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Pathological Cardiac Hypertrophy Alters Intracellular Targeting of PDE5 from Nitric Oxide Synthase-3 to Natriuretic Peptide Signaling

Manling Zhang, MD, PhD¹, Eiki Takimoto, MD, PhD¹, Dong-ik Lee, PhD¹, Celio X C. Santos, PhD², Taishi Nakamura, MD, PhD¹, Steven Hsu, MD¹, Aiyang Jiang, BS¹, Takahiro Nagayama, PhD¹, Djahida Bedja, MS¹, Yuan Yuan, MD, PhD¹, Philip Eaton, PhD³, Ajay M. Shah, MD, MPH², and David A. Kass, MD¹

¹Div of Cardiology, Dept of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD

²Cardiovascular Div, King's College London, British Heart Foundation Centre, London, United Kingdom

³The Rayne Institute, Kings College London, St. Thomas Hospital, London, United Kingdom

Abstract

Background—In the normal heart, phosphodiesterase type 5 (PDE5) hydrolyzes cGMP coupled to nitric oxide (specifically from NOS3) but not natriuretic peptide (NP) stimulated guanylyl cyclase. PDE5 is upregulated in hypertrophied and failing hearts and is thought to contribute to their pathophysiology. Since NO 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 NOS3 (N3⁻), and each model, the double cross, and controls were subjected to transaortic constriction (TAC). P5⁺ mice developed worse dysfunction and hypertrophy, and enhanced NP stimulation, whereas N3⁻ mice were protected. However, P5⁺/N3⁻ mice behaved similarly to P5⁺ despite the lack of NOS3-coupled cGMP generation, with PKG activity suppressed in both models. PDE5 inhibition did not alter ANP-stimulated cGMP in the resting heart, but augmented it in the TAC 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 due to the absence of NOS3 uncoupling). This highlights the importance of myocyte PKG activity as a protection to pathological remodeling.

Conclusions—These data provide the first evidence for functional retargeting of PDE5 from one compartment to another, revealing a role for NP-derived cGMP hydrolysis by this esterase in diseased heart myocardium. Retargeting likely impacts the pathophysiologic consequence and also therapeutic impact of PDE5 modulation in heart disease.

Address for Correspondence: David A. Kass, MD, Division of Cardiology, The Johns Hopkins Medical Institutions, Ross Building 858, 720 Rutland Avenue, Baltimore, MD, 21205, Tel: (410) 955-7153 Fax: (410)-502-2558, dkass@jhmi.edu.

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Keywords

cyclic nucleotides; hypertrophy; oxidative stress; phosphodiesterases receptors

Introduction

Phosphodiesterase type 5 (PDE5) is one of an eleven-member superfamily of enzymes responsible for the hydrolysis of cyclic nucleotides to their 5' monophosphate form. It was the first to be identified as relatively selective for cyclic guanosine monophosphate (cGMP), and is expressed in vascular smooth muscle particularly in the corpus cavernosum and pulmonary circulation. PDE5 inhibition stimulates protein kinase G-dependent vasodilation, forming the basis for its clinical use to treat erectile dysfunction and pulmonary hypertension¹. Recent studies have revealed PDE5 is also expressed in cardiac muscle²⁻⁵, and growing evidence supports its contribution to ventricular disease in experimental models^{6, 7} and in humans⁸. While expression is normally quite low, it rises considerably in experimental⁴ and human cardiac hypertrophy and failure^{5, 9, 10}, a change that has been linked to oxidative stress⁴. Higher levels of PDE5 expression can participate in cardiac pathophysiology, worsening remodeling and dysfunction after myocardial infarction⁵ or following sustained pressure-overload⁷. Such findings have led to clinical trials testing the utility of PDE5 inhibition in dilated cardiomyopathy (DCM)^{8, 11}, heart failure with a preserved ejection fraction (HFpEF)¹², and Duchenne and Becker muscular dystrophy¹³. Initial results support the efficacy of sildenafil in human DCM for improving breathlessness and exercise capacity^{14, 15}, and cardiac remodeling and function⁸, without altering systemic arterial pressures (i.e. afterload).

In the ventricular myocyte, cGMP is generated by two different pathways: by stimulating nitric oxide synthase (NOS) to generate NO and activate soluble guanylate cyclase (sGC)¹⁶, or by natriuretic peptide activation of particulate GC attached to the cognate receptor¹⁷. These are not redundant pathways, but display different constellations of distal targets and regulatory PDEs. In particular, myocyte PDE5 normally targets NOS3-sGC derived cGMP, while other PDEs (e.g. PDE1, PDE2) may target pGC related pools^{18, 19}. 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 inhibited pharmacologically^{2, 20}. Moreover, PDE5 inhibition enhances PKG activity upon NO but not ANP stimulation in the normal heart¹⁸, has no impact on membrane localized cGMP induced by ANP in rat myocytes¹⁹, and mimics NO- but not ANP modulated compartmentation of PKA isoform activity²¹.

In many forms of heart disease, NO-derived cGMP declines due in part to NOS uncoupling²² and sGC oxidation²³ whereas NP-derived cGMP is markedly enhanced. If increased PDE5 activity with HF remained solely targeted to NO-sGC derived pools, its impact would be likely 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 current study tested this hypothesis by studying mice with a tetracycline controllable myocyte-specific PDE5 overexpression (P5⁺)⁷ and crossing them into a background lacking NOS3 (N3⁻). We previously reported that P5⁺ mice exhibit worsened hypertrophy, fibrosis, and LV dysfunction when subjected to trans-aortic constriction (TAC), and this reverses once myocyte PDE5 expression is reduced (adding doxycycline to the drinking water to suppress transgene expression)⁷. N3⁻ mice, by contrast, develop concentric adaptive hypertrophy with severe TAC, coupled to a lack of oxidative stress that is otherwise generated by NOS uncoupling²², a process whereby NOS produces superoxide rather than NO²⁴. If PDE5 remained targeted only to NOS3-sGC

derived cGMP, one would predict a model lacking NOS3 but with myocyte PDE5 enhanced would behave like the N3⁻ itself. However, if alternative targeting to NP-pGC derived cGMP occurs, the pathophysiology should 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 organ and molecular levels, even despite low levels of myocardial oxidative stress.

Methods

Mouse Models and Pressure-Overload Stress

Bi-genic P5⁺ mice have been described previously⁷; they overexpress PDE5 in myocytes (α MHC promoter) but only in the absence of doxycycline (tet-off system). We used the line with 7 \times PDE5 protein overexpression. P5⁺ mice were further crossed into a N3⁻ background (Jackson Labs, C57BL/6) to generate tri-genic mice. Four groups were studied (mean age 2–3 months when heart function and molecular signaling are similar to controls): controls (littermates expressing NOS3 and lacking PDE5 transgene), P5⁺, N3⁻, and the cross (P5⁺/N3⁻). Mice were subjected to 7–8 days of trans-aortic constriction (TAC), a duration previously shown to induce marked chamber dilation/dysfunction and early mortality in P5⁺ mice⁷.

Cardiac Function

Intact cardiac function was assessed in conscious animals by echocardiography to derive indexes of LV dimension, fractional shortening, and wall thickness as described⁷.

Confocal Immunohistochemistry

The myocyte distribution of PDE5 followed previously reported protocols and equipment, using both a primary antibody to PDE5 (Cell Signaling) and to Flag (tagged with transgene, Sigma Aldrich)⁷. Myocyte size was detected by wheat germ agglutinin (WGA) staining⁷, using a semi-automated 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 (NFAT) signaling (regulator of calcineurin, *Rcan-1*, transient receptor potential canonical channel 6, *Trpc6*), and fibrosis/remodeling genes (transforming growth factor beta-1 *Tgfb1*, connective tissue growth factor, *ctgf*) were determined by real-time quantitative PCR (primers in Supplemental Methods). Cyclic GMP was assessed by EIA and PKG activity by fluorescent polarization assay as described⁷. 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 HPLC assay of 2-hydroxyethidium formation from frozen tissue extracts as described²⁵. Tissue level of reduced and oxidized glutathione (GSH/GSSG) was measured using a Bioxytech GSH/GSSG-412 kit (Oxis International Inc.). For measurement of GSSG, the thiol-scavenging reagent, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP), was added to the homogenization buffer to minimize oxidation of GSH to GSSG. Gene expression of NADPH oxidases, NOX2 and NOX4 was determined by qPCR.

Statistics

Results are presented as mean \pm SEM. For analysis between the four experimental groups, we used a 1-way ANOVA with a Tukey-Kramer multiple comparisons test. In experiments where the data distribution failed normality tests, a Kruskal-Wallis non-parametric test and Dunn's multiple comparison test was 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 employing a subsequent within-group 1-way ANOVA and multiple comparisons test as appropriate. The specific tests and significant differences between groups as well as relevant sample sizes are provided in the figure legends. Analysis was performed using 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 four groups (Supplemental Fig. S1). Resting PKG activity was lower in the two groups carrying the P5⁺ transgene in myocytes versus controls or N3⁻ (Supplemental Fig. S2). Normal basal PKG activation in hearts lacking NOS3 (N3⁻) is consistent with prior studies showing enhanced NP-signaling in this model²⁶.

In contrast to resting function, the cardiac response to TAC differed markedly among the four groups. Figure 1A displays representative echocardiograms, cross sectional histology, and WGA stained myocardium from hearts after TAC. LV dilation and function were worse and myocyte hypertrophy 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 activation⁷. Gene expression markers of calcineurin upregulation (*Rcan1*, *Trpc6*), and growth/fibrosis signaling (*tgfb1*, *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 an alternative pool of cGMP was being targeted, such as from NP signaling. Myocardial ANP and BNP gene expression rose in all models, with ANP increasing even more in P5⁺ and P5⁺/N3⁻ (Fig 3A). Corresponding PKG activity is shown in Fig. 3B. While TAC increased activity overall ($p < 0.05$ by 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 μ g), sildenafil (12.5 μ g), 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 SIL/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 WT 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 (Supplemental Fig. S3). Expression of both NOS1 (neuronal) and NOS2 (inducible) isoforms was similar at baseline in all four models. After TAC, NOS2 rose slightly more in mice expressing NOS3 versus those that did not ($p<0.05$, 3-way ANOVA). NOS1 by contrast remained similar among the groups, though expression was somewhat lower overall after TAC ($p<0.002$, 3-way ANOVA). Thus, while some changes were observed, they did not correlate with cardiac remodeling, 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^{2, 20}. This becomes diffuse if NOS3 activity is inhibited^{2, 20}, behavior also observed in canine dilated cardiomyopathy²⁷. We speculated that a similar change might accompany hypertrophy/dilation coupled with functional enzyme targeting. Figure 4A shows confocal images of myocytes from each model at baseline and after TAC. A striated pattern was present in CON and P5⁺ cells, but this became diffuse throughout the cell after TAC. Both N3⁻ and P5⁺/N3⁻ hearts displayed a diffuse pattern at baseline and after TAC. However, PKG activation was preserved in N3⁻ after TAC, despite a diffuse PDE5 distribution, likely due to persistent low levels of PDE5 activation in these hearts (Supplemental Fig. S4). In contrast, P5⁺/N3⁺ hearts had enhanced PDE5 activity due to the tet-off transgene. The TAC stress-specific change in localization of the PDE5 transgene was confirmed by staining with anti-Flag antibody (Fig. 4B), and immunoblot of PDE5 in myofibrillar sub-fractions, 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 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 (Fig. 4D).

PDE5 is itself potentially activated by PKG phosphorylation at S92, and in platelets this modification has been coupled to compartmentation²⁸. 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*²⁰. Myocytes isolated 48 hours later were examined by con-focal imaging. Neither mutant altered the normal striated pattern of PDE5 distribution (Supplemental Fig S5).

Hypertrophy/failure in P5⁺/N3⁻ hearts occurs despite minimal ROS 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 uncoupling²². PDE5 up-regulation has itself been linked to ROS⁴, and we hypothesized that consequent declines in cGMP/PKG signaling would further amplify ROS, perhaps contributing to the dysfunction/dilation observed in P5⁺ as well as P5⁺/N3⁻ hearts. The first prediction proved correct, but the second surprisingly not. Myocardial superoxide determined by HPLC assay for 2-OH DHE showed a >2-fold increase in P5⁺ over controls. Levels were below control in N3⁻ myocardium, yet were similarly low in P5⁺/N3⁻ (Figure 5A). These results were supported by the ratio of reduced/oxidized glutathione (GSH/GSSG) that reflects overall

redox. This ratio declined in Con and P5⁺, but was similar to control in models lacking NOS3 (Figure 5B). Despite differences in ROS, the expression of NADPH oxidases (NOX2 and NOX4) rose in all models after TAC, with NOX2 increasing equally and NOX4 rising more in both models with greater PDE5 expression and worse remodeling (Supplemental Fig. S6).

Discussion

The major new finding of this study is that cardiac stress remodeling associated with pressure-overload re-targets PDE5 hydrolytic activity to the cGMP pool generated via natriuretic peptide 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 are blunted by PDE5 upregulation, worsening maladaptive remodeling, but also increasing the therapeutic impact from PDE5 inhibition. Lastly, 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

In order 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 corresponding kinase and hydrolyzing phosphodiesterase. Such microdomains have been best studied for cAMP regulation in which all three components are often further coordinated by A-kinase anchoring proteins (AKAPs) which serve as complex scaffolds²⁹. PDEs also play a primary role in this compartmentation³⁰ by limiting cyclic nucleotide diffusion beyond a microdomain and/or preventing distal targeting. Among these, PDE4 appears particularly suited for regional regulation due to a variable N-terminal region that acts as a zip code, directing expression to specific locations³¹. Other PDEs are targeted by different and less well clarified mechanisms. Examples are the local confinement to the plasma membrane of cAMP coupled to β 2-AR stimulation by PDE3 and PDE4³², post-activation of the β 2-AR by PDE4D5 which shifts it from Gs to Gi-coupled signaling³³, and modulation of the ryanodine receptor by PDE4D3³⁴.

Compartmentation of cGMP signaling is equally important, although the molecular mechanisms remain less well understood in part due to challenges in creating sensitive fluorescent probes to detect cGMP or PKG activity in microdomains within adult myocytes. cGMP signaling can be detected at the plasma membrane by means of cGMP-sensitive olfactory ion channels, and this has yielded evidence of tight compartmentation. Membrane localized cGMP rises with ANP but not NO stimulation, and the ANP response is enhanced by inhibition of PDE2 but not PDE5¹⁹. This has functional consequences as PDE5 inhibition but not ANP blunts β -AR stimulated contraction in both myocytes and intact hearts, despite much greater increases in measurable myocardial cGMP from ANP stimulation¹⁸. Cyclic GMP compartmentation has been reported in studies conducted in neonatal myocytes as well, for example in the work by Stangherlin et al²¹ who found differential regulation by cGMP on cAMP-mediated PKA activation depending upon the guanylate cyclase stimulated, the PKA-isoform activated, and the specific PDE regulating the signaling. However, the exact details differ somewhat from adult cells, perhaps reflecting greater maturation of compartments as sarcomere structure develops. For example, they found both ANP and NO-stimulated cGMP regulated cAMP-PKA signaling²¹, with the former coupled

to PDE2. However, in adult myocytes, ANP has minimal effects on β -adrenergic modulation with or without PDE2 inhibition³⁵.

PDE5 retargeting as a mechanism for altered signaling

The normal myocyte localizes PDE5 in the cytosol as well as the sarcomere. In the latter location, its inhibition blunts β -AR stimulated contractility via β 3-AR coupled, PKG dependent troponin I phosphorylation³⁵. This is consistent with observed declines in cell shortening without concomitant changes in the calcium transient, i.e. calcium desensitization. As noted, NP \pm PDE2 inhibition does not mimic this effect, supporting the notion that myofilament PDE5 serves as a local containment mechanism for contractile regulation. There are only few data describing PDE5 compartmentation in other cell types, an example being an ER localized pool in platelets that regulates thrombin-mediated calcium²⁸. To 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 failure²⁷, and subsequently in mice lacking NOS3 or with chronic NOS inhibition^{2, 20}. In the latter case, altered localization was reversible by either 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 β -AR stimulation.

The present study reveals a second and arguably more important consequence of PDE5 dyslocalization; functional retargeting from one cGMP pool to another. This is particularly relevant to heart disease where 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 impact non sGC-derived cGMP pools. Further work is ongoing to assess different PDE5 sub-proteomes in normal and stressed hearts.

The decline in PKG activity under basal conditions in both the P5+ and P5+/N3-myocardium (Fig. 3B) might suggest that functional retargeting results from PDE5 upregulation itself, and not following pressure-overload stress. However, loss of NOS3 itself results in dispersion of PDE5 from the z-disk (Fig. 4A), supporting a key role of sGC-derived cGMP to normal PDE5 regulation. Without concomitant PDE5 upregulation, this does not impact basal PKG activity; however when both 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 (Fig 3D). This was only observed after TAC, further supporting the importance of stress-remodeling.

PDE5 retargeting could explain the efficacy of its inhibition even when this is used to treat late-stage hypertrophy/dilation disorders³⁶, where sGC and NOS activity are often blunted. Importantly, NP-derived cGMP signaling in the heart has also been shown to be anti-hypertrophic³⁷, and to inhibit RGS2, TRPC6, and other signaling pathways coupled to pathological remodeling³⁸. 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 NPR-A coupled cGMP generation despite increased gene expression³⁹ and unaltered or slightly reduced protein expression⁴⁰. Augmented PDE5 activity has been linked to renal and lung NP desensitization^{41, 42} 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 itself⁴³.

Retargeting of cyclic nucleotide signaling is not unique to the cGMP/PKG pathway, as studies have also reported changes in cAMP signaling under disease or pharmacological conditions. For example, Nikolaev et al⁴⁴ found β 2-ARs that normally lie within deep transverse-tubular membranes in the cardiac myocyte shifted to surface membrane crest territories where β 1-AR are usually found, in the diseased heart. This was coupled with loss of membrane containment of the cAMP signal, perhaps due to a change in the PDE microenvironment. PDE4A4 has been observed to move into accretion foci upon binding of the inhibitor rolipram to its catalytic site⁴⁵, although to 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 also move with cardiac stress. For example, Gq-agonist stimulation in vascular smooth muscle⁴⁶ and cardiac myocytes⁴⁷ results in rapid migration of PKG1 α from the cytosol to plasma membrane, coupled to activation and co-migration of regulator of G-protein signaling2. This blunts the Gq-stimulus, and appears coupled to early therapeutic efficacy of sildenafil to counter pressure-overload⁴⁷. More recently, soluble GC was observed to translocate from caveolin-3 enriched membrane domains into non-lipid raft domains²³. This had functional significance, as sGC in the latter was more oxidized and thus far less responsive to NO than that within Cav-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 this is that adult myocytes are required – as the compartmentation is lacking in the neonatal cells. Using the PDE5 tg -model to enhance pull down assays, we attempted a broad proteomic screen to define the interactome, and proteomic analysis with 2-D gel electrophoresis and mass spectroscopy revealed several heat shock proteins (HSC70 and HSP27) that were confirmed by IP. However, given the multitude of protein interactions, their role in localization is difficult to define. This analysis remains a work in progress.

While our models excluded NOS3 stimulation of sGC as a source of cGMP, it did not eliminate NOS1 or NOS2. However, neither were augmented in models lacking NOS3 before or after TAC, and their expression did not correlate with cGMP or PKG activity. Current data suggests myocyte NOS1 largely signals via cGMP-independent pathways⁴⁸, and though its decline with TAC could exacerbate disease and oxidative stress⁴⁹, differences among groups did not reach significance. As shown in prior work, NOS2 does not seem a major modulator of TAC-induced disease⁵⁰. Lastly, 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 behavior very similar between N3⁻ and WT controls administered L-NAME.

Conclusion

In conclusion, we have shown that upregulation of PDE5 in conjunction with pressure-overload stress and accompanying hypertrophy/dilation results in a change in the protein's myocyte sub-cellular distribution, retargeting from cGMP generated by NO-dependent sGC to that from NP-derived rGC. This likely plays a key role in the pathophysiological consequences of increased PDE5 expression observed in experimental and human heart disease, and also contributes to the ameliorative effects of its inhibition in heart diseases where NOS and sGC activity are impaired.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Clinical Summary

Phosphodiesterase type 5 (PDE5) hydrolyzes cyclic GMP and its inhibition is widely used to treat erectile dysfunction and pulmonary hypertension. Recent studies have expanded its role to the cardiomyocyte, as 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 NO-signaling declines whereas NP-generated cGMP rises. This poses a paradox for the inhibitor studies, since 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, PDE5 is retargeted to degrade NP-cGMP. Even in mice in which NOS3 is genetically deleted to remove the normal source of PDE5-targeted cGMP, upregulated myocyte PDE5 still worsened hypertrophy and LV 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 (ROS). However, this was not required for its pathological effects, as ROS was greatly reduced in mice with co-deletion of NOS3 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.

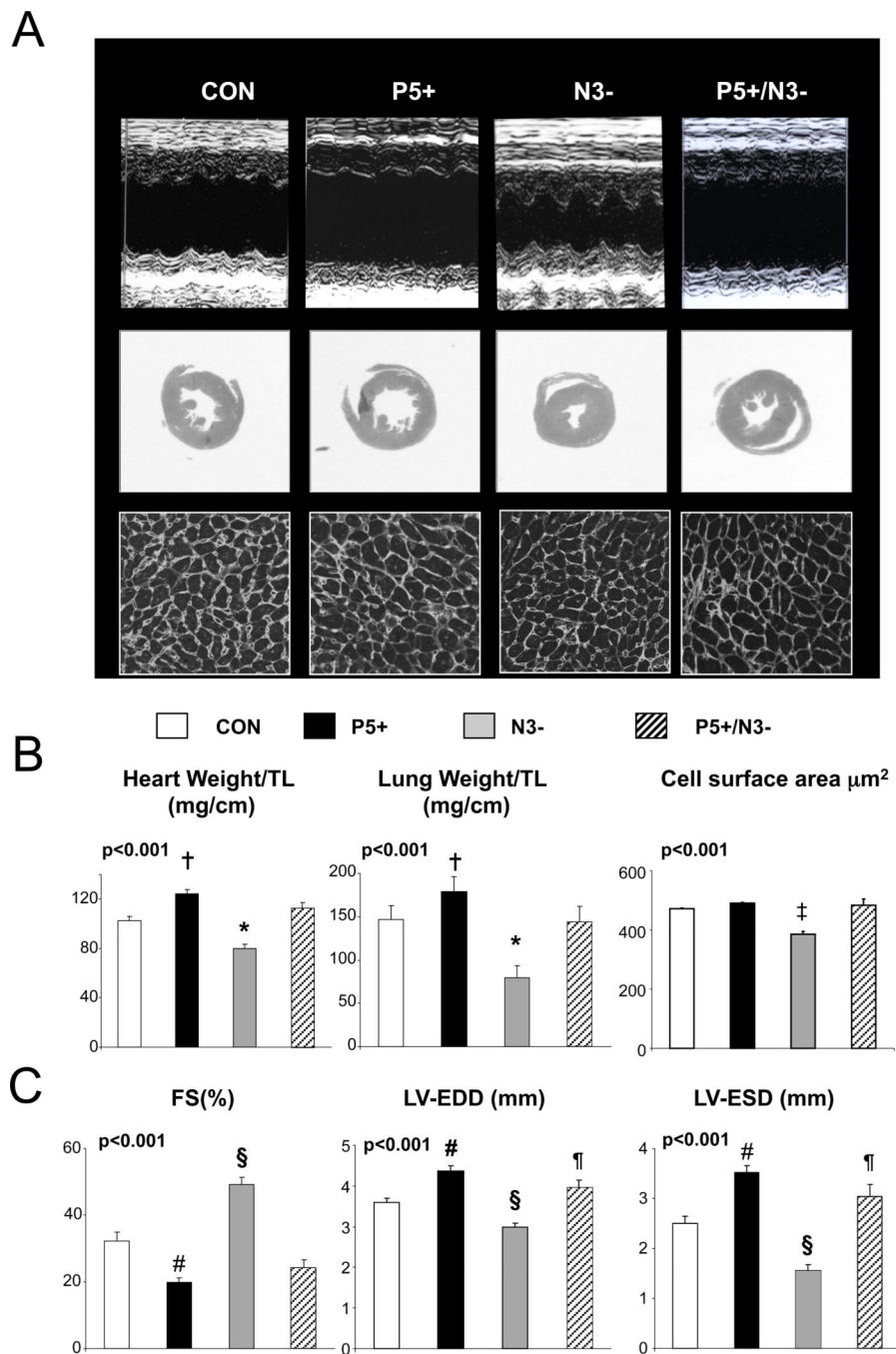


Figure 1. Influence of myocyte PDE5 upregulation with and without NOS3 expression on hearts subjected to trans-aortic constriction (TAC). A) *Top panels* show example M-mode echocardiograms, *Middle panels* show histologic cross-sections (H&E stain) at the mid-chamber level, and *lower panels* show myocyte cross sectional area (WGA stain, average of several hundred cells from multiple views in each heart, n=3–5 hearts per group). B) Summary results for heart and lung weight normalized to tibia length (TL), myocyte cross sectional area, fractional shortening (%FS), and left ventricular end-diastolic and end-systolic short axis dimension (LV-EDD, LVESV) for the four models after TAC (n=25, 15, 27, 15 for each model respectively, left to right). P-values shown at the upper left are results

for either a 1-way ANOVA or non-parametric Kruskal-Wallis test. Symbols above the bars denote results of multiple comparisons test: * $p < 0.05$ vs P5⁺ and P5⁺/N3⁻; † $p < 0.05$ vs control (Dunn's test); ‡ $p < 0.01$ vs all other groups; § $p < 0.001$ vs all other groups; # $p < 0.05$ vs CON; and ¶ $p < 0.05$ versus P5+; all Tukey-Kramer test.

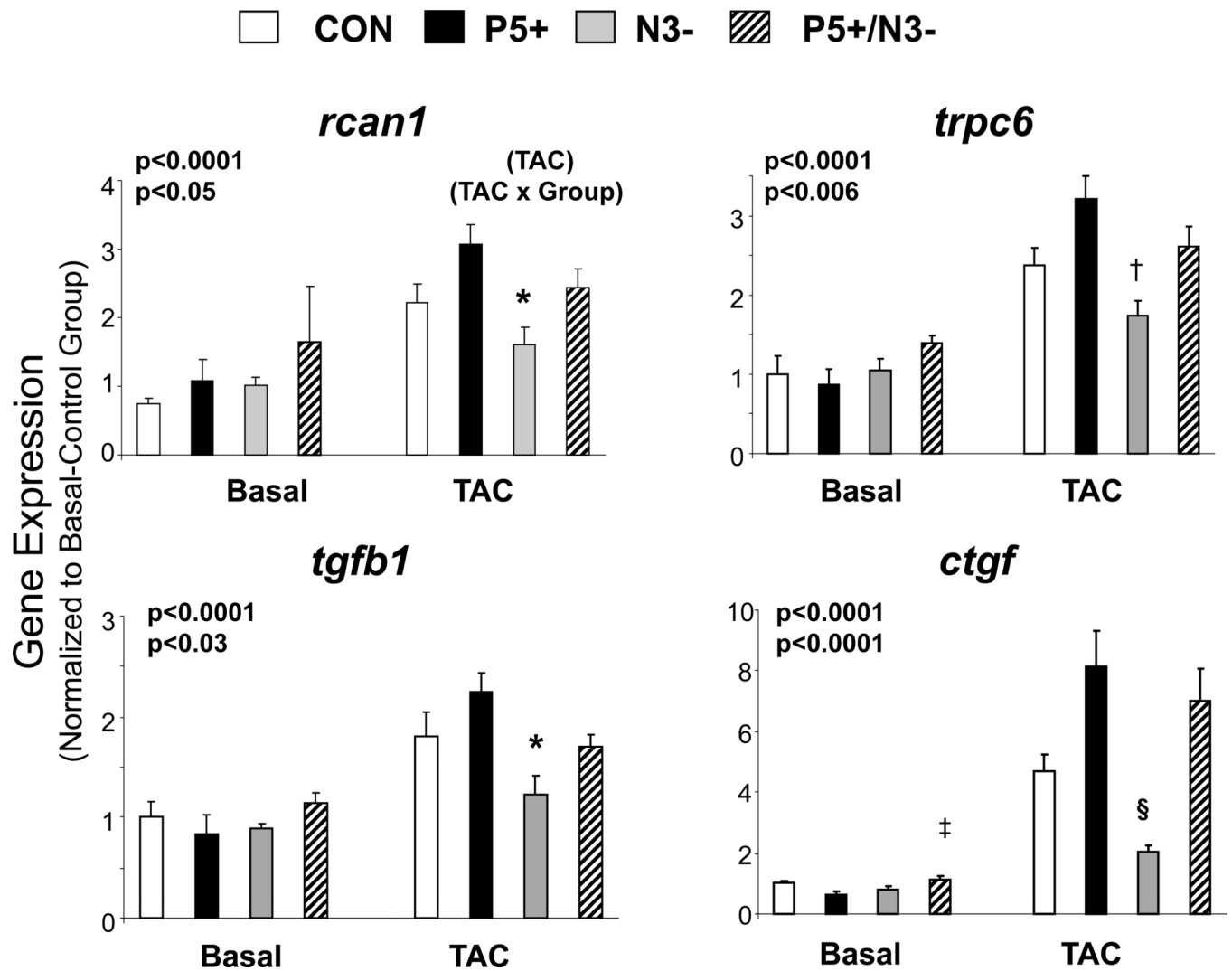
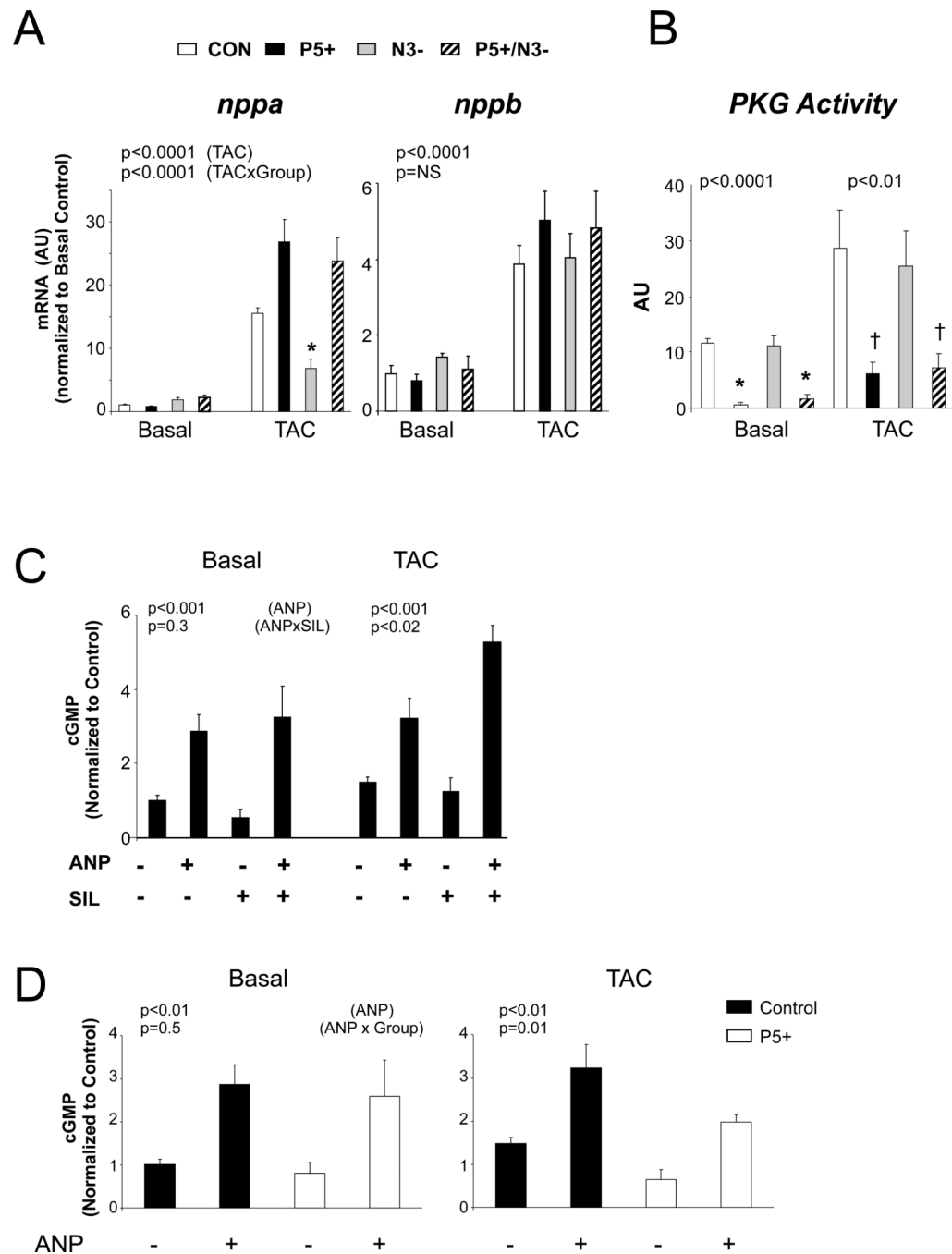


Figure 2.

Impact of myocyte PDE5 upregulation with or without NOS3 expression on myocardial genes coupled to hypertrophy/fibrosis signaling in hearts at rest and after TAC. Rcan-1 – regulator of calcineurin, trpc6 – transient receptor potential canonical channel 6, tgfb1 – transforming growth factor beta, ctgf – connective tissue growth factor. P values are for a combined 2-way ANOVA, with presence or absence of TAC and the experimental group being the two factors. Results for the influence of TAC (upper p-value) and interaction between TAC and group (lower value) are provided. Individual symbols are results of post-hoc Tukey-Kramer MCT. * $p < 0.01$ vs P5⁺, † $p < 0.002$ vs P5⁺, ‡ $p < 0.05$ vs P5⁺/N3⁻, § $p = 0.02$ vs P5⁺; § $p < 0.001$ vs P5⁺ and P5/N3. N=4–8 samples for each group and time point.

**Figure 3.**

PDE5 is retargeted to NP-derived pools in hearts subjected to TAC. A) mRNA expression for ANP (*nppa*) and BNP (*nppb*) genes at basal state and after TAC in four models. Both were markedly upregulated by TAC ($p < 0.001$) particularly in models with upregulated PDE5. $N3^-$ alone showed a blunted ANP response. $N=3-8/\text{group}$. P-values are results of a 2-way ANOVA, top value showing the effect from TAC effect, and the lower value the interaction of TAC \times experimental group. As in the prior figures, symbols denote results of multiple comparisons test. * $p < 0.05$ versus $P5^+$ and $P5/N3$. B) PKG activity increases with TAC, but is suppressed in hearts with enhanced PDE5 levels with or without NOS3 expression. $N=4-5/\text{group}$. P-values for 1-way ANOVA within each group (CON, TAC). *

p=0.01 versus CON and N3⁻, † p<0.05 versus CON and N3⁻ by Kruskal Wallis test. C) cGMP in myocardium from hearts exposed to ANP, sildenafil (SIL), or both. N=3–9/group. P-values from 2-way ANOVA: top value for effect from ANP, lower: ANP × SIL interaction. In sham controls, ANP increased cGMP similarly with or without SIL, whereas after TAC, the ANP+SIL response was greater than with ANP alone. D) cGMP in myocardium from CON or P5⁺ hearts before and after exposure to ANP, at basal condition and after TAC. P-values 2-way ANOVA: top ANP effect, bottom, ANP × Genotype interaction. N=3–9/group.

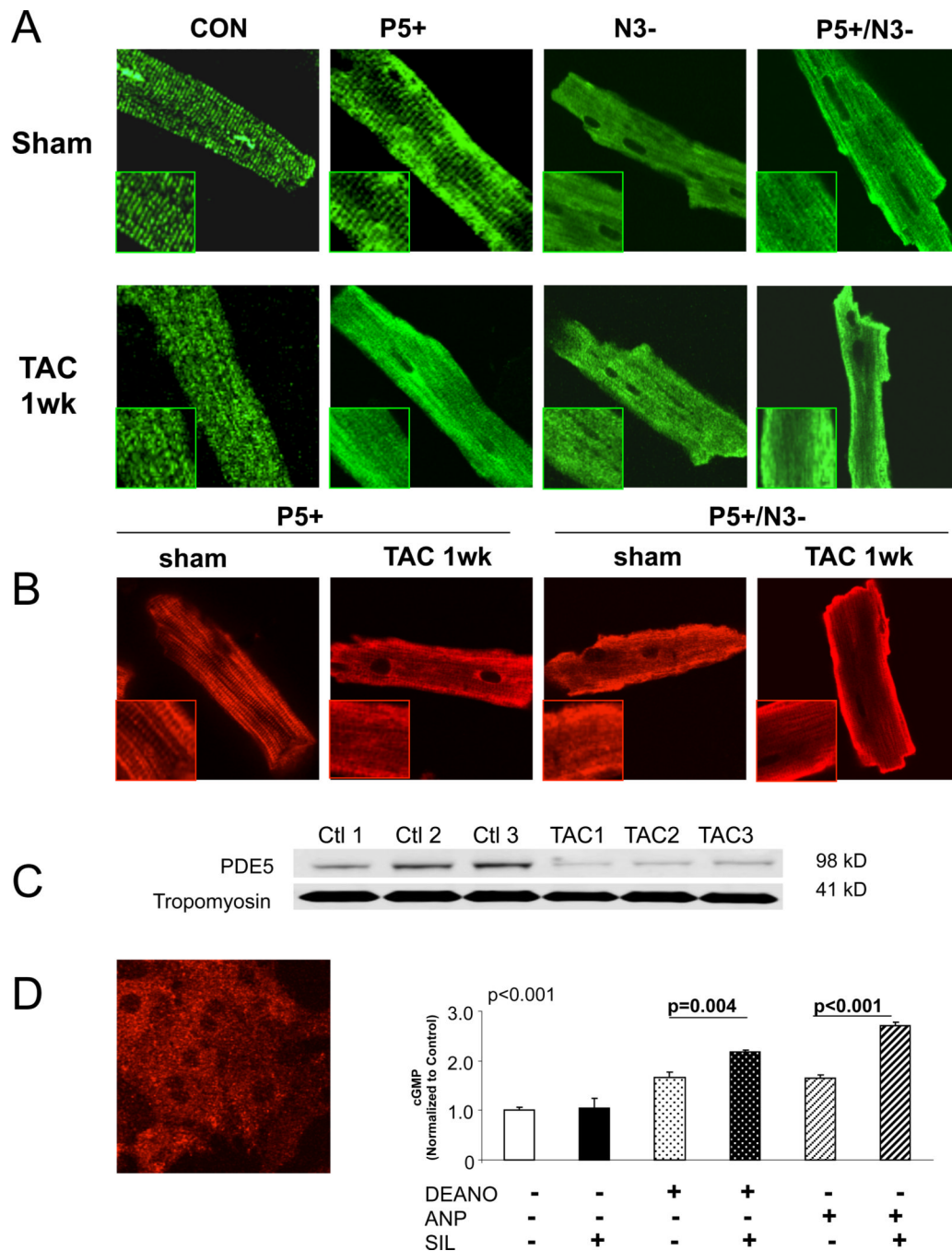


Figure 4. Altered myocyte PDE5a localization in myocytes from pressure-overload hearts. A) confocal images of myocytes from each experimental group at baseline and after TAC, staining for PDE5a. Control and P5⁺ cells show striated localization at rest, but diffuse distribution after TAC. Models lacking NOS3 show a diffuse pattern of staining before and after TAC. B) confocal images from P5⁺ and P5/N3 cells before and after TAC, using Flg Antibody to detect transgene only. This shows similar change in localization after TAC. C) Gel electrophoresis from myofibrillar sub-fraction probed with PDE5, and tropomyosin as a loading control. With TAC, there is a decline in PDE5 expression in this subfraction. D) PDE5 is diffuse in neonatal myocytes (left), and this is coupled to an enhancement of both

an ANP or NO-stimulated cGMP response (right). P values for unpaired t-test between groups.

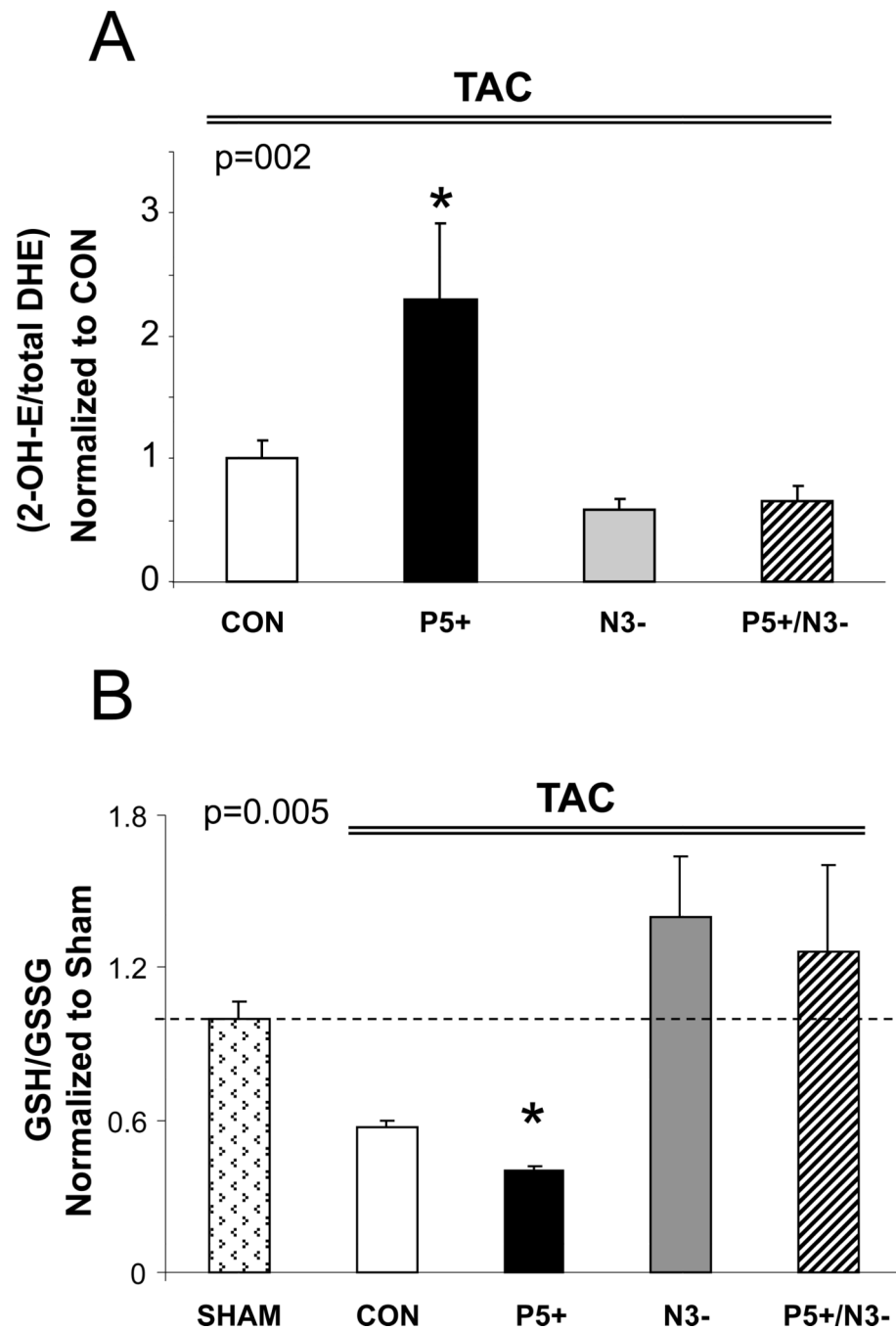


Figure 5. Enhanced PDE5 expression increases myocardial oxidative stress, but this is diminished in hearts lacking NOS3. A) Myocardial superoxide was detected by 2-OH-ET, and increased in P5⁺ tissue compared to control. Both N3⁻ and P5/N3 myocardium showed a similar and much lower level. * $p < 0.02$ vs all other groups. (n=8–10/group) B) Ratio of GSH/GSSG in the four models. The ratio was reduced most in P5⁺ (* $p < 0.05$ versus Sham) consistent with increased oxidative stress, but was at control levels in the two models lacking NOS3 (n=3–4/group). P values in graph are for 1-way ANOVA between groups.