PDE2-mediated cAMP hydrolysis accelerates cardiac fibroblast to myofibroblast conversion and is antagonized by exogenous activation of cGMP signaling pathways

C. Vettel,1,4* S. Lämmle,1,4* S. Ewens,1,4 C. Cervirgen,1,4 J. Emons,1,4 A. Ongherth,1,4 M. Dewenter,1,4 D. Lindner,2,5 D. Westermann,2,5 V. O. Nikolaev,3,4 S. Lutz,1,4 W. H. Zimmermann,1,4 and A. El-Armouche1,4,6

1Institute of Pharmacology, University Medical Center Göttingen, Germany; 2Department of General and Interventional Cardiology, University Heart Center Hamburg, University Medical Center Hamburg-Eppendorf, Germany; 3Department of Cardiology, University Medical Center Göttingen, Germany; 4German Center for Cardiovascular Research, partner site Göttingen, Germany; 5German Center for Cardiovascular Research, partner site Hamburg/Kiel/Luebeck, Germany; and 6Department of Pharmacology, Faculty of Medicine, University of Technology-Dresden, Germany

Submitted 29 October 2013; accepted in final form 28 January 2014

Fibrotic remodeling is a common feature of the diseased heart. Inflammatory processes, mechanical stress, and enhanced growth factor secretion result in the induction of a contractile subtype of fibroblasts, termed cardiac myofibroblasts (MyoCFs) (6, 20). MyoCFs share characteristics with both fibroblasts and smooth muscle cells, i.e., they are able to produce a variety of matrix proteins as well as to express, e.g., α-smooth muscle actin (α-SMA). This transformation is essential for postinjury scar formation and hence for organ integrity and function, e.g., after myocardial infarction. However, the unusual persistent, proliferative, and migratory properties of MyoCFs lead to an excessive accumulation of extracellular matrix that exceeds the scar area and over time promotes diastolic dysfunction and contributes to heart failure progression (25).

CAMP and cGMP have been shown to display antifibrotic properties by, e.g., inhibiting α-SMA/connective tissue growth factor (CTGF) expression and collagen synthesis (7, 18, 23). Both CAMP and cGMP are central regulators of the cardiovascular system often with opposing functions. For example, CAMP and its effector protein kinase A mediate the acute adaption of cardiac excitation-contraction coupling and cardiac growth, whereas CAMP is associated with vasodilatation and the activation of antihypertrophic signaling in response to nitric oxide (NO) or atrial natriuretic peptide (ANP) (26). To maintain the specificity of downstream target activation, localization and duration of CAMP/cGMP signals are tightly controlled. This spatiotemporal restriction of CAMP/cGMP concentrations is mainly achieved by phosphodiesterases (PDEs), which catalyze nucleotide hydrolysis. Until now, several different isoforms from the PDE families 1–5 and 8 have been described in the heart (12). Among those, the cGMP/cAMP-hydrolyzing PDE2 has the unique feature to be activated by cGMP via a cGMP-specific PDE motif located in the regulatory domain of the enzyme (15). The allosteric activation leads to an increase in PDE2-dependent CAMP hydrolysis by ~10-fold (24). By this mechanism, PDE2 allows cGMP-generating stimuli such as ANP or NO to negatively regulate CAMP levels. We recently showed that myocardial PDE2 is upregulated in human and experimental heart failure and that PDE2 upregulation in cardiomyocytes (CMs) could be a part of the well-known β-adrenergic receptor (β-AR) desensitization process, and hence its activation may prove beneficial for CM survival in terms of protection from chronic β-AR overstimulation (11).
In this study, we addressed the impact of PDE2 on cAMP- and cGMP-mediated signal transduction with regard to cardiac fibroblast (CF) conversion. We found that PDE2 overexpression enhances primarily cAMP degradation and induces fibroblast (CF) to MyoCF transformation, which led functionally to higher stiffness in fibroblast-derived engineered connective tissues (ECTs). However, despite the initial expectation of a cGMP-dependent increase in PDE2 activity and an enhancement of MyoCF induction, we found that cGMP-elevating stimuli ANP and NO-donor sodium nitroprusside (SNP) may fully antagonize PDE2-induced MyoCF phenotype.

MATERIALS AND METHODS

Reagents for cell culture were purchased from Gibco Life technologies. Cells were grown in a humidified incubator with 5% CO₂, 95% room air at 37°C. All reagents were of highest quality available and obtained from commercial sources: Biomol, Sigma-Aldrich, AppliChem, Carl Roth, Tocris, and Biotrend.

Animal care and culture of rat neonatal cardiac cells. Protocols for the care and use of the rats were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health and approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Germany). Neonatal rat heart cells were isolated from newborn rats (day 0 to 3) as previously described (27). Cells were preplated for 60 min to separate fibroblasts from the nonadhering CMs. For CF experiments, cells were used at passage 1.

Engineered connective tissues and recombinant adenoviruses. CFs were infected under serum-starved conditions with adenovirus (Ad)-enhanced green fluorescent protein (EGFP) or Ad-PDE2 for 4 h. Cells were harvested by trypsinization, and engineered connective tissues (ECT) were prepared as previously described (19, 28). After 4 days in culture, ECT was transferred into temperature-controlled organ baths for a stress-strain analysis. The recombinant adenoviruses were generated according to the method published by He et al. (3). For recombinant PDE2 adenovirus, the murine PDE2A3 transcript was cloned (NM_001008548.3). All viruses code in addition for EGFP.

Infections were carried out under serum-free conditions over a period of 48 h.

Immunoblot, immunofluorescence, cAMP/cGMP detection. Immunoblot analysis was carried out as recently described (22). The following antibodies were used: anti-α-smooth muscle-actin (1: 2,000), anti-α-tubulin (1:2,000) from Sigma-Aldrich, anti-PDE2 (1:200), anti-procollagen type1A1 (1:200), anti-CTGF (1:200), anti-GAPDH (1:1,000) from Santa Cruz Biotechnology, and anti-calsequestrin (1:2,500) from ThermoScientific. Immunofluorescence was carried out as recently described (22). cAMP/cGMP concentrations were determined with the acetylated protocol of the respective enzyme immunoassay kit (Biotrend). For cAMP measurements by fluorescence resonance energy transfer (FRET), CFs were infected with Ad-exchange protein directly activated by cAMP 2 (Epac2) camps for 48 h. Analysis was carried out according to recently published protocols (13, 14).

Statistics. Results are presented as means ± SE. Data sets were compared by Student’s t-test to assess differences between groups. P values of <0.05 were considered as statistically significant.

RESULTS

Overexpressed PDE2 induces the profibrotic factors α-SMA and CTGF that translates into a higher stiffness of ECT. Based on our initial finding of a PDE2 upregulation in total heart tissue, we first analyzed endogenous PDE2 expression in isolated neonatal rat CMs and CFs. To ensure a successful fractioning of these two cell populations, cell lysates were additionally probed for respective marker genes, calsequestrin for CMs and procollagen I for the CF population. As shown in Fig. 1, PDE2 expression was 2.5-fold higher in CFs compared with CMs (Fig. 1, A and B).

To better understand PDE2 function in CFs, we overexpressed this enzyme by using recombinant adenoviruses encoding for either PDE2 and EGFP or EGFP alone as control. PDE2 activity has been described for both cytosolic and membrane cell fractions most probably because of variances in the NH₂-terminus of the known splice variants PDE2A1–3 (17). Immunofluorescence staining showed that overexpressed PDE2 displayed a mostly cytosolic distribution in CFs and was additionally accumulated at perinuclear membranes (Fig. 2A). Expectantly, basal CAMP levels in total cell lysate were markedly abolished compared with those in controls (Fig. 2B). Moreover, α-SMA and CTGF expression were distinctly increased in PDE2-CFs by 3.5- and 2.3-fold, respectively (Fig. 2, C and D). Similar results were obtained when we analyzed α-SMA and CTGF by immunofluorescence, where PDE2 CFs showed greater stress fiber formation and higher abundance of CTGF containing vesicles (Fig. 2E).

To address functional consequences, we generated ECT, which comprise adenovirally transduced CFs embedded in a collagen matrix to form a tissue ring (Fig. 2G). After condensation, ECTs were stretched stepwise, and the passive force generated was measured at each ECT strain. PDE2 overexpression led to an increase in passive force, indicating a higher stiffness of the tissue (Fig. 2F).

β-AR receptor-induced accumulation of cAMP is partially inhibited by PDE2 but does not prevent PDE2-induced MyoCF phenotype. Several stimuli that promote the synthesis of cAMP have been shown to counteract TGF-β-induced fibroblast dif-
H1248  PDE2 INDUCES FIBROBLAST CONVERSION

A

PDE2  EGFP+DAPI  MERGE

B

![Graph showing cAMP levels](image)

C

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ad-EGFP</th>
<th>Ad-PDE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>CTGF</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>PDE2</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>GAPDH</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
</tbody>
</table>

D

![Bar chart showing relative α-SMA and CTGF expression](image)

E

![Immunofluorescent images](image)

F

![Graph showing passive force vs. stretch](image)

G

![Images of Ad-EGFP and Ad-PDE2](image)
differentiation. Among those are, e.g., prostaglandine-2 and the β1/β2-AR agonist isoprenaline (Iso) (8). To test the contribution of PDE2 to the degradation of β-AR-induced cAMP increase, we additionally transduced CFs with the cytosolically distributed Epac2-camps FRET biosensor for cAMP. As documented by the representative single trace of cyan fluorescent protein/yellow fluorescent protein (Fig. 3A), stimulation with Iso led to a modest but robust accumulation of cAMP in CFs. This increase was reduced by 75% under conditions of high PDE2 abundance and consequently restored when PDE2-specific inhibitor Bay 60-7550 was additionally applied (Fig. 3, A and B). We next analyzed α-SMA and CTGF expression after Iso stimulation. Control cells displayed a relatively low expression of both α-SMA and CTGF, which was not further reduced by Iso application (Fig. 3, C and D). Most importantly, the remaining Iso-induced cAMP pool in PDE2 transduced CFs did not counteract PDE2-mediated MyoCF induction.

PDE2-mediated fibroblast conversion is abolished by cGMP-generating stimuli. When intracellular cGMP concentrations exceed 500 nM, PDE2 is not only allosterically activated by this cyclic nucleotide but is also involved in its hydrolysis (24). Cellular cGMP is generated by either particulate or cytosolic

Fig. 2. Impact of PDE2 on basal cAMP synthesis, fibrotic markers, and tissue characteristics. Localization of recombinant PDE2 (A) and representative pictures of α-smooth muscle actin (α-SMA) and connective tissue growth factor (CTGF) expression (E) was visualized by immunofluorescence. Nuclei were stained with 4',6-diamino-2-phenylindole and viral enhanced green fluorescent protein (EGFP) expression was recorded as a control. B: basal cAMP concentration measured by enzyme immunoassay; n = 5. Ad, adenovirus. Representative immunoblots (C) and quantification of α-SMA and CTGF expression (D) after 24 h of stimulation with Iso normalized to tubulin; n = 3 with 2 to 3 replicates each. GFP, green fluorescent protein. Values are means ± SE; *P ≤ 0.05 vs. EGFP, basal.
Guanylyl cyclase. Therefore, we tested how PDE2 affects basal, ANP/particular guanylyl cyclase- and NO/soluble guanylyl cyclase-induced cGMP signal amplitudes and is in turn affected in its cAMP-hydrolyzing activity. Basal cGMP abundance was similar to those of control cells and hence not compromised by PDE2 (Fig. 4A). Both ANP and SNP led to a robust two- to threefold increase in cGMP, with a slightly reduced response in SNP/PDE2 compared with SNP/EGFP (2.2- and 3-fold, respectively). In regard to cAMP, both stimuli led to higher levels in control cells, whereas cAMP accumulation was either blunted (ANP) or decreasing (SNP) in PDE2-overexpressing fibroblasts (Fig. 4B).

We next addressed how elevated cGMP levels and the resulting loss in cAMP affect PDE2-induced MyoCF phenotype. ANP and SNP reduced basal α-SMA content but had no further impact on basal CTGF expression.
However, ANP, as well as SNP, completely normalized both α-SMA and CTGF expression in PDE2 cells (Fig. 4, C and D). Similar results were obtained when stress fiber formation and CTGF content were visualized by immunofluorescence (data not shown).

**DISCUSSION**

In our study, we show that PDE2 overexpression has two important consequences on CF physiology. First, PDE2 led to an expected decrease in cellular cAMP levels and, second, resulted in the induction of α-SMA and CTGF synthesis in the absence of specific profibrotic stimuli. It has been described that fibroblasts convert into MyoCFs under standard two-dimensional culture conditions mainly because of rigidity of the culture substrate and autocrine TGF-β stimulation (2, 10, 16). To maintain close to the physiological phenotype, we used passage 1 fibroblasts, where we observed moderate α-SMA and CTGF expression (see Fig. 2C). Our data suggest that under conditions of balanced pro- and antifibrotic signaling, basal cAMP generation is essential to prevent a fully fibroblast conversion. However, since these observations were obtained in a two-dimensional culture, the important question was whether this MyoCF induction would also occur in a more complex tissue-like environment and, consequently, affect its mechanistic properties. Cultivation of CFs in relaxed three-dimensional matrices has been described to cause a substantial downregulation of α-SMA (16, 21). Despite these more physiological conditions, PDE2 expression indeed led to greater stiffness (“passive force” due to chronic constriction based on matrix remodeling) of three-dimensional ECTs subjected to stress strain analyses. These measurements are, however, not an indicator of acute contractile responsiveness (“active force” due to MyoCF contraction), which was not determined here. Taken together, these results argue for a critical role of the PDE2-regulated cAMP pool in blocking signal pathways activated independently of cell surface interactions and allow a valid impression of how these molecular changes directly affect tissue characteristics.

As recently published, TGF-β-transformed CFs are less responsive to cAMP-generating stimuli due to a downregulation of adenyl cyclases and an upregulation of PDEs. The TGF-β-induced transformation was demonstrated to be reversible by a pharmacologically increased adenyl cyclase activity (9). Our FRET experiments showed a markedly reduced but not completely diminished response to β-AR stimuli in PDE2 cells. The question we posed in this context was whether this additional presence of cAMP generated by endogenous stimuli is sufficient to reverse PDE2-induced acceleration of CFs to MyoCF transition. However, in our setting, β-AR activation was neither able to compensate the loss in basal cAMP accumulation in PDE2-CFs nor showed any significant effect on α-SMA or CTGF in control cells. Our results are therefore well in line with the fact that β1/β2-blockers such as carvedilol or propranolol used to treat heart failure patients do not further enhance fibroblastic remodeling. Similar to cAMP, the signal molecule cGMP generated either by natriuretic peptide receptors or via NO has been shown to oppose the expression of α-SMA and CTGF in lung, renal, or CFs (1, 4, 5, 7). PDE2 is a dual-substrate PDE capable of hydrolyzing both cAMP and cGMP but is predominately activated by the latter. The initial expectation was therefore rather an enhancement of the MyoCF phenotype due to an increase in PDE2 activity and further reduced cAMP levels (Fig. 4B). Interestingly, the opposite was the case: ANP/SNP stimulation actually led to an inhibition of α-SMA and CTGF expression in PDE2-CFs, which despite diminished cAMP levels was similar to ANP/SNP-treated EGFP-CFs (Fig. 4, B and C). Thus our data suggest that exogenous activation of cGMP pathways is able to bypass PDE2-mediated degradation of cAMP and reverses the consequent induction of α-SMA and CTGF (Fig. 4D). To our knowledge this study provides the first comparative aspect between these two antifibrotic signaling pathways.

In summary, our findings emphasize the critical role of basal cAMP in the absence of cGMP-elevating stimuli and the PDEs regulating its accumulation. In the context of, e.g., cardiac disease, our data indicate that a PDE-dependent loss in cAMP accumulation could be overcome by higher cGMP levels, which allows a therapeutic option in the treatment of heart failure that can be considered beneficial not only for the fibroblast but also for CM population.

**GRANTS**

This study was supported by Deutsche Forschungsgemeinschaft Grants DFG EL 2705/1 (to A. El-Armouche), SFB 1002 (to V. O. Nikolaev, S. Lutz, W. H. Zimmermann, and A. El-Armouche), and IRTG 1816 (to C. Vettel, V. O. Nikolaev, S. Lutz, W. H. Zimmermann, and A. El-Armouche) and the German Centre for Cardiovascular Research.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


