Published in final edited form as:

J Mol Cell Cardiol. 2013 November ; 64: . doi:10.1016/j.yjmcc.2013.08.003.

Cyclic Nucleotide Phosphodiesterase 3A1 Protects the Heart Against Ischemia-Reperfusion Injury

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Abstract

Phosphodiesterase 3A (PDE3A) is a major regulator of cAMP in cardiomyocytes. PDE3 inhibitors are used for acute treatment of congestive heart failure, but are associated with increased incidence of arrhythmias and sudden death with long-term use. We previously reported that chronic PDE3A downregulation or inhibition induced myocyte apoptosis *in vitro*. However, the cardiac protective effect of PDE3A has not been demonstrated *in vivo* in disease models. In this study, we examined the role of PDE3A in regulating myocardial function and survival *in vivo* using genetically engineered transgenic mice with myocardial overexpression of the PDE3A1 isozyme (TG). TG mice have reduced cardiac function characterized by reduced heart rate and ejection fraction ($52.5 \pm 7.8\%$ vs. $83.9 \pm 4.7\%$) as well as compensatory expansion of left ventricular diameter (4.19 ± 0.19 mm vs. 3.10 ± 0.18 mm). However, there was no maladaptive increase of fibrosis and apoptosis in TG hearts compared to wild type (WT) hearts, and the survival rates also remained the same. The diminution of cardiac contractile function is very likely attributed to a decrease in beta-adrenergic receptor (β -AR) response in TG mice. Importantly, the myocardial infarct size ($4.0 \pm 1.8\%$ vs. $24.6 \pm 3.8\%$) and apoptotic cell number ($1.3 \pm 1.0\%$ vs. $5.6 \pm 1.5\%$) induced by ischemia/reperfusion (I/R) injury were significantly attenuated in TG mice. This was associated with decreased expression of inducible cAMP early repressor (ICER) and increased expression of anti-apoptotic protein BCL-2. To further verify the anti-apoptotic effects of PDE3A1, we

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6. Conflict of interest statement: None

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performed *in vitro* apoptosis study in isolated adult TG and WT cardiomyocytes. We found that the apoptotic rates stimulated by hypoxia/reoxygenation or H₂O₂ were indeed significantly reduced in TG myocytes, and the differences between TG and WT myocytes were completely reversed in the presence of the PDE3 inhibitor milrinone. These together indicate that PDE3A1 negatively regulates β -AR signaling and protects against I/R injury by inhibiting cardiomyocyte apoptosis.

Keywords

PDE3A; transgenic mice; myocardial injury; myocyte apoptosis; myocyte contractility

1. Introduction

cAMP regulates a wide variety of cellular functions in cardiomyocytes, from short-term cell contraction/relaxation to long-term cell growth/survival. Interestingly, increasing evidence indicates that there are multiple specially, temporally, and functionally distinct cAMP signaling domains in cardiomyocytes. β -AR mediated cAMP is one of the most well characterized signaling pathways, which plays a fundamental role in physiologically regulating cardiac performance by increasing heart rate as well as myocardial contractility and relaxation when demand for cardiac output increases (such as during exercise, fight-or-flight responses, or injury). Acute cAMP elevation upon β -AR stimulation activates protein kinase A (PKA). PKA subsequently phosphorylates and activates multiple substrates that influence contraction/relaxation of cardiomyocytes by regulating Ca²⁺ transients and contractile protein phosphorylation, including sarcolemmal Ca²⁺ channels, cardiac RyR2, phospholamban (PLB), and the contractile proteins such as troponin I (TnI) and troponin C (TnC) [1, 2]. However, chronic activation of β -AR, particular β 1-AR/cAMP signaling, appears to be detrimental through promoting pathological cardiac remodeling [3, 4].

Cyclic nucleotide phosphodiesterases (PDEs), by catalyzing the hydrolysis of cyclic nucleotides, play very important roles in regulating the amplitude, duration, and compartmentalization of intracellular cyclic nucleotide signaling. More than 60 different PDE isoforms encoded by 21 different genes have been identified and grouped into eleven broad families (PDE1-PDE11) [5]. PDE3 activity represents one of the major cAMP-PDEs in the human heart [6]. PDE3 inhibitors such as milrinone have been used clinically to treat congestive heart failure due to combined positive inotropic and peripheral vasodilatory effects of PDE3 inhibition [7, 8]. However, with long-term therapy, there is an increase in mortality, primarily as a result of arrhythmias and sudden death [9, 10]. The molecular mechanism(s) responsible for the chronic detrimental effects of PDE3 inhibition still remain poorly understood. PDE3 family members are encoded by two different genes, *PDE3A* and *PDE3B*. Both PDE3A and PDE3B are reported in cardiomyocytes. Recent experimental evidence from global PDE3A and PDE3B knockout mice has revealed that PDE3A but not PDE3B is responsible for the inotropic and chronotropic effects of PDE3 inhibitors [11]. PDE3A regulates cardiac contractility by modulating PLB-SERCA2 activity and subsequent sarcoplasmic reticulum Ca²⁺ uptake [12].

In addition to regulating cardiac hemodynamics, PDE3 might be also important in pathological cardiac remodeling. For example, decreased PDE3A expression has been found in various diseased hearts in human and rodent [13-16]. In cultured neonatal cardiomyocytes, inhibition of PDE3 activity or knockdown of PDE3A expression was associated with myocyte apoptosis, likely through sustained induction of a transcriptional repressor ICER (inducible cAMP early repressor) and thereby inhibition of anti-apoptotic molecule BCL-2 expression [13, 17, 18]. These observations lead to a hypothesis that

PDE3A expression/activity is critical in preventing myocyte apoptosis and cardiac damage. Therefore, in the present study, we investigated the specific role of the PDE3A1 isoform in cardiac function and myocardial injury by generating transgenic mice with cardiac myocyte-targeted overexpression of PDE3A1. Our data reveal that PDE3A1 negatively regulates cardiac chronotropic and inotropic effects, but more importantly, prevents ischemia/reperfusion (I/R)-induced myocardial infarction and myocyte apoptosis.

2. Methods

An expanded Methods section is available in the online Data Supplement

2.1 Transgenic mice

PDE3A1 transgenic mice were generated as we previously described [19]. The transgene was constructed by subcloning rat PDE3A1 cDNA between the 5.5-kb murine α -MHC promoter [20] and the human growth hormone polyadenylation sequence in a pBluescript-based vector developed by J. Robbins (Children's Hospital Research Foundation, Cincinnati, Ohio). Purified transgene fragment was microinjected into pronuclei of fertilized mouse oocytes (performed by University of Rochester Transgenic Core).

2.2. Ischemia/reperfusion injury

All animal procedures were performed in accordance with the National Institute of Health (NIH) and University of Rochester institutional guidelines. Mice were anesthetized with 2.0% isoflurane mixed with 40% oxygen and endotracheal intubation was performed with 20-gauge intravenous catheter. Ischemia was performed by ligating left anterior descending artery (LAD) at 1.5 to 2.0 mm below the tip of the left auricle. Occlusion of LAD was confirmed by the change of color and the elevation of ST segment on electrocardiogram. After 45-minute of occlusion, suture was untied for reperfusion, and chest cavity and skin incision were closed. Sham operation was performed via an identical procedure, except that the suture was just passed underneath LAD without occlusion. After 24-hour of reperfusion, LAD was re-occluded at the same position for ischemia-reperfusion surgery. The heart was perfused with 2% Evans Blue (Sigma-Aldrich) to delineate the risk area, and heart tissue slices were stained with 1% 2, 3, 5-Triphenyltetrazolium chloride (TTC) for the infarct areas. The area at risk (unstained by Evans blue dye) and the myocardial infarct area (unstained by TTC) were measured using NIH Image J software.

2.3. Adult mouse cardiomyocyte isolation

Adult mouse cardiomyocytes were isolated from the hearts of WT and TG mice by enzymatic dissociation using collagenase type II in a Langerdorff perfusion apparatus, according to a previously described protocol with modification [21].

2.4. Myocyte contractility

Isolated cardiomyocytes in 2 mM- Ca^{2+} Tyrode's solution and spread on the glass chamber under a microscope stage (Olympus IX71) connected to a field stimulator (MyoPacer, IonOptix). Sarcomere shortening and return velocity was measured at paced 0.5Hz and under room temperature using MyoCam-IonOptix (Milton, MA) software.

2.5. Myocyte apoptosis

To stimulate myocyte apoptosis, hypoxia/reoxygenation was performed as previously described with modification [22]. Briefly, cells were incubated in a hypoxia buffer in an airtight Plexiglas chamber with an atmosphere of 1% O_2 /5% CO_2 /94% N_2 at 37°C for 30 min, followed by culturing in myocyte culture medium in a normal culture incubator (5%

CO₂/95% O₂) at 37°C for 24 hours. Alternatively, cells in the normal myocyte culture medium were treated with 1 μM H₂O₂ to stimulate apoptosis.

2.6. Statistics

Data are expressed as mean ± SD. Comparisons between two groups were evaluated using student's t-test. One-way ANOVA followed Bonferroni/Dunn post-hoc test was used for multiple comparisons. P-values <0.05 were considered statistically significant. Survival analysis was performed by the Kaplan-Meier method.

3. Results

3.1. Transgenic mice with cardiac specific expression of PDE3A1

PDE3A1 is the primary gene product initially cloned from human myocardium [23] and the protein product corresponding to PDE3A1 appears to be the dominant one [24]. To determine the specific role of PDE3A1 isozyme in myocyte function, we created transgenic mice (TG) with cardiomyocyte-specific expression of PDE3A1 under the control of the rodent alpha-myosin heavy chain (α-MHC) promoter, a well-established myocardial specific promoter used in transgenic mice [19, 20, 25, 26]. As shown in Figure 1A, PDE3A1 protein levels in the hearts of TG mice were almost 10 fold higher than that in the age-matched control wild-type (WT) mice. Consistently, PDE3 activity was also increased about 10 fold in TG hearts (Fig. 1B). Global myocardial cAMP level in the TG heart was significantly reduced by 46% compared to that in WT hearts (Fig. 1C). In addition, there were no significant changes in the expression of PDE3B and two cardiac PDE4 isoforms in TG hearts (supplemental Fig. S1A). Because PKA is a critical downstream effector molecule of cAMP-mediated effects in myocytes, we measured the levels of PKA catalytic subunits. We found that PKA Cβ subunit but not Cα subunit was significantly upregulated (Supplemental Fig. S1B), which might be a compensatory change in response to PDE3A1 overexpression. This finding also suggests that PKA Cβ might be a predominant PKA isoform downstream of the PDE3A1/cAMP signaling. However, overall CREB phosphorylation was not significantly altered in TG hearts (supplemental Fig. S1C).

3.2. Cardiac function of TG mice

We first analyzed cardiac function by conscious echocardiogram in WT and TG mice at 2 and 10 months of age (Table 1). At 2 months of age, TG mice displayed reduced heart rate (662 vs. 435 bpm) and ejection fraction (EF%: 83.9 vs. 52.5) compared to WT mice. TG mice also showed increased diastolic left ventricular diameter (LVDd, 3.10 vs. 4.19 mm) and systolic left ventricular diameter (LVDs, 1.47 vs. 3.07 mm), which may represent compensatory ventricular chamber dilation. Interestingly, cardiac function did not worsen in aging mice, as shown by comparing mice at 2 and 10 months of age (Table 1).

Hemodynamic assessment of anesthetized mice confirmed the decreased heart rate and cardiac contractile function (LV dp/dt max, and LV dp/dt min) observed in TG mice compared to WT mice (Table 1). However, LV systolic pressure (LVP) was preserved (Table 1), indicating that TG mice do not have hemodynamic indications of heart failure. Indeed, TG mice had the same growth rates as WT mice, reflected by similar body weights (Table 1) and the same survival rates (Fig. 1H). In addition, there was no significance difference in exercise capacity between WT and TG mice in a 45-min forced swim test (Supplemental Fig. S2).

3.3. Cardiac morphology and gene expression of TG mice

We further characterized cardiac morphology and structural changes in TG mice. We found that TG hearts were larger than WT hearts as shown by the global sagittal view of hearts

(Fig. 1D) and heart weights (Fig. 1E, left). However, there was no significant difference in lung weights between two groups (Fig. 1E, right). We detected a small but significantly enlarged cardiomyocyte cross-sectional area in TG mouse hearts (Fig. 1F), which is likely due to compensatory myocardial hypertrophy to decreased cardiac contractive function and heart rate in TG mice. Surprisingly, there were no significant changes in collagen deposition (Fig. 1G) and apoptotic cells (Fig. 1H) in TG hearts. Similar observations were obtained in mice at 10 months of age (Supplemental Fig. S3).

Consistent with the increased myocyte size in TG mice, the hypertrophic markers ANP and β -myosin heavy chain (β MHC) were significantly induced in TG mice (Supplemental Fig. S3A, B and C). In contrast, β 1-AR and SERCA2, which are known to be downregulated in failing hearts, were not altered in TG mice (Supplemental Fig. S4A, D, and E). These results suggest that the expression of exogenous PDE3A1 in cardiomyocytes does not cause maladaptive cardiomyopathy.

3.4. Role of PDE3A1 in regulating cardiomyocyte contractility

To further investigate the negative inotropic and chronotropic effects of PDE3A1 overexpression in TG mice, we measured cardiac function under ISO stimulation in anesthetized mice by echocardiogram. ISO injection increased cardiac contractile function (EF%) in WT mice, which was significantly lower in TG mice (Fig. 2A). Administration of milrinone under baseline or ISO-stimulation conditions largely abolished the difference in contractility between WT and TG mice (Fig. 2A). Similarly, we observed that the difference in heart rate between WT and TG mice was also significantly reduced when PDE3 activity was inhibited by milrinone (Fig. 2B). These observations indicate that the reduction of cardiac inotropic and chronotropic effects in TG mice is primarily due to elevated PDE3 activity in TG mice.

We also examined cardiac phospholamban (PLB) and troponin I (TnI) phosphorylation, which are critical events in β -AR stimulated cardiac contraction. As shown in Figure 2C, both resting and ISO-stimulated PLB phosphorylation were significantly reduced in TG mice. However, in the presence of milrinone, the difference in PLB phosphorylation was abolished. We obtained very similar observations for TnI phosphorylation (Fig. 2D).

Furthermore, we investigated single adult cardiomyocytes isolated from WT and TG mice. We found that the basal sarcomere shortening in electrically paced cardiomyocytes was the same between WT and TG mice (Fig. 3A). However, the time constant (Tau) was significantly increased in TG cells (Supplemental Fig. S5A), suggesting a diastolic impairment in TG myocytes. ISO stimulation dose-dependently increased sarcomere shortening in WT myocytes (Fig. 3B), while failed to increase sarcomere shortening in TG myocytes (Fig. 3C). Similarly, 10 nM ISO significantly reduced the Tau value in WT myocytes but failed to change the Tau of TG myocytes (Supplemental Fig. 5B-C). The differences in sarcomere shortening and Tau between WT and TG myocytes were completely abolished by inhibition of PDE3 activity with milrinone but not by inhibition of PDE4 activity with rolipram (Fig. 3C and Supplemental Fig. 5C). This indicates that impaired ISO responsiveness is primarily attributed to elevated PDE3 activity in TG cardiomyocytes. The reason that Tau was increased in WT myocytes under a high concentration of ISO (100 nM) or ISO (10 nM) plus milrinone or rolipram remains unknown (Supplemental Fig. 5B-C). Nevertheless, these results together suggest that PDE3A1 plays a critical role in modulating β -AR-mediated myocyte contraction and relaxation.

3.5. Protective effect of PDE3A1 overexpression against cardiac ischemia/reperfusion injury

We have previously shown that chronic inhibition of PDE3 or downregulation of PDE3A expression induces apoptosis in cultured cardiomyocytes [13, 18]. We thus hypothesized that overexpression of PDE3A1 should prevent myocyte damage in ischemia/reperfusion (I/R)-induced injury. Therefore, we performed *in vivo* I/R injury on TG and WT mice by ligation of the LAD (ischemia) for 30 min followed by reperfusion for 24 hours. We found that myocardial infarct areas (MI) in TG mice were much lower than those in WT mice (1% vs. 21%) when the areas at risk (AAR) were similar (Fig. 4A-C). Consistently, the average apoptotic cell number was also significantly reduced in TG hearts measured by TUNEL staining (Fig. 4D). More importantly, the cardiac function (EF%) of pre-and post-injury was preserved in TG mice, but not in WT mice (Fig. 4E). These results demonstrate the cardiac protective effects of PDE3A1 *in vivo* through increased myocyte survival.

We have previously shown that in cultured rat neonatal cardiomyocytes, inhibition of PDE3A function is associated with myocyte apoptosis through sustained induction of the transcriptional repressor ICER (inducible cAMP early repressor) and consequent inhibition of anti-apoptotic molecule BCL-2 expression [13, 18]. We therefore analyzed BCL-2 and ICER protein levels in sham and I/R hearts from WT and TG mice. As shown in Figure 5A, basal BCL-2 levels were significantly higher in TG hearts than in WT hearts. As expected, I/R significantly reduced BCL-2 levels in WT hearts. However, the BCL-2 levels in TG mice after I/R remained as high as those in WT mice under pre-I/R. Consistently, in WT mice, ICER expression was very low during pre-I/R but was significantly upregulated during post-I/R, which did not occur in TG mice (Fig. 5B). The *in vivo* observation of ICER downregulation and BCL-2 upregulation in TG hearts is consistent with our previous *in vitro* finding that PDE3A positively regulates BCL-2 expression and myocyte survival through negatively regulating ICER.

3.6. Anti-apoptotic effect of PDE3A1 in isolated adult cardiomyocytes

To rule out the possibility that the cardiac protective effect is due to a change in hemodynamics (such as lower heart rate) in TG mice, we performed cell apoptosis studies using isolated adult WT and TG cardiomyocytes that have similar baseline contractility. Cell apoptosis was induced by HR (hypoxia/reoxygenation: hypoxia for 30 min followed by reoxygenation for 24 hours) or treatment with 1 μ M H₂O₂ for 24 hours, after which cell were fixed, and apoptosis measured by TUNEL staining. As shown in Figure S6 and 6A, the myocytes with TUNEL positive nuclei were significantly reduced in myocytes isolated from TG mice compared to WT mice, under both basal and H/R-stimulated conditions. However, the differences between TG and WT myocytes were not observed when PDE3 activity was inhibited by the PDE3 inhibitor milrinone, indicating that this anti-apoptotic effect is due to increased PDE3 activity in TG myocytes. Milrinone alone increased apoptosis in both TG and WT myocytes, which is consistent with our previous observation of increased apoptosis in milrinone-or PDE3A siRNA-treated neonatal rat cardiomyocytes [13]. We also obtained very similar results in H₂O₂-stimulated myocyte apoptosis for TG and WT myocytes (Fig. 6B).

4. Discussion

In the present study, we used transgenic mice with myocardial overexpression of PDE3A1 to delineate the specific role of the PDE3A1 isoform in regulating cardiomyocyte function *in vivo*. The major findings of this study are summarized as follows:

First, we found that myocardial overexpression of the PDE3A1 isoform in TG mice leads to decreased heart rate and myocardial contractile function (Table 1). In particular, PDE3A1 plays a critical role in negatively regulating β -AR-mediated myocyte contraction and relaxation (Fig. 2, 3 and S5), likely through attenuating PKA-mediated PLB and TnI phosphorylation (Fig. 2). Our findings are in accordance with recent findings from PDE3A and PDE3B knockout mice [11, 12] or PDE3 inhibition [27]. For example, the heart rate of PDE3A but not PDE3B KO mice is significantly increased compared to wild-type control mice [11]. In addition, ventricular contractile function was elevated in isolated hearts from PDE3A but not PDE3B deficient mice, and the increased cardiac contractility observed in PDE3A deficient hearts is associated with elevated PLB phosphorylation and SERCA2 activity [12]. Although the role of PDE3 activity in regulating heart rate and contractile function is well recognized because of the remarkable chronotropic and inotropic effects of PDE3 inhibitors in human and animal hearts [28, 29], the specific PDE3 isoform involved had not been well characterized until recently. Thus, our current study using a gain-of-function approach, in conjunction with previous studies using a loss-of-function approach, have demonstrated a critical role for PDE3A, particularly the PDE3A1 isozyme, in modulating β -AR/cAMP signaling and cardiac contractile function.

Additionally, TG mice appear to undergo adaptive remodeling, including ventricular chamber dilation and slight myocyte hypertrophy (Table 1 and Fig. 1, and S4), probably as an adaptive response to their reduced cardiac contractility. Interestingly, there was no sign of maladaptive cardiomyopathy (such as cardiac fibrosis, myocyte apoptosis, and suppressed expression of SERCA2 and β 1-AR) detected in TG mice up to one year of age (Table 1, Fig. 1, S3 and S4). This suggests that increased PDE3A1 protects against pathological cardiac remodeling. PDE3A1 overexpression in the heart has somewhat similar effects as chronic blockade of β -AR signaling, which has been shown to reduce heart rate and cardiac contractility, but ameliorate a number of adverse process of pathological cardiac remodeling in heart failure patients and in animal models of heart failure [30, 31]. The increased fetal gene expression in TG hearts appeared to be paradoxical because fetal gene expression has long been associated with pathological cardiac hypertrophy. However, uncoupling between fetal gene expression and pathological hypertrophic growth has also been reported. For example, transgenic mice with cardiac-targeted overexpression of the α_{1A} -AR did not develop pathological hypertrophy but showed significantly increased fetal gene expression [32]. In addition, a similar paradoxical increase in fetal gene expression was reported in mice and in cultured cardiomyocytes treated with β -AR blockers [33]. Therefore, the connection between fetal gene expression and maladaptive cardiac remodeling deserves to be re-evaluated.

Finally and most importantly, we found that I/R-induced myocardial infarction and myocyte apoptosis are significantly reduced in TG hearts, accompanied with blocked ICER induction and preservation of BCL-2 expression (Fig. 4 and 5). The protective effects against cardiac injury seen in TG mice appear to be at least partially attributable to the anti-apoptotic effect of PDE3A1 in cardiomyocytes. This is because isolated adult cardiomyocytes from TG mice are more resistant to H/R- or H₂O₂-induced myocyte apoptosis in a PDE3 activity-dependent manner (Fig. 6). These are also in line with our previous *in vitro* findings from cultured neonatal cardiomyocytes. For example, we showed that chronically inhibiting PDE3 activity by pharmacological inhibitors or downregulating PDE3A expression by siRNAs induced cardiomyocyte apoptosis, via PKA-mediated stabilization of ICER and subsequent attenuation of BCL-2 expression [13, 18]. In animal and human failing hearts, PDE3A was found to be downregulated [13, 14], suggesting that PDE3A might be critical in protecting against cardiomyocyte apoptosis after myocardial injury or during pathological cardiac remodeling. Thus, our data provide the first *in vivo* evidence for a protective effect of PDE3A1 against cardiomyocyte apoptosis and cardiac injury.

Taken together, our findings reveal dual functions for PDE3A1-cAMP signaling in the myocardium. On one hand, PDE3A1 plays a critical role in controlling heart rate and regulating β -AR-mediated myocyte contractility. On the other hand, PDE3A1 is also important in regulating cardiomyocyte apoptosis. These findings strongly support the notion that chronic elevation of cAMP due to PDE3A1 inhibition is detrimental to cardiomyocytes. This may partially explain increased mortality due to arrhythmias and sudden death associated with long-term clinical use of PDE3 inhibitors [9, 34] because of the link between arrhythmias and myocyte apoptosis [35-37]. However, a number of previous studies have also shown that transient exposure(s) to a PDE3 inhibitor (such as milrinone, olprinone, or amrinone) prior to ischemia protects the heart from myocardial infarction [38, 39]. This may well be that the transient PDE3 inhibition activates similar signaling events occurring during an ischemia, which mimics ischemic preconditioning and thereby renders the myocardium resistant to injury from a subsequent prolonged episode of I/R. Indeed, increased cAMP levels and decreased PDE activities have been reported during ischemic preconditioning [40, 41], and PDE3 inhibition has been implicated in preconditioning protection [42, 43]. Thus, although the chronic PDE3 inhibition during prolonged I/R is detrimental to the heart, the transient PDE3 inhibition can confer protection through a preconditioning mechanism. Similar paradoxical observations have been also found with other signaling molecules. For example, NO preconditioning fights against NO-induced cardiomyocyte apoptosis [44]. Reactive oxygen species (ROS) are detrimental mediators of myocardial apoptosis and post-I/R injury, while the transient increase of ROS during preconditioning results in cardioprotection [45]. Nonetheless, the effect of ischemic preconditioning in TGPDE3A1 mice deserves to be further evaluated in the future.

Although in TG hearts PDE3A1 levels and activity are increased about 10 fold, the phenotypes of TG myocytes are unlikely to have resulted from non-specific effects due to a high level of PDE3A1 overexpression, as the findings reported here are consistent with the findings from PDE3A knocking down or inhibition. For example, overexpressing PDE3A1 led to reducing cardiac contractility (Fig. 2 & 3) and attenuating myocyte apoptosis (Fig. 4-6), while PDE3A knockdown or inhibition of PDE3 activity led to increasing cardiac contractility [12] and increased cardiomyocyte apoptosis [13] (Fig. 6). It, of course, would be ideal to evaluate whether I/R-induced cardiomyocyte apoptosis and cardiac injury are exaggerated in PDE3A knockout mice *in vivo* in the future. In addition, the phenotypes of TG mice correlate with the PDE3A1-overexpression, which is not likely to have been modified by the insertion position of the PDE3A1 transgene. This is because the functional changes between WT and TG mice are largely abolished by PDE3 inhibitor milrinone. In this study, we reported a protective effect of PDE3A1 overexpression in an acute I/R injury model. However, the protective role of PDE3A1 in the chronic myocardial injury model is still not clear, which deserves a further investigation.

There are multiple cAMP pools in the heart, and many of them appear to play distinct roles in the regulation of cardiac contractility and protection. For example, catecholamines, prostaglandin 2 (PGE2), glucagon-like peptide 1 (GLP-1), and adenosine all are able to elevate myocyte cAMP. cAMP generation through β -AR activation by catecholamine stimulates profound myocyte contraction, while cAMP elevation by PGE2 or GLP-1 has limited [46] or negative effect [47] on myocyte contraction, respectively. Chronic stimulation of β 1-AR/cAMP signaling by catecholamine is pro-apoptotic to myocyte, while cAMP signaling through activation of GLP-1 receptors [48, 49] and adenosine receptors [50, 51] are anti-apoptotic and protective against I/R. Moreover, cAMP produced by adenylyl cyclase 5 (AC5) and AC6 also have different cardiac effects. It appears that AC5-derived cAMP plays a detrimental effect [52, 53], while AC6-derived cAMP plays a protective effect in pathological cardiac remodeling [54, 55]. Therefore, understanding the specific role

of individual cAMP signaling is important. Our study indicates that PDE3A1 is a critical negative regulator of β -AR signaling in the cardiac myocyte.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

5. Sources of Funding: This research was supported by grants from the National Institutes of Health (HL088400 and HL111291 for Dr. Yan, HL108551 for Dr. Abe, the American Heart Association (0740021N for Dr. Yan), and Japanese KAKENHI (Grant-in-Aid for Young Scientists (B) no. 23790867 for Dr. Oikawa).

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Non-standard Abbreviations

AAR	area at risk
β-AR	beta-adrenergic receptor
BCL-2	B-cell lymphoma 2
CSA	cross-sectional area
LV dp/dt max	the maximum rate of left ventricular pressure rise
LV dp/dt min	the maximum rate of left ventricular pressure decrease
EF	ejection fraction
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HW	heart weight
LAD	left anterior descending
ICER	inducible cAMP early repressor
I/R	ischemia/reperfusion
ISO	isoproterenol
MI	myocardial infarction
Mil	milrinone
LV	left ventricle
LVDd	left ventricular diastolic diameter
LVDs	left ventricular systolic diameter
LVP	left ventricular systolic pressure
LW	lung weight
PDE3A1	phosphodiesterase 3A1
PKA	cAMP-dependent protein kinase type A
PLB	phospholamban
p-PLB	phosphorylated phospholamban
p-TnI	phosphorylated TnI
Rol	rolipram

SERCA2	sarcoplasmic reticulum calcium ATPase type 2
TG	transgenic mice
TnC	troponin C
TnI	troponin I
TL	tibia length
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling assay
WT	wild-type mice

Highlights

- Myocardial overexpression of PDE3A1 in mice reduces heart rate and contraction
- PDE3A1 negatively regulates beta-adrenergic receptor (β -AR) stimulated myocyte contraction
- PDE3A1 protects the heart from ischemia-reperfusion injury
- PDE3A1 inhibits cardiomyocyte apoptosis

Figure 1.

Cardiac morphological analysis of WT and TG mice. (A) PDE3A1 protein expression in hearts measured by Western blotting. (B) PDE3 activity in hearts was measured by PDE assay using 1 μM cAMP as a substrate. Milrinone-inhibitable activity was considered to be PDE3 activity (n=3). (C) Basal cAMP levels in ventricular tissues of WT and TG mice measured by enzymatic immunoassay (EIA) (n=3). (D) Representative H&E-stained frontal sections of mouse hearts. Scale bars: 1mm. (E) Heart weight (HW) and lung weight (LW) normalized to tibia length (TL) (n=5). (F) Representative wheat germ agglutinin (WGA)–fluorescein isothiocyanate–stained left ventricle sections of mouse hearts (green) showing

cardiomyocyte cross-sectional area (CSA), and quantified results of CSA (averaged from more than 200 myocytes per section per animal, n=5). **(G)** Representative images of collagen deposition (blue) in left ventricle of mouse hearts depicted by Masson's trichrome staining, and quantification of percentage fibrosis (n=5). **(H)** Quantification of TUNEL positive nuclei in left ventricle of mouse hearts (n=3). **(I)** Survival rate of WT (n=9) and TG mice (n=11) up to 10 months of age. Values are mean $\pm$ SEM *P<0.05 vs WT mice. ns: no significant difference.

Figure 2.

(A-B) Isoproterenol (ISO)-induced cardiac contractile function in WT and TG mice. LV EF % (A) and heart rate (B) were measured by echocardiogram at baseline and three minutes following intraperitoneal injection of 0.1 mg/kg ISO. Milrinone (Mil, 10mg/kg) was intraperitoneally injected ten minutes before echocardiography. Values are mean $\pm$ SEM (n=5 to 13). ** P<0.01 vs. WT. (C-D) Western blotting results showing the levels of phosphorylated-PLB (p-PLB) (C) and phosphorylated-TnI (p-TnI) (D). Band intensities were quantified by densitometry. Fold changes normalized to total PLB or TnI were calculated. Values are mean $\pm$ SEM (n=3 each group). *P<0.05 vs. WT.

Figure 3.

Contractility measurements in isolated adult mouse cardiomyocytes. **(A)** Basal sarcomere shortening of cardiomyocytes paced at 0.5Hz. **(B)** ISO-induced sarcomere shortening. Sarcomere shortening was measured 5 minutes after 1, 10, or 100 nmol/L ISO treatment. **(C)** Effects of PDE inhibitors on sarcomere shortening. Cardiomyocytes were pretreated with Mil (10 μ mol/L) or rolipram (Rol, 10 μ mol/L) for 10 minutes followed by ISO (10 nmol/L) stimulation. Sarcomere shortening was measured 5 minutes after isoproterenol stimulation. Values mean $\pm$ SEM represent averages of 6-10 cells from 3 mice per condition. * P <0.01 compared to WT.

Figure 4.

Cardioprotective effect of PDE3A1 overexpression against ischemia/reperfusion (I/R) injury. (A) Representative photographs of post-I/R mouse heart slices perfused with Evans Blue and stained with triphenyltetrazolium chloride (TTC). Mice were subjected to myocardial ischemia for 45 min and reperfusion for 24 hours through ligation of the left anterior descending artery. Hearts were then perfused with 2% Evans Blue to delineate the area at risk (AAR), transversely cut into 4-5 slices, and stained with TTC to access the myocardial infarct area (MI). AAR was the unstained area by Evans blue dye and MI was the unstained area by TTC. (B-C) Quantification of percentage of AAR in the total LV or MI in AAR, AAR and MI were assessed using NIH Image J software. (C) Quantification of TUNEL positive nuclei in AAR of the corresponding mice. (D) LV ejection fraction of pre and post I/R assessed by M-mode echocardiography. Values are mean $\pm$ SEM (n=5-8 each group). *P<0.05 vs. WT.

Figure 5.

Western blotting results showing the expression of BCL-2 (**A**) and ICER (**B**) in LV of WT and TG mice pre- and post-I/R. Loading was confirmed by immunoblotting for GAPDH. Band intensities were quantified by densitometry. Fold changes normalized to GAPDH were calculated. Values are mean $\pm$ SEM (n=3-4 each group). *P<0.05 vs. WT.

Figure 6.

Apoptotic analysis of isolated adult cardiomyocytes. Adult mouse cardiomyocytes were isolated from ventricles of WT or TG mice and subjected to normoxia (control) or hypoxia/reoxygenation (H/R) (hypoxia for 30 min and reoxygenation for 24 hours) or treated with 1 μ M H₂O₂, in the presence or absence of 30 μ M of milrinone. Apoptotic myocytes were detected by TUNEL staining. Myocytes with nuclear TUNEL staining were counted. **(A-B)** Quantitative data showing the percentage of TUNEL positive myocytes. Values are mean $\pm$ SD (n=5 for WT and n=4 for TG). For each sample, 200-400 myocytes were counted. * P<0.05, ns: no significant difference.

Table 1
Cardiac function of WT and TG mice

	2 months		10 months	
	WT (n=5-10)	TG (n=5-12)	WT (n=8)	TG (n=10)
HR, bpm	662 ± 21	435 ± 45 *	707 ± 32	451 ± 23 †
AWd, mm	0.89 ± 0.07	0.77 ± 0.11 *	0.99 ± 0.11	0.89 ± 0.10 †
PWd, mm	0.86 ± 0.06	0.78 ± 0.06 *	1.03 ± 0.16	0.94 ± 0.08 †
LVDs, mm	1.47 ± 0.20	3.07 ± 0.32 *	1.54 ± 0.24	3.27 ± 0.43 †
LVDd, mm	3.10 ± 0.18	4.19 ± 0.19 *	3.28 ± 0.27	4.38 ± 0.41 †
 EF, %	83.9 ± 4.7	52.5 ± 7.8 *	85.1 ± 7.8	50.2 ± 8.3 †
 BW, g	26.0 ± 1.7	26.0 ± 1.6	35.5 ± 3.3	36.1 ± 2.1
LVP, mmHg	90.1 ± 2.5	93.2 ± 4.6		
LV dp/dt max, mmHg/sec	5963 ± 467	4265 ± 94 *		
LV dp/dt min, mmHg/sec	-5240 ± 251	-3866 ± 152 *		

HR: heart rate; AWd: anterior wall diameter; PWd: posterior wall diameter; LVDd: left ventricular diastolic diameter; LVDs: left ventricular systolic diameter; EF: ejection fraction; LVP: left ventricular systolic pressure; LV dp/dtmax, the maximum rate of left ventricular pressure rise; LV dp/dt min, the maximum rate of left ventricular pressure decrease; Values are expressed as mean ± SD. n indicates number of mice;

* P<0.05 vs WT at 2 months,

† P<0.05 vs WT at 10 months.