the sub-cellular distribution of either or both sGC subunits. LV extracts from Sham and TAC mice were sub-fractionated into cytosol and membrane compartments and analyzed by immunoblot (Figure 2*A*).[36] GAPDH and Cav3 served as markers for cytosol and membrane fractions, respectively. In both Sham and TAC hearts, the total cytosol-to-membrane ratio of sGCα_1 expression was similar, with approximately 66% in cytosol, 33% in the membrane (Fig. 2*B*). This is consistent with prior studies performed in rat myocardium[19] and frog cardiomyocytes[18]. In contrast, sGCβ_1 distribution was slightly altered by TAC. In Sham hearts, sGCβ_1 was nearly equally divided between cytosol and membrane fractions (47±3.4% in cytosol, 53±3.4% in membrane). In TAC hearts, sGCβ_1 distribution was predominantly cytosolic, with about 60% of total sGCβ_1 localized in the cytosol and 40% in the membrane fraction ($P = 0.018$ versus Sham).

Confocal immunohistochemistry of formaldehyde perfusion-fixed LV tissue revealed co-localization of sGCα_1 with Cav-3 in Sham hearts at both plasmalemma and T-tubular membranes (Figure 2*C*). In TAC hearts, sGCα_1 remained present at both membrane sites though co-localization with Cav3 appeared slightly disrupted. By contrast, sGCβ_1 was distributed diffusely throughout the myocardium in a longitudinal pattern, displaying some co-localization with Cav3. This did not appear altered by TAC.

Pressure overload stress alters membrane microdomain localization of sGCβ_1 subunit

To more directly examine whether sGC localization *within* plasma membrane compartments is altered by TAC, we determined the distribution of sGCα_1 and sGCβ_1 in microdomains of Sham and TAC hearts (Figure 3). Sham and TAC LV homogenates were subjected to detergent-free, discontinuous 35–5% sucrose density gradient centrifugation to separate caveolae-enriched lipid raft microdomains (F4 and F5, Cav3$^+$LR) from non-enriched domains (F11, NLR). Each sucrose density gradient fraction was run in equal volume on SDS-PAGE electrophoresis (Figure 3*B*). Protein concentrations determined by Bradford assay confirmed that total protein distribution was weighted towards heavier fractions (F6 and higher) lacking Cav3 in both control and TAC hearts (Figure 3*C*). In Sham controls, sGCα_1 and sGCβ_1 were detected in caveolae-enriched and non-enriched microdomains. In Sham controls, the distribution of sGCα_1, sGCβ_1, and Cav3 across fractions revealed sGCα_1 and sGCβ_1 to be relatively higher in Cav3$^+$LR after normalizing to total protein within the respective fraction. However, in TAC hearts, the proportion of sGCβ_1 present in Cav3$^+$LR declined (Figures 3*D*, *E*).

Pressure-overload cardiac stress differentially reduces NO-induced sGC activity in caveolae-enriched lipid raft and non-enriched microdomains

To test whether membrane microdomain localization of sGC influenced its functional response to NO, $Cav3^+$LR and NLR fractions were assayed for NO and BAY 60-2770 stimulated cGMP generation (Figure 4). As before, measurements were made in the presence of IBMX, so cGMP generation could be stably detected. Baseline cGMP in respective caveolae-enriched or non-enriched microdomains were similar between Sham and TAC (Table 1). However, cGMP levels were ~3-fold higher in caveolae-enriched microdomains (P= 0.01 for Sham, P = 0.04 for TAC). More strikingly, NO-stimulated cGMP production was markedly greater in caveolae-enriched versus non-enriched fractions, and this was observed in Sham and TAC similarly (note logarithmic scale). However, NO-induced sGC activity was also uniformly higher in Sham $Cav3^+$LR and NLR fractions compared to corresponding domains in TAC hearts (P<0.05 on paired Student's t-test for all NLR measurements versus respective $Cav3^+$LR). In contrast to NO stimulation, the sGC response to BAY 60-2770 was >10-fold greater in NLR than $Cav3^+$LR fractions, and this held similarly for Sham and TAC hearts. In NLR fractions, the BAY 60-2770 response exceeded that to DEA/NO, whereas in $Cav3^+$LR fractions, the two responses were similar. This pattern persisted after TAC. BAY 60-2770 activates sGC in a heme-independent manner, and this response is enhanced by heme oxidation,[30, 37] supporting greater sGC oxidation in NLR fractions, be they in Sham or TAC hearts. With TAC, the concordant reduction in both NO and BAY 60-2770 responsiveness of $Cav3^+$LR fraction is consistent with a shift of sGC away from caveolae-enriched into non-enriched microdomains. Two-way ANOVA of the cGMP responses to DEA/NO in the caveolae-enriched and non-enriched microdomains of Sham and TAC hearts revealed that both pressure-overload and microdomain localization impacted sGC NO-responsiveness (P<0.001 for both), with a borderline interaction effect (P=0.06). This disparity is further quantified in Table 2. An increase in the BAY 60-2770-to-DEA/NO relative responses occurs with heme-oxidation. Within $Cav3^+$LR fractions, this relative response ratio was unaltered by TAC; however, it dramatically rose in NLR fractions.

Loss of caveolin 3 blunts sGC NO responsiveness specifically in lipid raft fractions

To further test whether caveolae represent enhanced microdomains for NO-inducible sGC activity, we studied sGC responsiveness in hearts of caveolin-3 null (Cav3KO) mice. The model develops spontaneous mild hypertrophy by 4 months of age[38]. For whole myocardial analysis, we examined 6-month old hearts, which had a <10% higher LV/BW ratio, well below that induced by TAC (c.f. Fig 1*A*). Myocardial DEA/NO-induced sGC activity was nearly 90% lower in Cav3KO versus WT (Figure 5*A*), whereas the BAY 60-2770 response was identical between groups (Figure 5*A*). This appeared similar to what we observed with TAC, and supports greater sGC oxidation in the absence of a Cav3-microdomain pool. There was a trend towards reduced protein expression of $sGC\alpha_1$ in Cav3KO (p=0.052), while sGCβ1 was unchanged over controls (Figure 5*B*). However, as the BAY 60-2770 response was similar between groups, total sGC heterodimer was likely unaltered. We next examined sGC activity in membrane microdomains, using the same fractions F4/F5 versus F11 to define the two compartments. The DEA/NO response was much less in Cav3KO over

WT in the F4/F5 fraction (Figure 5*B*), whereas both responses were lower but equal in the F11 (NLR) fraction. Interestingly, the response to BAY 60-2770 was also reduced in Cav3KO F4/F5 fractions, while in the F11 NLR fraction, it was higher (as in controls and TAC) and similar to littermate controls. The marked decline in the BAY 60-2770 response in F4/F5 fractions in Cav3KO was not due to an absence of sGC in this microdomain, as Western blots of sucrose density gradient fractions (Figure 5*D*) revealed the presence of each subunit in both microdomains (F4/F5 and F11). This suggests that Cav3 provides an important facilitating function to NO/heme-dependent and independent sGC activation.

Discussion

This study investigated whether and how the activation of myocardial soluble guanylyl cyclase is impacted by pressure-overload induced cardiac hypertrophy. The results highlight several novel and important findings. First, sGC is profoundly modified in the hypertrophied heart, rendering it less NO-responsive yet equally activated by the NO- and heme-independent agonist BAY 60-2770 as compared to controls. This supports the presence of oxidation of sGC, which while previously documented in the pulmonary and systemic hypertension[29, 30], has not been previously reported in the myocardium. Secondly, we reveal the presence of a substantial proportion of myocardial sGC in the plasma membrane within microdomains that confer differential activation properties in the normal heart. Specifically, sGC within Cav3-enriched microdomains is more responsive to NO-activation, and relatively protected from oxidation. By contrast, sGC in non-lipid raft (non-Cav3 containing) domains is less NO responsive, and more oxidized. Caveolae-localization of sGC as a mechanism to augment NO responsiveness is consistent with prior work showing co-localization of sGC with NOS3 and PKG[19, 20] and NO-cGMP signaling within plasmalemmal caveolae. Third, we link the two behaviors, showing that this sub-membrane distribution of sGC is dynamic, being diminished in $Cav3^{+}$LR domains by pressure-overload, resulting in a shift from NO to BAY 60-2770 sGC-responsiveness. Thus, while heme-oxidation contributes to the overall decline of NO activation of sGC in the hypertrophied heart, this modulation occurs predominantly in non-caveolae enriched microdomains. Lastly, we show that the presence of Cav3 within lipid rafts is an important regulator of both NO/heme-dependent and -independent sGC activation. Viewed together, these observations shed new light on how abnormal subcellular distribution and oxidation of sGC can contribute to depressed cGMP-related signaling in cardiac disease.

Despite its name and differentiation from particulate GC coupled to natriuretic receptors, soluble GC is also detectable in membrane fractions. The $\alpha_2\beta_1$ sGC isoform is found in placenta, epithelia, and neurons, and has been shown in rat neuronal synaptic membrane[21]. The $\alpha_1\beta_1$ heterodimer is more ubiquitously expressed,[34] and is also detected in the plasmalemma of vascular endothelial cells,[19] platelets,[19] neuroblastoma cells,[39] and sarcolemma of skeletal, [22, 23] smooth,[40] and cardiac muscle tissue[18, 19]. The specific function of membrane-associated versus cytosolic $sGC\alpha_1\beta_1$ has been relatively little studied. Zabel et al[19] first identified a translocation of sGC to caveolin-enriched domains in endothelial cells when NO synthesis was stimulated by calcium, and this in turn enhanced cGMP-generation. Castro et al also observed an NO-sGC-cGMP signal in cardiomyocyte sub-plasmalemma by expressing an olfactory cyclic nucleotide gated membrane channel,

though this was considered the particulate (e.g. NP-coupled) GC[41], and not sGC. Other studies have revealed compartmentalized NO-stimulated sGC-cGMP pools that blunt beta-adrenergic stimulation modulated by PDE5a[42], PDE2[43], and β3-adrenergic signaling[43, 44], suggesting co-localization at the plasma membrane. Whether this functional compartment includes sGC has not been tested. In another study, bovine tracheal smooth muscle showed membrane-associated $sGC\alpha_1\beta_1$ produces the first of two sequential cGMP signals involved in muscarinic activation, involving sGC translocation from cytosol to the plasma membrane.[40] How sGC moves remains unclear, though it is found in a complex with NOS3 and heat shock protein 90 (HSP90)[45], and HSP90 can act as a migration chaperone. Clarification of this mechanism awaits further investigation.

The present study is the first to identify differential NO/heme-dependent and -independent activation properties of sGC localized within or external to $Cav3^+$LR microdomains. Strikingly, while sGC heme-oxidation appears prevalent in the NLR fraction with TAC, this appears not the case in $Cav3^+$LR. The cause for an apparent protection against oxidation of sGC in the $Cav3^+$LR versus greater oxidation in NLR domains remains unknown. Interestingly, superoxide dismutase 1 has been reported to localize to caveolae in vascular endothelial cells[46] and may confer targeted anti-oxidant effects. Another study found thioredoxin reductase 1 binds to caveolin-1[47], though in this instance the interaction was suggested to impede rather than enhance anti-oxidant activity. As NOS is also localized to this microdomain and can be the target of oxidation depressing its function[13], it seems plausible that a protected $Cav3^+$LR microdomain would facilitate NO-sGC interaction and cGMP generation, as we observed. The Cav3KO results further suggest that Cav3 itself plays an important role to this preservation, whether heme/NO dependent or not. The latter may relate to a structural organization of the proteins within caveolae, that become disrupted by elimination of Cav3. Cav3KO mice develop mild cardiac hypertrophy, LV dilatation, and reduced systolic function[38], whereas cardiac-specific overexpression of Cav3 attenuates pressure-overload hypertrophy[48], both supporting a cardioprotective role of Cav3. Whether other members of the caveolae sub-proteome also change with chronic cardiac stress (or perhaps aging) remains unknown, but raises an intriguing possibility in the context of abnormal signal transduction in various forms of cardiac hypertrophy and disease.

The exact stoichiometry of sGC subunits in the myocyte microdomains remains unknown, and this is non-trivial to quantify given the isolation procedures involved. Furthermore, we recognize that expression of each isoform does not determine their net balance in a heterodimer, and that this balance may itself alter activation. The shift of β_1 more than α_1 from the Cav3-microdomain suggests that some change in heterodimer composition may have occurred. In the rat brain, post-natal development has been associated with decreased sGC activity despite unaltered sGC subunit expression, and for the cerebrum, this has correlated with less $sGC\alpha_1\beta_1$ heterodimerization.[49] In the human heart, $sGC\alpha_1$ gene expression is nearly 3-fold higher than $sGC\beta_1$ ($sGC\alpha_2$ is undetectable).[34] Our findings support this, suggesting $sGC\beta_1$ may determine sGC NO-responsiveness via hetero-dimerization with a relative surplus of the α_1 subunit.

Pressure-overload has been previously shown to depress NOS activity via functional uncoupling[13] and to augment phosphodiesterase-mediated cGMP hydrolysis[50]. Based on the

current data, pressure-overload also results in sGC oxidation and disrupted localization away from Cav3^{+}LR microdomains –further depressing NO stimulated responsiveness. Each mechanism can play a role in reducing effective cGMP signaling and thus contribute to cardiac stress maladaptations. As we cannot yet directly manipulate sGC membrane translocation, its *in vivo* effects on cardiac stress responses admittedly remain speculative. The new results do not conflict with prior observed benefits from PDE5 or PDE1 inhibition[50, 51] or from reversing NOS-uncoupling by tetrahydrobiopterin (BH4)[52]. In the former case, the effect was due to enhancing cGMP once generated, which in turn increased PKG activity. By contrast, BH4 reduced NOS-derived ROS and enhanced NO synthesis, but this did not lead to increased PKG activation. It is possible that persistent sGC dysfunction contributed to this observation. Further studies will be required to test whether reverse remodeling of the hypertrophied heart can restore normal sGC NO-sensitivity and relocalize it to Cav3^{+}LR. The present results should help frame such research by highlighting a new sGC regulatory mechanism and providing insights into the potential utility of heme-independent sGC activators for the treatment of heart disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

β-AR	β–adrenergic receptor
Cav3	caveolin-3
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
cGK	cyclic GMP dependent protein kinase
DEA/NO	diethylamine NONOate
FS	fractional shortening
IBMX	3-isobutyl-1-methylxanthine
KO	knock out
LR	lipid raft
LV	left ventricle
NLR	non-lipid raft

NO	nitric oxide
NOS3	endothelial NO synthase
ODQ	1H-[1,2,4] oxadiazolo[4,3-a] quinox-alin1-one
PDE	phosphodiesterase
PKG	protein kinase G
pGC	particulate guanylyl cyclase
sGC	soluble guanylyl cyclase
TAC	transverse aortic constriction
WT	wild type

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Novelty and Significance

What is Known?

- Soluble guanylyl cyclase (sGC) increases its conversion of guanosine triphosphate to cyclic guanosine monophosphate (cGMP) upon nitric oxide (NO) activation.
- cGMP mediates a wide spectrum of cardiovascular processes including cardiac contractility and chronic cardiac hypertrophy.
- sGC activity is depressed in cardiovascular disease, though the mechanism of reduced cyclase activity are poorly understood.

What New Information Does This Article Contribute?

- Pressure-overload cardiac stress results in the oxidation of myocardial sGC, rendering it less responsive to NO in the hypertrophied heart.
- sGC within caveolae-enriched membrane microdomains of both normal and hypertrophied hearts are relatively protected from oxidation, demonstrating enhanced NO-responsiveness relative to sGC within non-caveolae-enriched microdomains.
- Submyocardial distribution of sGC is dynamic, with pressure-overload cardiac stress inducing a relocalization of membrane associated sGC away from caveolae.
- Caveolin 3 itself is important in order to observe NO-responsive sGC, and its deletion reduces sGC NO- and heme-independent activation in lipid-raft domains as well.

Reduced NO-cGMP signaling contributes to the pathophysiology of cardiovascular disease and has been attributed to depressed nitric oxide synthase activity via functional uncoupling and to diminished cGMP signaling via augmented phosphodiesterase-mediated cGMP hydrolysis. In pulmonary and systemic hypertension, oxidation of sGC depresses cyclase activity in the vasculature, further diminishing cGMP signaling. Here we provide evidence that sGC activity is markedly reduced in pressure-overload hypertrophied myocardium not due to reduced expression but rather to oxidation that suppresses both NO and heme-dependent activity. Furthermore, both cyclase oxidation and its NO-activation are differentially impacted depending on the subcellular membrane localization of sGC. sGC is found in both cytosol and membrane at near equal levels. When residing in caveolae-enriched membrane lipid rafts, sGC is more responsive to NO-activation and relatively protected from oxidation as compared with sGC in non-lipid-raft domains. Pressure-overload triggers a re-localization of sGC out of caveolae-enriched lipid rafts to non-lipid rafts, and this reduces NO responsiveness while increasing oxidation of the enzyme. Furthermore, caveolin-3, the major coat protein of caveolae, regulates both NO/heme-dependent and –independent sGC activation in lipid rafts. These findings link dynamic subcellular localization with differential enzyme oxidation and activity, revealing a novel regulatory mechanism of sGC activity. Our

work also suggests the caveolae microdomain as a potential therapeutic target for cardiac hypertrophy and heart failure.

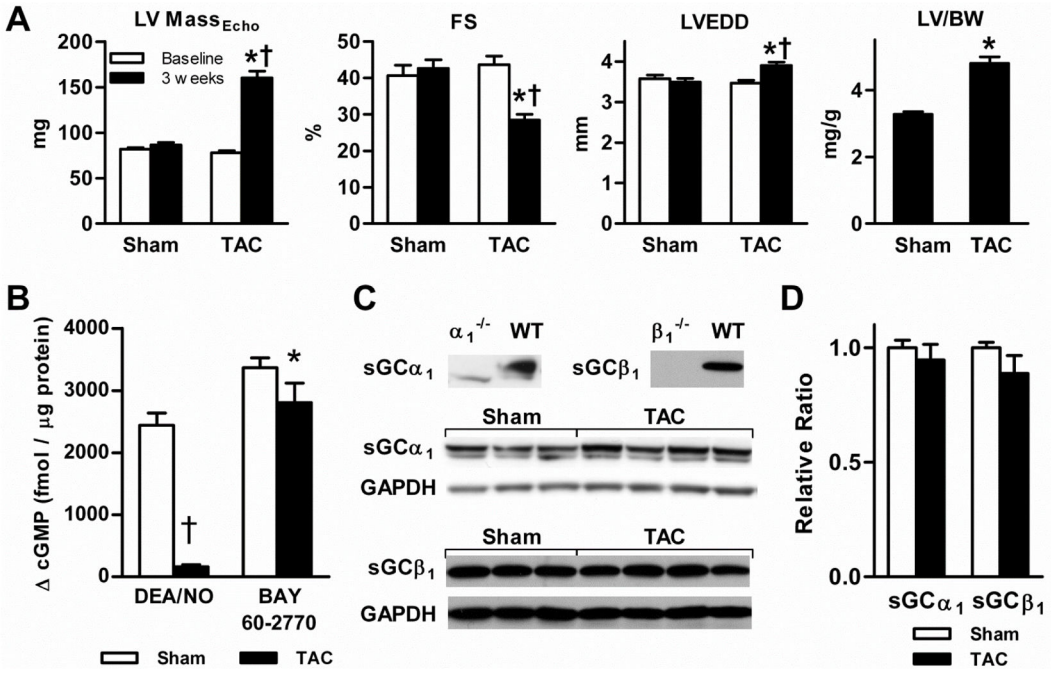

Figure 1.

Hypertrophied hearts have reduced sGC activity despite unchanged expression levels of sGCα_1 and β_1 subunits. **A,** LV mass, fractional shortening, and LV end-diastolic diameter of Sham (N=31) and TAC (N=48) mice at baseline and 3 weeks post-surgery as measured by echocardiography. LV mass to body weight ratio. * P <0.0002 versus baseline; † P <0.002 versus respective Sham. **B,** sGC activity of total protein from Sham and TAC hearts in response to NO-donor DEA/NO 1mmol/L (N=15 for Sham, 14 for TAC) and NO- and heme-independent sGC activator BAY 60-2770 0.1mmol/L (N=7 for Sham, 6 for TAC). * P <0.01 versus respective DEA/NO; † P <0.00001 versus respective Sham. **C** and **D,** Western immunoblots and normalized densitometry analysis of total sGCα_1 and sGCβ_1 expression in Sham (N=9 for α_1, N=6 for β_1) and TAC (N=10 for α_1, N=6 for β_1). Data are mean±SEM.

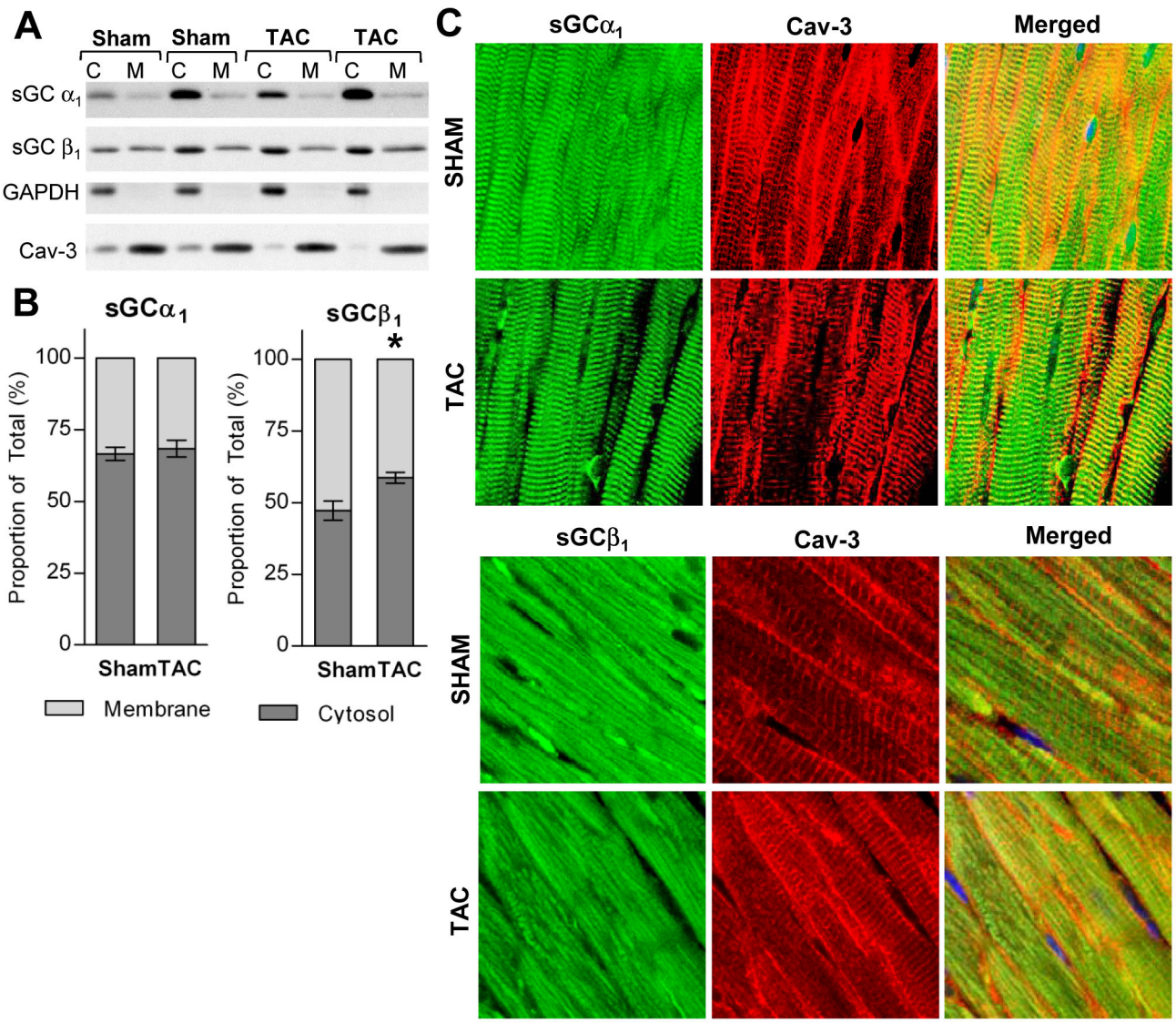

Figure 2.

Submyocardial distribution of sGC subunits is differentially altered by pressure-overload stress. **A,** Western blots of cytosol (C) and membrane (M) fractions. **B,** Summary analysis of C-M distribution of sGCα_1 and β_1 in TAC versus Sham hearts (N=9 each for α_1, N=5 each for β_1). * P <0.02 versus Sham. **C,** Confocal imaging of LV tissue immunostained for sGCα_1 or sGCβ_1 (green) and Cav3 (red). Images at 100x.

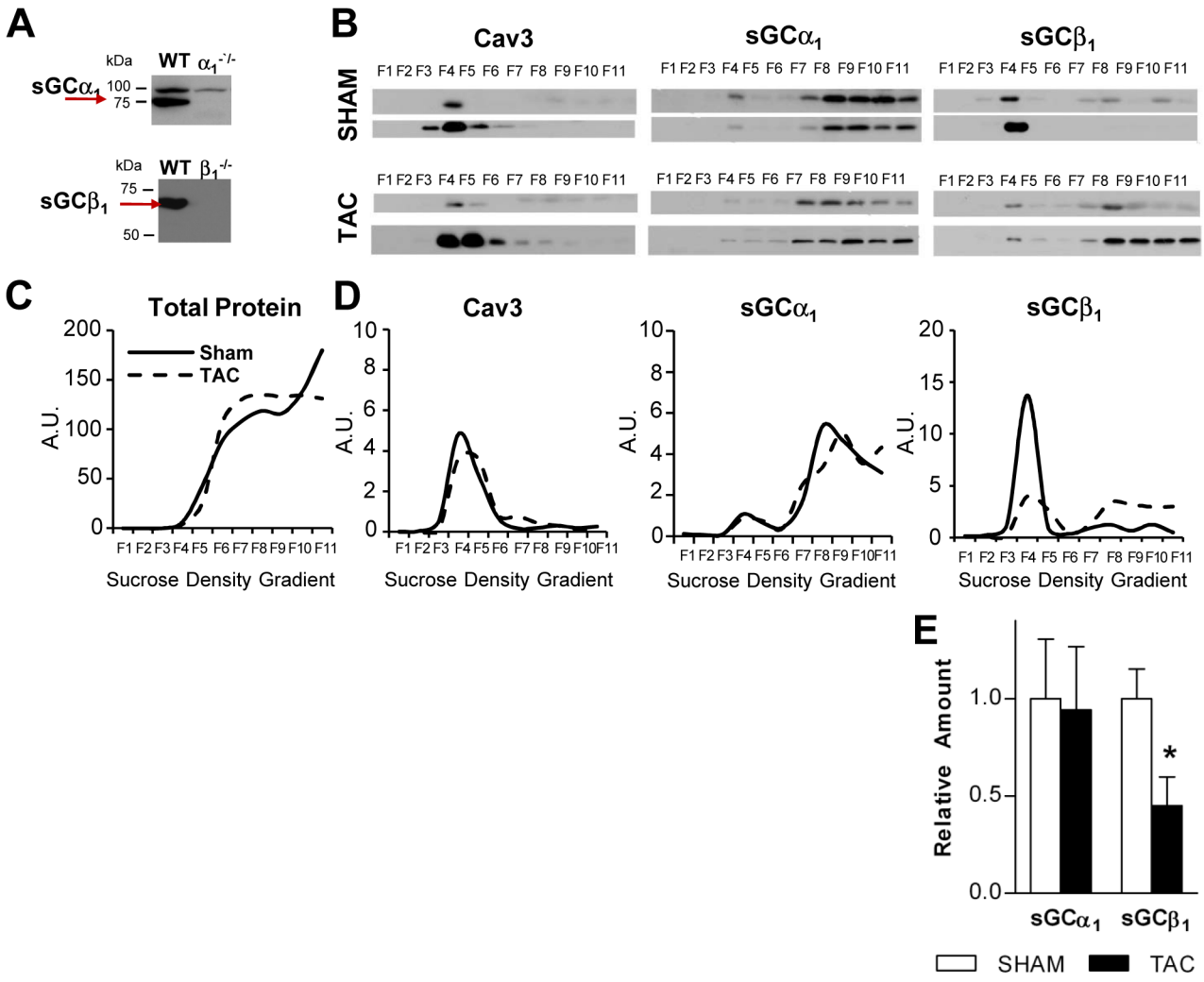

Figure 3.

Caveolae-localization of sGCb$_1$ subunit is disrupted in hypertrophied hearts. Caveolae-enriched lipid raft microdomains (F4 and F5, Cav3$^+$LR) and non-lipid raft (F11, NLR) fractions were separated using detergent-free discontinuous 35–5% sucrose density gradient centrifugation. **A,** Western blot of sGC$\alpha_1{}^{-/-}$ and sGC$\beta_1{}^{-/-}$ hearts as negative controls. **B**, Representative Western blots of all sucrose density gradient fractions of Sham (N=4) and TAC (N=6) using anti- sGCα_1, sGCβ_1, and Cav3 antibodies. **C,** Relative distribution of total protein across all 11 fractions of Sham and TAC hearts, as determined by Bradford assay. **D,** Relative distribution of Cav3, sGCα_1, and sGCβ_1 confirm a localization of sGCα_1, and sGCβ_1 in Cav3$^+$LR (F4,F5). Graphs are based upon densitometry analysis of Westerns.. **E,** Relative amount of sGCα_1 and β_1 in Cav3$^+$LR in TAC versus Sham. * *P* =0.037 versus Sham.

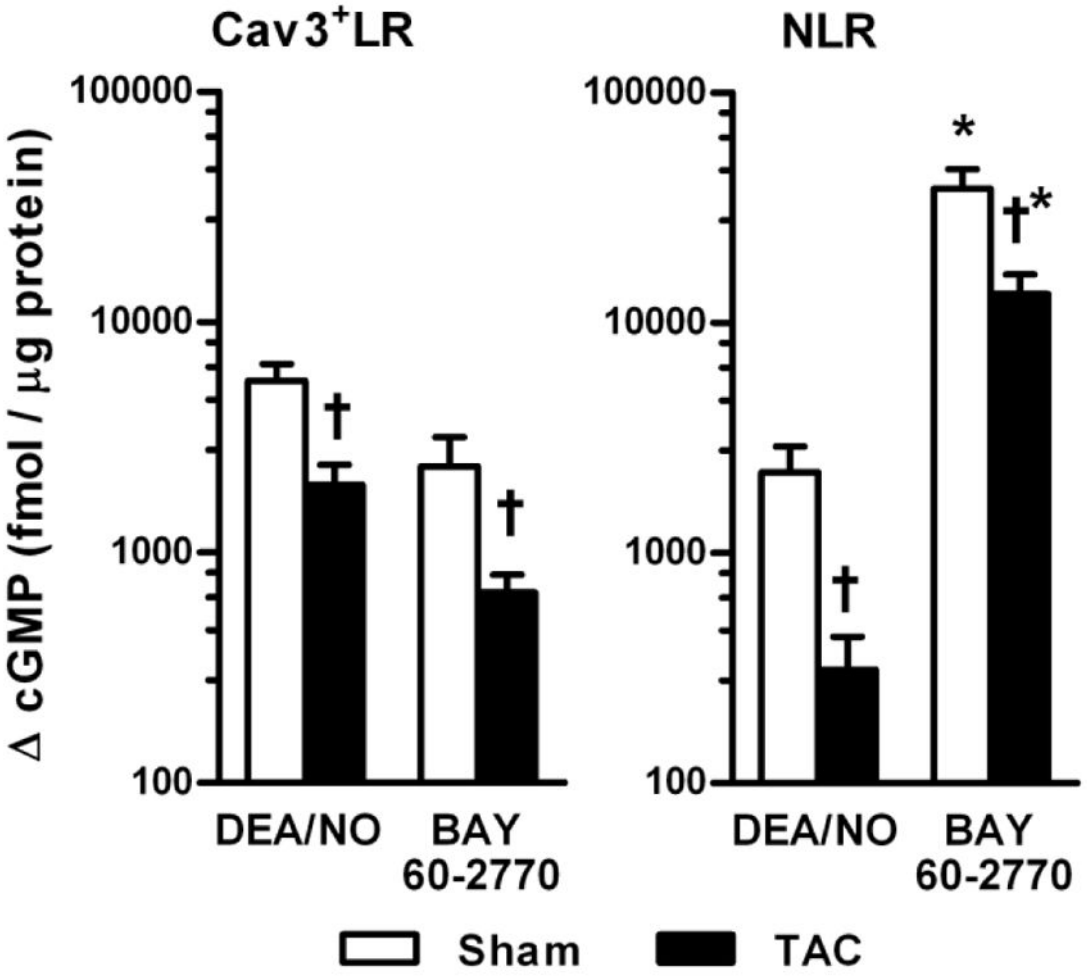

Figure 4.

Activation of sGC by NO-donor DEA/NO and NO- and heme-independent sGC activator BAY 60-2770 in Cav3^{+}LR and NLR fractions of Sham and TAC hearts. DEA/NO N=7 for Sham, 6 for TAC. BAY 60-2770 N=4 per group. * P<0.0003 versus respective DEA/NO. † P<0.05 versus respective Sham in Cav3^{+}LR, P<0.03 versus respective Sham in NLR.

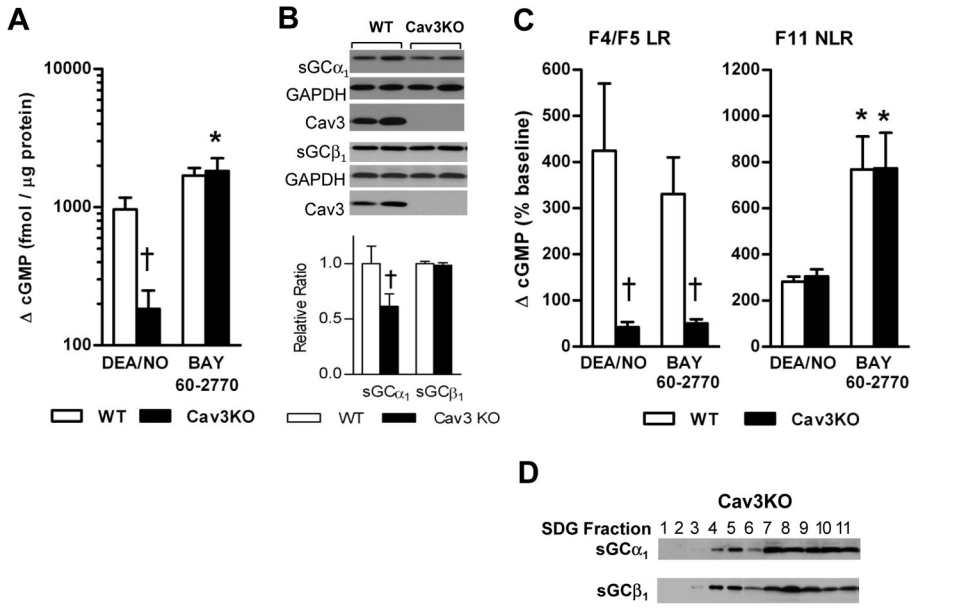

Figure 5.

NO-inducible sGC activity is blunted in Cav3KO hearts. **A,** sGC activation by DEA/NO and BAY 60-2770 in total LV protein of WT and Cav3KO (DEA/NO N=10, BAY 60-2770 N=4, per genotype). * $P<0.0001$ versus DEA/NO, † $P<0.003$ versus WT. **B,** Westerns of total LV protein, lower panel with summary data, n=6 per bar. † $P=0.052$ versus WT. **C,** sGC activation in F4/F5 lipid raft (LR) versus F11 (NLR) fractions (n=4 per drug, per genotype). * $P<0.05$ versus DEA/NO, † $P<0.04$ versus WT. **D,** Representative western blot of Cav3KO sucrose density gradient (SDG) fractions.

Table 1

Baseline cGMP levels (fmol/μg, mean ±SEM)

	Sham	TAC	*P*
Total	170.1±24.8	102.8±8.1	0.02
Cav3$^+$LR	1220.2±209.6	754.4±86.8	0.08
NLR	199.4±34.1*	297.8±88.7*	0.3

*P<0.05 on paired Student's t-test versus respective Cav3$^+$LR

Table 2

Differential sGC responsiveness within microdomains of normal and hypertrophied hearts

	Cav3$^+$LR		NLR	
Relative Response	**Sham**	**TAC**	**Sham**	**TAC**
BAY 60-2770: DEA/NO	1.0	0.6	3.5	17.4

Relative response ratios were calculated using the % change in cGMP levels within respective microdomains of Sham and TAC hearts. Values are normalized to the response ratio of Sham Cav3$^+$LR fraction. Increasing BAY 60-2770:DEA/NO ratios reflect enhanced response to BAY 60-2770 and diminished response to DEA/NO, resulting from heme-oxidization of sGC.