Heart Failure

Pathological Cardiac Hypertrophy Alters Intracellular Targeting of Phosphodiesterase Type 5 From Nitric Oxide Synthase-3 to Natriuretic Peptide Signaling

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Background—In the normal heart, phosphodiesterase type 5 (PDE5) hydrolyzes cGMP coupled to nitric oxide–(specifically from nitric oxide synthase 3) but not natriuretic peptide (NP)–stimulated guanylyl cyclase. PDE5 is upregulated in hypertrophied and failing hearts and is thought to contribute to their pathophysiology. Because nitric oxide signaling declines whereas NP-derived cGMP rises in such diseases, we hypothesized that PDE5 substrate selectivity is retargeted to blunt NP-derived signaling.

Methods and Results—Mice with cardiac myocyte inducible PDE5 overexpression (P5⁺) were crossed to those lacking nitric oxide synthase 3 (N3⁻), and each model, the double cross, and controls were subjected to transaortic constriction. P5⁺ mice developed worse dysfunction and hypertrophy and enhanced NP stimulation, whereas N3⁻ mice were protected. However, P5⁺/N3⁻ mice behaved similarly to P5⁺ mice despite the lack of nitric oxide synthase 3–coupled cGMP generation, with protein kinase G activity suppressed in both models. PDE5 inhibition did not alter atrial natriuretic peptide–stimulated cGMP in the resting heart but augmented it in the transaortic constriction heart. This functional retargeting was associated with PDE5 translocation from sarcomeres to a dispersed distribution. P5⁺ hearts exhibited higher oxidative stress, whereas P5⁺/N3⁻ hearts had low levels (likely owing to the absence of nitric oxide synthase 3 uncoupling). This highlights the importance of myocyte protein kinase G activity as a protection for pathological remodeling.

Conclusions—These data provide the first evidence for functional retargeting of PDE5 from one compartment to another, revealing a role for natriuretic peptide–derived cGMP hydrolysis by this esterase in diseased heart myocardium. Retargeting likely affects the pathophysiological consequence and the therapeutic impact of PDE5 modulation in heart disease. (Circulation. 2012;126:942-951.)

Key Words: nucleotides, cyclic ■ hypertrophy ■ oxidative stress ■ phosphodiesterases ■ natriuretic peptides ■ nitric oxide synthase ■ protein kinase G

Phosphodiesterase type 5 (PDE5) is one of an 11-member superfamily of enzymes responsible for the hydrolysis of cyclic nucleotides to their 5’ monophosphate form. The first to be identified as relatively selective for cGMP, PDE5 is expressed in vascular smooth muscle, particularly in the corpus cavernosum and pulmonary circulation. PDE5 inhibition stimulates protein kinase G (PKG)–dependent vasodilation, forming the basis for its clinical use for treating erectile dysfunction and pulmonary hypertension. Recent studies have revealed that PDE5 is also expressed in cardiac muscle, and growing evidence supports its contribution to ventricular disease in experimental models and in humans. Although expression is normally quite low, it rises considerably in experimental and human cardiac hypertrophy and failure, a change that has been linked to oxidative stress. Higher levels of PDE5 expression can participate in cardiac pathophysiology and in worsening remodeling and dysfunction after myocardial infarction or after sustained pressure overload. Such findings have led to clinical trials testing the utility of PDE5 inhibition in dilated cardiomyopathy, heart failure with a preserved ejection fraction, and Duchenne and Becker muscular dystrophy. Initial results support the efficacy of sildenafil in human dilated cardiomyopathy for improving breathlessness and exercise capacity and cardiac function.
p916
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In the ventricular myocyte, cGMP is generated by 2 different pathways: by stimulating nitric oxide (NO) synthase (NOS) to generate NO and to activate soluble guanylate cyclase (sGC) or by activation of natriuretic peptide receptor coupled GC. These pathways are not redundant but display different constellations of distal targets and regulatory PDEs. In particular, myocyte PDE5 normally targets NOS3-sGC–derived cGMP, whereas other PDEs (eg, PDE1, PDE2) may target particulate GC–related pools. PDE5 inhibition in the normal heart (including human) and myocytes suppresses acute β-adrenergic–stimulated contractility; however, this is not observed when NOS3 is genetically deleted or NOS is inhibited pharmacologically. Moreover, PDE5 inhibition enhances PKG activity upon NO but not atrial natriuretic peptide (ANP) stimulation in the normal heart, has no impact on membrane-localized cGMP induced by ANP in rat myocytes, and mimics NO- but not ANP-modulated compartmentation of protein kinase A isofrom activity.

In many forms of heart disease, NO-derived cGMP declines in part as a result of NOS uncoupling and sGC oxidation, whereas NP-derived cGMP is markedly enhanced. If increased PDE5 activity with heart failure remained solely targeted to NO-sGC–derived pools, its impact would likely be diminished given less available substrate. However, if the esterase could be retargeted to NP-cGMP pools, its pathophysiological role and the therapeutic impact from its inhibition would be enhanced. The present study tested this hypothesis by studying mice with a tetracycline-controllable myocyte-specific PDE5 overexpression (P5+) and crossing them into a background lacking NOS3 (N3−). We previously reported that P5+ mice exhibit worsened hypertrophy, fibrosis, and left ventricular (LV) dysfunction when subjected to transaortic constriction (TAC) and that this reverses once myocyte PDE5 expression is reduced (by adding doxycycline to the drinking water to suppress transgene expression). N3− mice, in contrast, develop concentric adaptive hypertrophy with severe TAC, coupled to a lack of oxidative stress that is otherwise generated by NOS uncoupling, a process whereby NOS produces superoxide rather than NO. If PDE5 remained targeted only to NO-sGC–derived cGMP, one would predict a heart lacking NOS3 would be unaffected by PDE5 upregulation. However, if alternative targeting to NP–particulate GC–derived cGMP occurred, the pathophysiology would be worse. Our results support the latter, providing the first evidence for functional PDE retargeting to a completely different cyclic nucleotide pool with in vivo consequences. We further reveal that suppressing myocyte PKG activity exacerbates pressure-overload remodeling at the organ and molecular levels despite low levels of myocardial oxidative stress.

Methods

Mouse Models and Pressure-Overload Stress

Bigenic P5+ mice have been described previously; they overexpress PDE5 in myocytes (α-myosin heavy chain promoter) but only in the absence of doxycycline (tet-off system). We used the line with 7× PDE5 protein overexpression. P5+ mice were further crossed into a N3− background (C57BL/6; Jackson Laboratories) to generate trigenic mice. Four groups were studied (mean age, 2–3 months when heart function and molecular signaling are similar to that of controls): controls (littermates expressing NOS3 and lacking PDE5 transgene), P5+, N3−, and the cross (P5+/N3−). Mice were subjected to 7 to 8 days of TAC, a duration previously shown to induce marked chamber dilation/dysfunction and early mortality in P5+ mice.

Cardiac Function

Intact cardiac function was assessed in conscious animals by echocardiography to derive indexes of LV dimension, fractional shortening, and wall thickness as described.

Confocal Immunohistochemistry

The myocyte distribution of PDE5 followed previously reported protocols and equipment using a primary antibody to both PDE5 (Cell Signaling) and Flag (encoded in the transgene; Sigma Aldrich). Myocyte size was detected by wheat germ agglutinin staining using a semiautomated method to assess mean cross-sectional area.

Molecular Analysis

Myocardial expression of fetal genes (nppa, nppb), genes related to nuclear factor of activated T-cell signaling (regulator of calcineurin [Rcan-1], transient receptor potential canonical channel 6 [Trpc6]), and fibrosis/remodeling (transforming growth factor β-1 [Tgfβ1], connective tissue growth factor [ctgf]) were determined by real-time quantitative polymerase chain reaction (Primers are given in the online-only Data Supplement). cGMP was assessed by enzyme immunoassay and PKG activity by fluorescent polarization assay as described. PDE5 protein expression in subcellular and cytosolic compartments was determined in myocardial tissue subjected to gradient centrifugation.

Oxidative Stress

Myocardial superoxide formation was determined by high-performance liquid chromatography assay of 2-hydroxyethidium 2-OH-E+ formation from frozen tissue extracts incubated with dihydroethidium (DHE) as described. Tissue level of reduced glutathione (GSSG) and oxidized glutathione (GSSG) was measured with a Bioxytech GSH/GSSG-412 kit (Oxis International Inc). For measurement of GSSG, the thiol-scavenging reagent 1-methyl-2-vinylpyridinium trifluoromethanesulfonate was added to the homogenization buffer to minimize oxidation of GSH to GSSG. Gene expression of the NADPH oxidases NOX2 and NOX4 was determined by quantitative polymerase chain reaction.

Statistics

Results are presented as mean±SEM. For analysis among the 4 experimental groups, we used a 1-way ANOVA with a Tukey-Kramer multiple-comparisons test. In experiments in which the data distribution failed normality tests, a Kruskal-Wallis nonparametric test and a Dunn multiple-comparisons test were performed. All P values reported for within-group differences are adjusted for multiple comparisons. We also used 2-way ANOVA to test for TAC-group or drug-drug interactions, often using a subsequent within-group 1-way ANOVA and multiple-comparisons test as appropriate. The specific tests and significant differences between groups and the relevant sample sizes are provided in the figure legends. Analysis was performed with Systat version 10.2.

Results

Cardiac Function and Remodeling Are Worse After TAC in P5+ Mice With or Without NOS3

Baseline cardiac morphology, function, and LV and lung mass were similar in all 4 groups (Figure I in the online-only
Data Supplement). Resting PKG activity was lower in the 2 groups carrying the P5\(^+\) transgene in myocytes compared with controls or N3\(^-\) (Figure II in the online-only Data Supplement). Normal basal PKG activation in hearts lacking NOS3 (N3\(^-\)) is consistent with prior studies showing enhanced NP signaling in this model.\(^{26}\)

In contrast to resting function, the cardiac response to TAC differed markedly among the 4 groups. Figure 1A displays representative echocardiograms, cross-sectional histology, and wheat germ agglutinin–stained myocardium from hearts after TAC. LV dilation and function were worse and myocyte hypertrophy was increased in P5\(^+\). N3\(^-\) hearts were protected, displaying no dilation but rather concentric hypertrophy, with less net increase in LV mass and myocyte enlargement. However, in P5\(^+\)/N3\(^-\) mice, the protection afforded by an absence of NOS3 was lost, and LVs again developed marked dilation, dysfunction, and myocyte and organ hypertrophy. Summary data are provided in Figure 1B.

The differences in chamber remodeling were accompanied by corresponding disparities in molecular signaling that is targeted by PKG activation.\(^{7}\) Gene expression markers of calcineurin upregulation (Rcan1, Trpc6) and growth/fibrosis signaling (tgb1, ctgf) were significantly enhanced after TAC, particularly in models with PDE5 overexpression (Figure 2).
Importantly, this occurred whether NOS3 was expressed in the heart or not.

PDE5 Is Functionally Retargeted to NP-Derived cGMP in TAC-Stressed Hearts

The finding that PDE5 upregulation worsened organ and molecular responses to TAC despite a lack of NOS3 suggested that an alternative pool of cGMP such as from NP signaling was being targeted. Myocardial ANP and brain NP gene expression rose in all models, with ANP increasing even more in P5+/N3− (Figure III in the online-only Data Supplement), supporting retargeting. To test this more directly, sham and TAC mice were administered ANP (10 μg), sildenafil (12.5 μg), or both19 (Figure 3A). Corresponding PKG activity is shown in Figure 3B. Although TAC increased activity overall (P<0.05, 2-way ANOVA), there remained striking differences between groups, with activation remaining reduced in both models with enhanced PDE5 activity. This suggested that NP-generated cGMP was being hydrolyzed. To test this more directly, sham and TAC mice were administered ANP (10 μg), sildenafil (12.5 μg), or both19 (Figure 3C). In sham controls, myocardial cGMP rose similarly with ANP alone or when combined with sildenafil. However, after TAC, cGMP rose disproportionately more with the combination than with ANP itself (P<0.02 for SII/ANP interaction).

A potential concern with any transgenic model is that the overexpressed protein may exhibit promiscuous targeting. To test this, we administered ANP to both wild-type and P5+ hearts at baseline and after TAC (Figure 3D). ANP augmented cGMP similarly in both models at baseline, indicating that PDE5 overexpression alone did not result in off-target cGMP hydrolysis. After TAC, however, the ANP-stimulated rise in cGMP was blunted more in P5+ hearts (P=0.01 for interaction of ANP and genetic model), supporting retargeting.

We also examined gene expression levels for the other NOS isoforms (Figure III in the online-only Data Supplement). Expression of both NOS1 (neuronal) and NOS2 (inducible) isoforms was similar at baseline in all 4 models. After TAC, NOS2 rose slightly more in mice expressing NOS3 than in those that did not (P<0.05, 3-way ANOVA). In contrast, NOS1 remained similar among the groups, although expression was somewhat lower overall after TAC (P<0.002, 3-way ANOVA). Thus, although some changes were observed, they did not correlate with cardiac remodeling or cGMP or PKG activity.

Functional Retargeting of PDE5 Is Accompanied by a Loss of Normal Myocyte Localization

Cardiomyocyte PDE5 is normally distributed throughout the cell, with enhanced striated pattern reflecting sarcomere localization.220 This becomes diffuse if NOS3 activity is inhibited,220 behavior also observed in canine dilated cardiomyopathy.27 We speculated that a similar change might accompany hypertrophy/dilation coupled to functional enzyme re-targeting. Figure 4A shows confocal images of myocytes from each model at baseline. A diffuse staining pattern was present in control and P5/N3 hearts had both diffuse and enhanced localization.2,20 This becomes diffuse if NOS3 activity is inhibited,220 behavior also observed in canine dilated cardiomyopathy.27 We speculated that a similar change might accompany hypertrophy/dilation coupled to functional enzyme re-targeting. Figure 4A shows confocal images of myocytes from each model at baseline after TAC. A striated pattern was present in control and P5+ cells, but this became diffuse throughout the cell after TAC. Both N3− and P5+/N3− hearts displayed a diffuse pattern at baseline as well as after TAC, consistent with the lack of NOS3. Despite this diffuse distribution, PKG activity was preserved in N3− hearts likely because of persistent low levels of PDE5 activation (Figure IV in the online-only Data Supplement). In contrast, P5+/N3+ hearts had both diffuse and enhanced PDE5 activity owing to the tet-off transgene, so PKG activity was less. The TAC stress-specific change in localization of the PDE5 transgene was confirmed by staining with anti-Flag antibody (Figure 4B), and by immunoblot of PDE5 in myofibrillar subfractions with tropomyosin used as a loading control (Figure 4C).

When neonatal myocytes are stained for PDE5, they show a diffuse staining pattern (Figure 4D) analogous to that
observed in adult myocytes after TAC, suggesting that this latter change may be part of fetal recapitulation. Interestingly, diffuse staining in neonatal cells was also coupled to dual targeting of PDE5 to NO- and ANP-stimulated cGMP pools (Figure 4D).

PDE5 is itself potently activated by PKG phosphorylation at S92, and in platelets, this modification has been coupled to compartmentation. We therefore speculated that loss of this signal from NOS3-PKG activation might lead to unmooring of the enzyme from its sarcomeric location. PDE5 mutants with either inactivated (S92A) or pseudophosphorylated (S92D) modifications fused with DsRed tag were incorporated into adenoviral vectors and injected into the heart in vivo. Myocytes isolated 48 hours later were examined by confocal imaging. Neither mutant altered the normal striated pattern of PDE5 distribution (Figure V in the online-only Data Supplement).

Hypertrophy/Failure in P5+/N3− Hearts Occurs Despite Minimal Reactive Oxygen Species Activation

In N3− hearts, protection against maladaptive (dilated) hypertrophy is coupled to the absence of NOS3-derived reactive oxygen species that otherwise occurs via NOS uncoupling. PDE5 upregulation itself has been linked to reactive oxygen species, and we hypothesized that declines in cGMP/PKG signaling due to such upregulation would further amplify reactive oxygen species, perhaps contributing to worse dysfunction/dilation observed in P5+ and P5+/N3− hearts. The first prediction proved correct, but the second surprisingly did not. Myocardial superoxide determined by high-performance liquid chromatography assay for 2-OH-E showed a 2-fold increase in P5+ over controls. Levels were below control in N3− myocardium from hearts exposed to ANP, sildenafl (SIL), or both. n=3 to 9 per group. P values from 2-way ANOVA: top, value for effect from ANP; bottom, for ANP-by-SIL interaction. In sham controls, ANP increased cGMP similarly with or without SIL, whereas the ANP+SIL response was greater after TAC than with ANP alone. P values 2-way ANOVA: top, ANP effect; bottom, ANP-by-genotype interaction. n=3 to 9 per group.

Discussion

The major new finding of this study is that cardiac stress remodeling associated with pressure overload retargets PDE5 hydrolytic activity to the cGMP pool generated via NP
signaling rather than solely from NOS3-NO-sGC–dependent cGMP as found in the normal heart. This supported by NP-PDE5A interaction data in hearts subjected to TAC and by the finding that genetic deletion of NOS3 did not eliminate the adverse impact of PDE5 upregulation. Retargeting was accompanied by a redistribution of the protein from a sarcomeric pattern to one that is diffuse, similar to that observed in neonatal myocytes. As a result, potentially protective effects from NP signaling were blunted by PDE5 upregulation, worsening maladaptive remodeling but also increasing the therapeutic impact from PDE5 inhibition. Finally, we show that myocyte PKG is a key suppressor of maladaptive cardiac hypertrophy and dilation, even in settings with reduced oxidative stress.

**Compartmentation of Cyclic Nucleotide Signaling**

For cyclic nucleotides to perform their broad array of intracellular signaling roles, concentrations and protein targeting must be constrained within microdomains. This is accomplished by compartmentalizing the cyclase with its corre-
and increased in P5

Functional consequences because PDE5 inhibition but not

Figure 5. Enhanced phosphodiesterase 5 (PDE5) expression increases myocardial oxidative stress, but this is diminished in hearts lacking nitric oxide synthase 3 (NOS3). A, Myocardial superoxide was detected by 2-OH-E\(^*\) normalized to total DHE and increased in P5\(^+\) tissue compared with control (CON). Both N3\(^-\) and P5/N3 myocardium showed a similar and much lower level. \(P<0.02\) vs all other groups. \(n=8–10\) per group. B, Ratio of reduced and oxidized glutathione (GSH/GSSG) in the 4 models. The ratio was reduced most in P5\(^-\) (\(P<0.05\) vs sham), consistent with increased oxidative stress, but was at control levels in the 2 models lacking NOS3. \(n=3–4\) per group. \(P\) values in graph are for 1-way ANOVA between groups.

PDE5 Retargeting as a Mechanism for Altered Signaling

The normal myocyte localizes PDE5 in the cytosol and the sarcomere. In the latter location, its inhibition blunts \(\beta\)-AR–stimulated contractility via \(\beta\)-AR–coupled, PKG-dependent troponin I phosphorylation.\(^{18}\) This is consistent with observed declines in cell shortening without concomitant changes in the calcium transient, ie, calcium desensitization. As noted, NP with or without PDE2 inhibition does not mimic this effect, supporting the notion that myofilament PDE5 serves as a local containment mechanism for contractile regulation. Few data describe PDE5 compartmentation in other cell types, an example being an endoplasmic reticulum–localized pool in platelets that regulates thrombin-mediated calcium.\(^{28}\)

To the best of our knowledge, the cardiac myocyte is the only cell type in which redistribution of the enzyme has been reported, first in a canine model of heart failure\(^{27}\) and subsequently in mice lacking NOS3 or with chronic NOS inhibition.\(^{2,20}\) In the latter case, altered localization was reversible either by chronically restoring NOS activity or by activating sGC even with persistent NOS inhibition.\(^{2,20}\) Importantly, only when PDE5 was localized to the sarcomere did its inhibition blunt \(\beta\)-AR stimulation.

The present study reveals a second and arguably more important consequence of PDE5 dysloccalization: functional retargeting from one cGMP pool to another. This is particularly relevant to heart disease in which NP-stimulated cGMP often rises markedly whereas NOS-derived cGMP declines. Staining patterns did not reveal enhancement at the plasma membrane, and whether direct protein interaction of PDE5 with the NPRA (or NPRB) receptor occurs remains unknown. However, the functional results showed that a diffuse cytosolic diffusion was sufficient to affect non–sGC-derived cGMP pools. Further work is ongoing to assess different PDE5 subproteomes in normal and stressed hearts.

The decline in PKG activity under basal conditions in both the P5\(^+\) and P5\(^-\)/N3\(^-\) myocardium (Figure 3B) might suggest that functional retargeting results from PDE5 upregulation itself, not after pressure-overload stress. However, loss of NOS3 itself results in dispersion of PDE5 from the z disk (Figure 4A), supporting a key role of sGC-derived cGMP to normal PDE5 regulation. Without concomitant PDE5 upregu-
lation, this does not affect basal PKG activity; however, when they are combined, this activity declines substantially as observed. In addition, the P5+ model itself did not display basal PDE5 hydrolytic interaction with ANP-stimulated cGMP pools (Figure 3D). This was observed only after TAC, further supporting the importance of stress remodeling.

PDE5 retargeting could explain the efficacy of its inhibition even when it is used to treat late-stage hypertrophy/dilation disorders, in which sGC and NOS activity is often blunted. Importantly, NP-derived cGMP signaling in the heart has also been shown to be antihypertrophic and to inhibit RGS2, TRPC6, and other signaling pathways coupled to pathological remodeling. More promiscuous targeting by upregulated and dyslocalized PDE5 would suppress this modulation and enhance the impact of PDE5 inhibition. Enhanced NP targeting is also consistent with studies performed in kidney that found reduced NPRA-coupled cGMP generation despite increased gene expression and unaltered or slightly reduced protein expression. Augmented PDE5 activity has been linked to renal and lung NP desensitization, and our findings now support this in the diseased heart as well. Impaired PKG activity linked to upregulated and retargeted PDE5 could further impair ANP signaling by suppressing the PKG-dependent phosphorylation/activation of the NPRA itself.

Retargeting of cyclic nucleotide signaling is not unique to the cGMP/PKG pathway; studies have also reported changes in cAMP signaling under disease or pharmacologic conditions. For example, Nikolaev et al found that β2-ARs that normally lie within deep transverse tubular membranes in the cardiac myocyte had shifted to surface membrane crest territories where β1-ARs are usually found in the diseased heart. This was coupled with loss of membrane containment of the cAMP signal, perhaps caused by a change in the PDE microenvironment. PDE4A4 has been observed to move into accretion foci on binding of the inhibitor rolipram to its catalytic site, although to the best of our knowledge, retargeting of PDE4 as a component of heart disease has not been reported.

Movement and functional retargeting of PDE5 can be added to other members of the PKG signalosome that move with cardiac stress. For example, Gq-agonist stimulation in vascular smooth muscle and cardiac myocytes results in rapid migration of PKG1α from the cytosol to plasma membrane, coupled to activation and comigration of the regulator of G-protein signaling 2. This blunts the Gq stimulus and appears coupled to the early therapeutic efficacy of sildenafil to counter pressure overload. More recently, sGC was observed to translocate from caveolin-3–enriched membrane domains into nonlipid raft domains. This had functional significance in that sGC in the latter was more oxidized and thus far less responsive to NO than that within caveolin-3 domains, and pressure overload induced a shift out of the latter, contributing to reduced NO responsiveness.

Study Limitations
The proteins involved with PDE5 migration remain unknown. One of the difficulties of studying PDE5 migration is that adult myocytes are required because the compartmentation is lacking in the neonatal cells. Using the PDE5tg-model to enhance pull-down assays, we attempted a broad proteomic screen to define the interactome, and proteomic analysis with 2-dimensional gel electrophoresis and mass spectrometry revealed several heat shock proteins (HSC70 and HSP27) that were confirmed by immunoprecipitation. However, given the multitude of protein interactions, their role in localization is difficult to define. This analysis remains a work in progress.

Although our models excluded NOS3 stimulation of sGC as a source of cGMP, they did not eliminate NOS1 or NOS2. However, neither was augmented in models lacking NOS3 before or after TAC, and their expression did not correlate with cGMP or PKG activity. Current data suggest that myocyte NOS1 largely signals via cGMP-independent pathways, and although its decline with TAC could exacerbate disease and oxidative stress, differences among groups did not reach significance. As shown in prior work, NOS2 does not seem to be a major modulator of TAC-induced disease. Finally, we recognize that N3 mice reflect an embryonic knockout that may induce adaptations to alter signaling. However, prior studies regarding PDE5 regulation of the β-AR cascade have revealed very similar behavior in N3− and wild-type controls administered N-nitro-l-arginine methyl ester.

Conclusions
We have shown that upregulation of PDE5 in conjunction with pressure-overload stress and accompanying hypertrophy/dilation results in a change in the myocyte subcellular distribution of protein, retargeting from cGMP generated by NO-dependent sGC to that from NP-derived receptor coupled GC. This likely plays a key role in the pathophysiological consequences of increased PDE5 expression observed in experimental and human heart disease and contributes to the ameliorative effects of its inhibition in heart diseases in which NOS and sGC activity is impaired.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Phosphodiesterase type 5 (PDE5) hydrolyzes cGMP, and its inhibition is widely used to treat erectile dysfunction and pulmonary hypertension. Recent studies have expanded its role to the cardiomyocyte because PDE5 inhibitors also suppress remodeling and improve function in heart diseases such as hypertrophy, infarction, and dilated heart failure. These preclinical data have spawned clinical trials in heart failure. In the normal myocyte, PDE5 degrades cGMP generated by nitric oxide–stimulated soluble guanylate cyclase but has virtually no impact on natriuretic peptide (NP)–derived cGMP. Yet in heart failure, cGMP from nitric oxide signaling declines whereas NP-generated cGMP rises. This poses a paradox for the inhibitor studies because, if normal PDE5 cGMP-targeting persisted in heart failure, its impact and efficacy of inhibitors should both decline. Here, we reveal an explanation, showing in the hypertrophied/dilated heart that PDE5 is retargeted to degrade NP-cGMP. Even in mice in which nitric oxide synthase 3 is genetically deleted to remove the normal source of PDE5-targeted cGMP, upregulated myocyte PDE5 still worsened hypertrophy and left ventricular dilation/dysfunction to pressure-overload coupled to increased NP-cGMP hydrolysis. This was associated with physical displacement of PDE5 from its normal sarcomere localization to a diffuse cytosolic distribution. Enhanced myocyte PDE5 expression also stimulated reactive oxygen species. However, this was not required for its pathological effects because reactive oxygen species was greatly reduced in mice with codeletion of nitric oxide synthase 3 despite similar adverse remodeling to pressure overload. PDE5 retargeting likely contributes to cardiac NP desensitization and suggests a combined benefit from PDE5 inhibition and NP stimulation in cardiac disease.
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Supplemental Material

Methods:

Primers used for quantitative rtPCR analysis:

TaqMan primers and probes for mouse Rcan1 (NM_001081549), Tgfb1 (NM_011577), Ctgf (NM_010217), Trpc6(NM_013838) and Gapdh (NM_008084) were purchased from Applied Biosystems. For SYBR green method, the following primers were used: Mouse Nppa(NM_008725) 5’-TCGTCTTTGGCCTTTTGCT-3’ (forward) and 5’-TCCAGGTGGTCTAGCAGTTCT-3’ (reverse); Mouse Nppb(NM_008726) 5’-AAGTCC TAGCCAGTCTCCAGA -3’ (forward) and 5’-GAGCTGTCTCTGTGGCCATTTC-3’ (reverse); Mouse Nos1(NM_008712) 5’-AGAAGCAGCGTCTGCTGGTCCTCAG-3’ (forward), 5’-CTGTATCCGGTTGAGCCAGGAG-3’ (reverse); Mouse Nos2(NM_010927) 5’-CAGCTGGGCTGTACAAACCT-3’ (forward), 5’-CATTGGAAGTGAAGCGTTTC-3’ (reverse). Primers for mouse NOX2, forward 5’ GGG CTA TTC AAT GCT TGT GGC TGT 3’, reverse 5’ TCT TCA CTG GCT GTA CCA AAG GGT 3. Primers for mouse NOX4, forward 5’ TCA TGG ATC TTT GCC TCG AGG GTT 3’, reverse 5’ TCC AGG TCT GTG GGA AAT GAG CTT 3’.
Supplemental Figure Legends

S1) Resting heart function, and heart and lung weights normalized to tibia length for the four experimental groups. There was no difference in cardiac size, wall thickness, or function among models (n=3-7/group).

S2) Basal PKG activity in each of the four experimental models. Activity was much lower in the both models (P5* and P5/N3) with enhanced myocyte PDE5 expression (n=3-6/group). P<0.0001 for 1-way ANOVA, * p<0.001 versus CON and N3- models.

S3) Gene expression of NOS1 and NOS2 isoforms at baseline and after TAC in the four experimental groups. 1-way ANOVA within group (basal versus TAC) yielded no significant differences among experimental models (p value ranging 0.18 to 0.4). 3-way ANOVA (+/- TAC, +/-NOS3, +/- PDE5 over-expression) yielded a borderline significant effect of NOS3 expression on the TAC-induced increase in NOS2 (p=0.047), and overall effect of TAC on lowering NOS1 expression (p=0.001). n=3-9 per group.

S4) PDE5 cyclic G esterase activity assessed in WT versus N3- hearts before and after TAC. Activity was increased in WT after TAC, but this was significantly less so in the N3- heart. * p<0.01 versus sham, † p<0.02 interaction of genotype and TAC(n=4-12/group).

S5) Confocal immunohistochemistry of adult myocytes transfected in vivo with a DSred-PDE5 mutant fusion protein (S92A, and S92D). Cells were then isolated and infected cells
identified by fluorescence. Neither mutation (gain or loss of phosphorylation capacity) altered the localization of PDE5 to the sarcomere.

S6) NOX4 and NOX2 gene expression in the four experimental models – at baseline and after TAC. NOX2 expression rose in all four models similarly, whereas NOX4 increases most in the two models with enhanced PDE5 expression, and was lowest in N3- (n=3-11/group). P-values are for 2-way ANOVA. Top value reflects overall impact of TAC on expression, lower value is the interaction between TAC and experimental model. * p<0.05 versus P5+; † p<0.05 versus P5+ and P5+/N3-. 
Supplemental Figure S1
PKG activity (myocardium)

Arbitrary Units

CON  P5+  N3-  P5+/N3-
Supplemental Figure S3

Gene Expression (Normalized to Basal-Control)

Nos1/GAPDH mRNA

Basal

TAC

Nos2/GAPDH mRNA

Basal

TAC

CON  P5+  N3-  P5+/N3-

Supplemental Figure S3
Supplemental Figure S4

PDE5 cGMP-esterase Activity Normalized to Basal-control

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Ds-Red PDE5A S92A  

Ds-Red PDE5A S92D
**Supplemental Figure S6**

**NOX2**

- mRNA Normalized to GAPDH and to Baseline Control
- p<0.001 (TAC)
- p=NS by (TACxGroup)

**NOX4**

- mRNA Normalized to GAPDH and to Baseline Control
- p<0.001 (TAC)
- p<0.001 (TACxGroup)