Differential Expression of PDE5 in Failing and Nonfailing Human Myocardium

Xiaoyin Shan, PhD; Michael P. Quaile, PhD; Jeffery K. Monk, BS; Benjamin French, PhD; Thomas P. Cappola, MD; Kenneth B. Margulies, MD

Background—Recognizing that inhibitors of phosphodiesterase type 5 (PDE5) are increasingly employed in patients with pulmonary hypertension and right ventricular (RV) failure, we examined PDE5 expression in the human RV and its impact on myocardial contractility.

Methods and Results—Tissue extracts from the RV of 20 patients were assayed for PDE5 expression using immunoblot and immunohistochemical staining. Tissues were selected from groups of nonfailing organ donors and transplant recipients with endstage ischemic cardiomyopathy or idiopathic dilated cardiomyopathy. Among dilated cardiomyopathy patients, subgroups with mild or severe RV dysfunction and prior left ventricular assist devices were analyzed separately. Our results showed that PDE5 abundance increased more than 4-fold in the RVs of the ischemic cardiomyopathy compared with the nonfailing group. In dilated cardiomyopathy, PDE5 upregulation was more moderate and varied with the severity of RV dysfunction. Immunohistochemical staining confirmed that cardiac myocytes contributed to the upregulation in the failing hearts. In functional studies, PDE5 inhibition produced little change in developed force in RV trabeculae from nonfailing hearts but produced a moderate increase in RV trabeculae from failing hearts.

Conclusions—Our results showed the etiology- and severity-dependent upregulation of myocyte PDE5 expression in the RV and the impact of this upregulation on myocardial contractility. These findings suggest that RV PDE5 expression could contribute to the pathogenesis of RV failure, and direct myocardial responses to PDE5 inhibition may modulate the indirect responses mediated by RV afterload reduction. (Circ Heart Fail. 2012;5:79-86.)

Key Words: pde5 ■ cgmp ■ heart failure ■ myocardium ■ contractility

Phosphodiesterases (PDE) are a superfamily of enzymes that hydrolyze cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) to AMP and GMP, respectively. Both cyclic nucleotides are essential second messengers in regulating the contraction and relaxation of vascular smooth muscle cells and cardiac myocytes.1 CGMP exerts its functions through downstream effectors, including protein kinase G, cGMP-binding proteins, and cyclic nucleotide-gated channels.2,3 PDE5 is a subtype of PDEs that preferentially hydrolyzes cGMP. The functional enzyme exists as a homodimer with each monomer containing both regulatory cGMP-binding GAF domains and a catalytic domain.4 Previous studies have shown that PDE5 activity can be regulated through phosphorylation and cellular cGMP levels.5–7 PDE5 has been shown to exist in many tissue types with high abundance in smooth muscle.8 By hydrolyzing cGMP, PDE5 modulates the cellular level of the secondary messenger that critically regulates the tone of the smooth muscle, and PDE5 inhibitors are increasingly employed as therapy for ameliorating pathological increases in pulmonary arterial resistance.9

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While the function of PDE5 in the pulmonary vasculature has been well-characterized, the physiological roles of myocardial PDE5 expression are more obscure, partially because of the much lower PDE5 mRNA expression in the myocardium.10 In animal studies using PDE5 inhibitors to explore cardiac functions of PDE5 and its potential as a drug target in treating cardiac hypertrophy,11–16 the effects of PDE5 inhibition have been inconsistent: beneficial under certain hypertrophic circumstances but not under the others.11,14–16 Also, the beneficial effects of PDE5 inhibition tend to be sensitive to the magnitude of cardiac stresses used in creating the animal models.13 In humans, 2 previous studies have reported increased PDE5 abundance in the left ventricle (LV) of patients with heart failure,17,18 and 1 study found increased right ventricular (RV) PDE5 in the hearts of neonates and young children with congenital heart anomalies.14 In each study, the impact on cardiac function has been explored in animal models, with increased PDE5 expression tending to depress myocardial...
contractile function. Inhibition of PDE5 has been reported to be beneficial for patients with pulmonary arterial hypertension who are prone to RV failure and for patients with stable systolic heart failure.9,19 PDE5 inhibitors are pulmonary vasodilators and may augment RV function via reduced afterload; however, a direct inotropic action of PDE5 inhibitors on human myocardium has not been established at this point. Also, the effects of disease severity and etiologies of cardiomyopathy on PDE5 expression have not been explored to date. Accordingly, the present study examined PDE5 expression in the RV of hearts with severe ischemic cardiomyopathy (ICM) and nonischemic dilated cardiomyopathy (DCM) with mild or severe RV dysfunction. Our data support a functionally significant increase in PDE5 abundance in failing RV myocardium. These findings indicate direct myocardial effects of PDE5 inhibitors during their in vivo administration to patients with RV failure and support a possible therapeutic role in the absence of increases in RV afterload.

Methods

Chemicals
All chemicals used were purchased from Sigma-Aldrich (Saint Louis, MO) unless otherwise indicated.

Human Tissue Procurement
The Institutional Review Board at the University of Pennsylvania approved the use of human tissues in this study, and all patients provided prospective informed consent. RV tissues were obtained from explanted nonfailing (NF) and failing human hearts at the time of organ donation or heart transplantation. High potassium cardioplegia was administered immediately before or after cardiectomy, and hearts were promptly transported to the laboratory on wet ice where they were immediately processed for molecular, histological, or physiological characterization.

Echocardiography
Quantification of 2-dimensional and Doppler echocardiographic data (including left heart chamber dimensions, LV ejection fraction, and

### Table. Patient Characteristics

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RVD Grade indicates right ventricular dysfunction grade; Est PA Syst, estimated pulmonary artery systolic pressure; TR Grade, tricuspid regurgitation grade; LVEDD, left ventricular end diastolic diameter; LVEF, left ventricular ejection fraction; MR Grade, mitral regurgitation grade; LVAD Dur, duration of left ventricular assist device implementation; A, amiodarone; ACE, angiotensin-converting enzyme inhibitors; ARB, angiotensin II receptor blockers; BB, beta-adrenergic blocking agents; D, diuretic; Dig, digoxin; H, hydralazine; Mil, milrinone; N, nitrates; S, statins; W, warfarin.
the degree of mitral and tricuspid regurgitation) were performed in the standard manner. RV function was quantified by measuring the tricuspid annular plane systolic excursion as described previously. Tricuspid annular plane systolic excursion values <1.5 cm were considered severe RV dysfunction, values 1.5 to 1.8 cm were considered moderate RV dysfunction, values 1.81 to 2.1 cm were considered mild RV dysfunction, and values >2.1 cm were considered normal. The Doppler estimation of pulmonary artery systolic pressure was determined using the peak TR velocity in the standard manner.

Protein Extraction and Immunoblot Analysis

About 100 mg frozen human RV and LV tissues were minced briefly into small chunks and homogenized using the FastPrep System (BIO 101, Inc, Vista, CA) with 3 mm zirconium beads (OPS Diagnostics, LLC, Lebanon, NJ) in 20 mmol/L Tris pH7.5/100 mmol/L NaCl/1 mmol/L EDTA/1 mmol/L PMSF/ protease inhibitor cocktail (Sigma). Triton X-100 was added to the homogenates to the final concentration of 1% and incubated on ice for 30 minutes. At the end of the incubation, the homogenates were centrifuged at 13 kG force at 4°C for 15 minutes. The supernatants were collected, and the protein content in each sample was determined, using the BCA assay (Pierce, Rockford, IL). An equal amount of protein from each sample was used for SDS-PAGE. PDE5 and GAPDH expression levels in the samples were assayed according to a standard immunoblotting procedure, using a polyclonal rabbit anti-PDE5 antibody (Cell Signaling Technology, Danvers, MA) and a polyclonal rabbit anti-GAPDH antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). ECL substrates (GE Healthcare, Piscataway, NJ) were used to

Figure 1. Phosphodiesterase type 5 (PDE5) expression in right ventricular (RV) tissues of ischemic cardiomyopathy (ICM), dilated cardiomyopathy (DCM), and nonfailing (NF) human hearts. Immunoblot analysis was carried out to examine PDE5 protein expression levels in the right ventricular (RV) tissue extracts using an anti-PDE5 antibody. In each blot, a group of NF samples was included as a control for the disease groups, and abundance of GAPDH was also determined to control for total protein content in each sample. A, DCM with mild RV dysfunction versus NF hearts; B, DCM with severe RV dysfunction with or without antecedent left ventricular (LV) assist device support versus NF hearts; and C, ICM versus NF hearts. In B, D, and F, GAPDH-normalized PDE5 protein levels, measured by densitometry of protein bands in the blots, were used to compare the relative abundance of the protein in the samples. Sample orders in each group are listed in Table 1.
Muscle chamber was incremented gradually to 1.75 mmol/L CaCl₂.

4 patients with an LVAD still required milrinone for RV medications at the time of their transplantation. Of note, 3 of who provided heart tissue received multiple cardiovascular time of tissue procurement. Each of the transplant recipients organ donors had normal RV and LV function based on RVD, and DCM with severe RVD, DCM with mild RV dysfunction (RVD), DCM with severe DCM with mild RV dysfunction. NF control hearts from DCM with severe pre-explant support grouped into 5 categories: NF, ICM, endstage idiopathic DCM with mild RV dysfunction. Isometric twitches were recorded before and after administration of the PDE5 inhibitor, MY-5445, at 10 μmol/L.

Immunohistochemistry
Isolated human RV tissues were immediately fixed in 4% formaldehyde, followed by paraffin-embedding. Immunohistochemical staining was carried out using the ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Briefly, sections were deparaffinized and rehydrated. A citrate buffer, 10 mmol/L, citric acid, 0.05% Tween 20, pH 6.0, was used to unmask antigens at 100°C for 20’, and the endogenous peroxides was quenched by incubating the sections in PBS/2% H₂O₂ for 15’ at resistance training. After blocking with 10% normal goat serum/ PBS/0.05% T-20, PDE5 and myosin heavy chain expression in the sections were detected with antibodies against the proteins. The rabbit polyclonal anti-PDE5 was from Abcam (Cambridge, MA), and the mouse monoclonal antimyosin heavy chain antibody was from Developmental Studies Hybridioma Bank (Iowa City, IA). Horseradish-peroxidase–conjugated secondary antibodies to the rabbit and mouse were from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). DAB was used to visualize myosin heavy chain staining, and hematoxylin was used for nuclei counterstaining (Vector Laboratories) while DAB-Ni was used to visualize PDE5 staining. Background controls employed only the secondary antibodies.

Contractile Response to PDE5 Inhibition
Without antecedent fixation or freezing, thin subendocardial myocardial trabeculae were isolated from the RV free wall and mounted in a slackened position in a custom-designed isometric muscle chamber (Scientific Instruments, Heidelberg, Germany), as previously described. The Ca²⁺ concentration of physiological buffer within the muscle chamber was incremented gradually to 1.75 mmol/L, CaCl₂. After a 60-minute equilibration period, acute studies were conducted at 37°C and at a stimulation frequency of 0.5 Hz. Experimental trabeculae length (L) was set to 80% of the difference between L₀ and Lₘₐₓ. Isometric twitches were recorded before and after administration of the PDE5 inhibitor, MY-5445, at 10 μmol/L.

Statistical Methods
For immunoblots, PDE5 levels normalized to GAPDH were compared between patient groups using Kruskal-Wallis and Mann-Whitney tests, and correlation between levels in LV and RV was assessed using Spearman rank correlations. For muscle strip experiments, linear mixed-effects models with a group-by-time interaction term were used to compare mean contractile parameters before and after PDE5 inhibition in NF and failing hearts. Strip-specific and subject-specific random intercepts were used to account for the temporal correlation of responses within strips over time and the correlation because of clustering of multiple strips within a subject. All analyses were completed using Stata 11.0 (StataCorp LP, College Station, TX).

Results
Table summarizes the clinical characteristics of individuals providing myocardial tissue for these studies. Subjects were grouped into 5 categories: NF, ICM, endstage idiopathic DCM with mild RV dysfunction (RVD), DCM with severe RVD, and DCM with severe RVD and pre-explant support with an LV assist device (LVAD). NF control hearts from organ donors had normal RV and LV function based on echocardiography with minimal medication exposure at the time of tissue procurement. Each of the transplant recipients who provided heart tissue received multiple cardiovascular medications at the time of their transplantation. Of note, 3 of 4 patients with an LVAD still required milrinone for RV support at the time of their transplants. Among the patients with ischemic cardiomyopathy, the severity of RV dysfunction varied from mild to severe.

Expression Levels of PDE5 in the RV
To understand the regulation of PDE5 expression in response to cardiomyopathies, we examined PDE5 protein levels in the RV of hearts grouped according to the 5 categories listed above. Extracts from these tissues were subjected to immunoblot analysis (Figure 1A, 1C, and 1E) using antibody against PDE5 (see Methods for details). GAPDH level in each sample was also measured by immunoblot and used as a reference for normalization. A set of NF samples were loaded on each gel to allow the correction of gel-to-gel variations. The PDE5 level in each sample was obtained by

Figure 2. Phosphodiesterase type 5 (PDE5) expression in different categories of failing human hearts. A, Right ventricular (RV) PDE5 abundance was elevated in heart failure compared with NF controls; Mann-Whitney P = 0.003. B, Comparison of different RV failure subgroups suggests heterogeneity in the degree of PDE5 elevation in the failing RV, depending on the degree of RV failure; Kruskal-Wallis P = 0.004. Data are in arbitrary units normalized to GAPDH abundance (see Figure 1). PDE5 expression level in the left ventricular (LV) was determined in the same manner as that of the RV. The GAPDH-normalized PDE5 levels in paired RV and LV samples from each heart was plotted in panel C, demonstrating a lack of correlation of RV and LV PDE5 expression levels; Spearman rho = 0.04; P = 0.85.
normalizing the band intensity of PDE5 with the band intensity of GAPDH (Figure 1B, 1D, and 1F). A comparison of PDE5 levels in the NF and failing group, including all etiologies, is shown in Figure 2A. PDE5 levels in each disease subgroup and NF group are shown in Figure 2B.

As shown in Figure 1 and 2, our results revealed that there is a significant change in PDE5 protein level among the failing hearts compared to NF. Specifically, PDE5 expression increased in the RV of ICM group compared with the NF group (4.6-fold and $P<0.001$, $n=9$ for ICM group and $n=4$ for the NF group). For the DCM group, a 2-fold increase in PDE5 level was observed when RV dysfunction was mild ($P<0.008$, $n=4$ for each group); when RV dysfunction was severe, a bigger increase in PDE5 expression was detected (3-fold and $P<0.003$, $n=4$ for each group); however, for DCM hearts with LVAD support, PDE5 expression in the RV was not statistically different from that of the NF group.

Comparison of different RV failure subgroups suggests heterogeneity in the degree of PDE5 elevation in the failing RV, depending on the degree of RV failure (Kruskal-Wallis, $P=0.004$). Interestingly, across all samples and subgroups, PDE5 abundance in the RV was not statistically different from that of the NF group. Comparison of different RV failure subgroups suggests heterogeneity in the degree of PDE5 elevation in the failing RV, depending on the degree of RV failure (Kruskal-Wallis, $P=0.004$). Interestingly, across all samples and subgroups, PDE5 abundance in the RV was not correlated with PDE5 abundance in the LV (Figure 2C). These results suggest that responses of PDE5 expression to cardiomyopathy were etiology-dependent, with ICM having more impact on the PDE5 expression level than DCM. LVAD support did not appear to reduce the RV PDE5 expression associated with severe RVD.

Localization of PDE5 in the Myocardium
The adult myocardium is composed of various cell types, including cardiac myocytes, fibroblasts, endothelial cells, and vascular smooth muscle cells. The endothelial and smooth muscle cells are known to express PDE5. To assess if cardiac myocytes contributed to the observed change of the PDE5 protein level in the RV tissue from hearts of ICM and DCM with mild RVD, we carried out immunohistochemical staining of PDE5 in paraffin-embedded tissue sections of RV fixed immediately after procurement (Figure 3A). Polyclonal rabbit anti-PDE5 antibody was used to detect the presence of the protein in the tissue. Monoclonal mouse antisarcomeric myosin heavy chain antibody was used to identify cardiac myocytes in consecutive sections (Figure 3C).

As shown in Figure 3A, vascular PDE5, a positive control, can be readily detected (see solid arrows) in the RVs of NF, DCM, and ICM. In the area primarily occupied by myocytes, staining of comparable intensity, as that of vasculature, can be observed in the ICM sample. The connective tissue regions remain unstained (see open arrows), demonstrating the specificity of the staining assay. In the case of DCM from a heart with mild RVD, the PDE5 staining in the myocyte region is less intense compared with that of vasculature and lower in frequency compared with the ICM. This corresponds well with the observed PDE5 protein levels in ICM and DCM tissue samples by immunoblot. In the NF tissue, PDE5 staining is most intense in the vasculature; myocyte PDE5 staining intensity is considerably less, suggesting relative less contribution from myocyte PDE5 expression. The expression of PDE5 in cardiomyocytes was also shown by previous studies of Nagendran and colleagues and Pokreisz and colleagues. Taken together, the data indicate that more myocytes from the RV of ICM and DCM express PDE5.
protein than from RV of NF and that PDE5 from cardiac myocyte thus contributes substantially to the total tissue PDE5 content. Furthermore, the intensity of immunohistochemical staining among different samples correlated well with that observed with immunoblotting, suggesting that the difference in PDE5 protein levels between NF and ICM or DCM hearts can be attributed, at least in part, to the differences in the respective myocytes.

**PDE5 Inhibition Increases Contractility in Isolated RV Trabeculae**

We next examined whether PDE5 directly modulates contractility in RV trabeculae obtained from 3 ICM, 1 DCM, and 2 NF human hearts, with multiple trabeculae isolated from each RV using a PDE5 inhibitor, MY-5445. As shown in Figure 4, PDE5 inhibition had a negligible effect on the isometric force generation in trabeculae from the NF RV (Figure 4A, 4C); however, a positive inotropic effect was evident in trabeculae from the failing RV compared with the baseline (Figure 4B, 4C). Diastolic force, already elevated in the failing trabeculae, tended to increase further with PDE5 inhibition but decreased in the NF trabeculae (Figure 4D). The greater effect of PDE5 inhibition in the failing heart was also reflected in the rate of force development (Figure 4E) and force decline (Figure 4F). The association of increased contractile effects because of PDE5 inhibition with the differential PDE5 expression in the ICM myocardium suggests that the upregulated PDE5 in the failing tissues is a functionally important regulator of basal contractility in these hearts.

**Discussion**

PDE5 is a cGMP-specific PDE that plays an important role in regulating cellular cGMP levels. The present study demonstrates that RV PDE5 abundance is increased in failing human hearts compared with NF myocardium. Increased PDE5 expression was most apparent in failing hearts from patients with ICM where RV myocardium exhibited more than 4-fold increases in PDE5 protein abundance. Localization...
tion of PDE5 via immunohistochemical staining demonstrated that increased expression was likely a result of increases in cardiac myocyte PDE5, with vascular smooth muscle PDE5 expression providing background levels in both NF and failing hearts. Among individuals with non-ICM, degrees of PDE5 upregulation in the RV seem to be dependent on the severity of RV dysfunction. Our studies showing differential responses to acute PDE5 inhibition in isolated RV trabeculae provides the first direct evidence that increased PDE5 expression in failing human myocardium may modulate RV contractility independent of preload or afterload.

By selecting samples from different disease groups and examining the expression of PDE5 in the RV, we obtained insights into factors regulating PDE5 expression in the human myocardium. Unlike previous human studies focusing only on non-ICM,\textsuperscript{14,17,18} our comparison of ICM versus non-ICM revealed significantly greater RV PDE5 expression in ICM. Moreover, the presence of variable degrees of RV dysfunction provided an opportunity to demonstrate that the severity of RV dysfunction affects the degree of RV PDE5 upregulation among patients with DCM. As with previous studies,\textsuperscript{14} immunohistochemical staining demonstrated that increased overall myocardial PDE5 abundance in failing hearts was likely due to increased cardiac myocyte PDE5 expression with relatively unchanged expression in vascular smooth muscle. Thus, our data are generally in agreement with the result of earlier studies but reveals further potential factors that may contribute to the regulation of PDE5 expression during disease progression.

Taken together, intergroup comparisons indicate that the etiology and severity of RV cardiomyopathy affect PDE5 expression while LVAD support does not significantly alter RV PDE5 expression. Accordingly, it is reasonable to speculate that 1 or more of the many hypertrophic signaling pathways activated in the failing myocardium\textsuperscript{24} directly or indirectly contribute to increased PDE5 expression in the failing RV. For example, recent studies by Mokai and colleagues\textsuperscript{25} demonstrated that angiotensin II infusion induces increased PDE expression (including PDE5) during the early phases of myocardial hypertrophy, while other studies have linked oxidative stress to increased myocardial PDE5 expression.\textsuperscript{18}

Previously, no direct association between the upregulation of PDE5 and contractility alteration in human tissues have been reported. In studies that examined PDE5 upregulation in failing human myocardium, animal models were used to understand the impact on tissue functions.\textsuperscript{14,17,18} Assessing impacts of the upregulation on human tissue could, to a certain degree, verify the relevance of the results from the animal studies, given the reported difference between human and animal in metabolizing cGMP.\textsuperscript{26} Our results reported here showed that PDE5 inhibitor MY5445 acutely increases the contractility of muscle strips isolated from the failing hearts independent of effects on preload or afterload. Our finding that acute PDE5 inhibition increases contractility and prior studies indicating that PDE5 inhibition protects against cardiac hypertrophy in animal models\textsuperscript{14,19,27,28} support the possibility that increased PDE5 expression may exacerbate RV dysfunction and hypertrophy in the failing heart. Important caveats concerning this possibility relate to difference in time frame and compensatory responses in our studies versus clinical trials. For example, our data demonstrate functional actions of acute PDE5 inhibition during an interval of less than 1 hour while clinical trials examine responses over much longer intervals. With respect to compensatory responses, a strength of using isolated myocardial trabeculae is that intrinsic contractile responses to PDE5 can be assessed without potentially confounding changes in preload, afterload, perfusion, or neurohormonal influences. In vivo, these influences may change and shape the overall myocardial response to PDE5 inhibition.

Together, our findings suggest that PDE5 inhibition in patients with pulmonary arterial hypertension may have both direct (via inotropy) and indirect (via reduced afterload) positive effects on RV contractility. Such findings also suggest that upregulated myocardial PDE5 expression may contribute to the progression of heart failure and support an emerging therapeutic role for PDE5 inhibitors for both RV and LV failure.\textsuperscript{19}

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Disclosures
None.

References

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CLINICAL PERSPECTIVE

The prognosis of patients with pulmonary artery hypertension is closely linked to the ability of the right ventricle (RV) to adapt to the increased vascular load. Even among patients with left ventricular dysfunction, the presence and severity of RV dysfunction are powerful predictors of adverse prognosis. In pulmonary artery hypertension, pulmonary vasodilators (including endothelin antagonists, prostacyclin analogues, and inhibitors of phosphodiesterase 5 [PDE5]) have each demonstrated efficacy in clinical trials. The ongoing Phosphodiesterase 5 Inhibition to Improve Quality of Life And EXercise Capacity in Diastolic Heart Failure (RELAX) Trial is exploring the therapeutic potential of PDE5 antagonism as a treatment for heart failure with preserved left ventricular ejection fraction. In this context, the present studies demonstrating severity-dependent increases in RV PDE5 expression indicate that PDE5 inhibitors may directly modulate myocardial NO/cyclical guanosine monophosphate signaling in the failing RV in addition to their indirect benefits via reduced RV afterload. Though we observed moderate acute positive inotropic effects of PDE5 inhibition in RV trabeculae from failing human hearts, the biology of myocardial NO/cyclical guanosine monophosphate signaling suggests that myocardial PDE5 inhibition should also promote cardioprotective effects that may be particularly relevant to the RV. If verified by further experimentation, targeting upregulated PDE5 in the RV might be appropriate even in the absence of elevated pulmonary vascular resistance.