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# Inhibition of TGF-β induced lung fibroblast to myofibroblast conversion by phosphodiesterase inhibiting drugs and activators of soluble guanylyl cyclase

Torsten R. Dunkern [a,*], Daniel Feurstein [b], Giovanni A. Rossi [c], Federica Sabatini [c], Armin Hatzelmann [a]

[a] *Department of Biochemistry Inflammation, ALTANA Pharma AG, Byk-Gulden-Str.2, 78467 Konstanz, Germany*
[b] *Faculty of Biology, University of Konstanz, Jacob-Burckhardtstr.25, 78457 Konstanz, Germany*
[c] *Pulmonary Disease Unit, G. Gaslini Institute, Largo G. Gaslini 4, 16148 Genoa, Italy*

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**Abstract**

Pulmonary fibroblast to myofibroblast conversion is a pathophysiological feature of idiopathic pulmonary fibrosis and COPD. This conversion is induced by transforming growth factor (TGF)-β derived from epithelial cells as well as activated macrophages that have infiltrated the lung. Preventing this conversion might be a favourable therapeutic approach. Within this study we examined the activity of different members of the phosphodiesterase (PDE) family in primary human lung fibroblasts and various lung fibroblast cell lines both before and after TGF-β induced differentiation to myofibroblasts as reflected by the expression of alpha-smooth muscle actin. We showed that the predominant PDE activities in lung fibroblasts are attributed to PDE5, PDE1 and to a smaller extent to PDE4. cyclic GMP (cGMP)-hydrolyzing activity declines by about half after differentiation to myofibroblasts in all pulmonary fibroblasts investigated, which is accompanied by a down-regulation of PDE5 protein. Lung fibroblast to myofibroblast differentiation is blocked by treatment with the PDE4 inhibitor piclamilast alone, depending on the TGF-β concentration applied, and in combination with prostaglandin $E_2$ ($PGE_2$) in a synergistic manner. Despite the high PDE5 activity the PDE5 inhibitor sildenafil by itself as well as in combination with brain natriuretic peptide or the nitric oxide-donor DETA-NONOate shows no inhibiting effects. However, combining sildenafil with the guanylyl cyclase (GC) activator BAY58-2667 and ODQ (which sensitizes GC for activation by BAY58-2667) suppressed TGF-β induced differentiation. In summary, our data indicate that drugs interfering with the cyclic AMP (cAMP)-as well as with the NO-cGMP-pathway offer the therapeutic opportunity to prevent the differentiation of pulmonary fibroblasts to myofibroblasts in lung fibrosis.


*Keywords:* Lung fibroblast; Fibrosis; Phosphodiesterase; Sildenafil; TGF-β

## 1. Introduction

Fibrosis, to a various extent, is a hallmark of different respiratory diseases like idiopathic pulmonary fibrosis, asthma and chronic obstructive pulmonary disease (COPD). In COPD there is a complex remodelling process in the peripheral lung, resulting in emphysema and fibrosis of the small airways. Patients with idiopathic pulmonary fibrosis suffer from massively interspersed interstitial fibrotic foci in the lung containing palisades of fibroblasts, myofibroblasts and huge amounts of extracellular matrix deriving from these cells (Pardo and Selman, 2002). During the different stages of fibrosis progression the phenoptype of lung fibroblasts changes from a migratory phenotype, to a proliferative and profibrotic one, which is called a myofibroblast. The myofibroblast is characterized, besides having a strong production of extracellular matrix (e.g. collagen), by the expression of alpha-smooth muscle actin, which is not present in fibroblasts and contributes to the altered mechanical characteristics of the lung (Adler et al., 1989). The myofibroblast somehow has a hybrid phenotype between a fibroblast and a smooth muscle cell. For a long time, a well-accepted theory stated that a common pathogenic mechanism underlies all fibrotic lung diseases. This was thought to include an initial inflammation accompanied by a

* Corresponding author. Tel.: +49 7531 84 3121; fax: +49 7531 84 93121.
*E-mail address:* torsten.dunkern@altanapharma.com (T.R. Dunkern).

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strong accumulation of intra-alveolar macrophages providing e.g. large amounts of transforming growth factor (TGF)-β, inducing the conversion of fibroblasts to the contractile myofibroblasts. Therefore enhanced TGF-β concentrations have been detected in various fibrotic diseases including idiopathic pulmonary fibrosis (Khalil et al., 1991; Broekelmann et al., 1991), sarcoidosis (Salez et al., 1998) and cystic fibrosis (Wojnarowski et al., 1999). It has also been shown that TGF-β is expressed by macrophages of the terminal airway and alveoli (De Boer et al., 1998). However, it became evident that anti-inflammatory treatment with glucocorticoids did not improve the outcome of idiopathic pulmonary fibrosis (Mapel et al., 1996) and that fibrosis results from an epithelial injury followed by an abnormal wound healing process (for review see Selman et al., 2001). Under those conditions, especially in the late stage of the fibrotic lung disease, TGF-β was detected in bronchiolar epithelial cells, epithelial cells of the honeycomb cysts and particularly in activated hyperplastic type II pneumocytes. Thus, epithelial cells seem to be the main source of profibrotic TGF-β (Khalil et al., 1991, 1996).

Cyclic nucleotide phosphodiesterases (PDEs) comprise a superfamily of related proteins which can be subdivided into 11 isoenzymes based on their amino acid sequences, sensitivity to different activators and inhibitors as well as their ability to hydrolyze either preferentially cyclic AMP (cAMP), cyclic GMP (cGMP) or both (Francis et al., 2001). Inhibitors of PDE4, which specifically modulate intracellular cAMP concentrations, and inhibitors of cGMP-specific PDE5 are in clinical development for the treatment of respiratory diseases (Lee et al., 2005; Lipworth, 2005). Thus, the PDE4-selective inhibitor roflumilast is currently in phase III clinical trials for asthma and COPD, whereas the PDE5-selective sildenafil is approved for the treatment of pulmonary arterial hypertension and is in phase II trials for COPD. Up to now the therapeutic value of PDE family-specific inhibitors in the treatment of lung fibrosis by prevention of the differentiation of fibroblasts to myofibroblasts has not been evaluated in detail.

In this study we analyzed the activity of phosphodiesterases 1, 2, 3, 4 and 5 in three human lung fibroblast cell lines (HFL-1, GM06114 and IMR-90) as well as primary human lung fibroblasts before and after TGF-β1-induced fibroblast to myofibroblast conversion by measuring the enhanced expression of the myofibroblast marker alpha-smooth muscle actin. We addressed the question whether inhibition of phosphodiesterase 4 and 5 alone, inhibition of PDE4 in combination with prostaglandin $E_2$ ($PGE_2$), or inhibition of PDE5 in combination with guanylyl cyclase (GC) activators might interfere with the TGF-β1-induced differentiation of fibroblasts to myofibroblasts. We provide evidence that TGF-β1-induced alpha-smooth muscle actin expression in human lung fibroblasts can be suppressed by inhibition of PDE4 alone and in a synergistic fashion in combination with $PGE_2$. In contrast, inhibition of PDE5 by itself did not affect alpha-smooth muscle actin expression but when combined with the soluble GC (sGC) activator BAY58-2667 plus ODQ (1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one; sensitizes the sGC for activation by BAY58-2667) or BAY41-2272, alpha-smooth muscle actin expression was affected.

## 2. Materials and methods

### 2.1. Materials

The selective PDE3 inhibitor motapizone (Borbe et al., 1986) was a generous gift from Sanofi-Aventis (formerly Rhone-Poulenc Rorer, Cologne, Germany). The PDE2 inhibitor 9-(6-phenyl-2-oxohex-3-yl)-2-(3,4-dimethoxybenzyl)purin-6-one (abbreviated as PDP) is example 100 from US patent 5861396 (Bayer AG) and was resynthesized at ALTANA Pharma AG. The selective PDE4 inhibitor piclamilast RP73401 (Benzamide,3-cyclopentyloxy-4-methoxy-*N*-(3,5-dichloro-4-pyridyl; abbreviated as RP; Raeburn et al., 1994) and the PDE5 inhibitor sildenafil were prepared at the chemical facilities of ALTANA Pharma AG. The activator of the soluble guanylyl cyclase BAY41-2272 (3-(4-Amino-5-cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1*H*-pyrazolo[3,4-*b*]pyridine) was purchased from ALEXIS Biochemicals. BAY58-2667 (4-[(4-Carboxy-butyl)-{2-[2-(4-phenethyl-benzyloxy)-phenyl]-ethyl}-amino)-methyl]-benzoic acid) was provided by the chemical facilities of ALTANA Pharma AG. Human recombinant TGF-β1 was purchased from R&D Systems GmbH (Minneapolis, USA), $PGE_2$ and ODQ (1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one) from Sigma-Aldrich GmbH (Taufkirchen, Germany) and DETA-NONOate from Cayman Chemicals (Ann Arbor, Michigan, USA). Monoclonal anti-alpha-smooth muscle actin antibody is a product of Sigma-Aldrich GmbH (Taufkirchen, Germany). The polyclonal rabbit anti-ERK2 (extracellular signal-regulated kinase 2) antibody and the polyclonal rabbit anti-connective tissue growth factor (CTGF) antibody were purchased from Santa Cruz Biotechnology Inc. (California, USA). Peroxidase-coupled secondary antibodies used for western blotting are a product of Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). cAMP ELISA kits are a product of Assay Designs (Ann Harbor, Michigan, USA).

### 2.2. Cell culture

Normal human lung fibroblasts (NHFL, Cambrex Bio Science, Wakersville, MD, USA) were cultured in Fibroblast Basal Medium (Cambrex Bio Science, Wakersville, MD, USA) plus 10% heat-inactivated fetal calf serum and 2 mM L-glutamine at 37 °C in an atmosphere containing 5% $CO_2$ up to passage six. The cell lines HFL-1, IMR-90 (European Collection of Cell Cultures, Salisbury, UK) and GM06114 (Coriell Institute for Medical Research, New Jersey, USA), were cultured in Dulbecco's Modified Eagle Medium plus 4500 mg/l glucose (Gibco, Invitrogen life technologies, Grand Island, N.Y., USA), 2 mM L-glutamine, 1 mM sodium pyruvate and 10% heat-inactivated fetal calf serum.

### 2.3. Measurements of phosphodiesterase isoenzyme activities and preparation of cellular extracts

Cells ($1–3 \times 10^6$) were washed twice in phosphate buffered saline (PBS) and resuspended in 1 ml homogenization buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM

$KH_2PO_4$, 10 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 5 mM pepstatin A, 10 mM leupeptin, 50 mM phenylmethyl-sulfonyl fluoride, 10 mM soybean trypsin inhibitor, 2 mM benzamidine, pH 8.2). Thereafter, cells were disrupted by sonification (Bandelin sonifier, Sonopuls HD2070, 3×15 s) and lysates were used for phosphodiesterase (PDE) activity measurements. PDE activities were assessed in cellular lysates as described by Thompson and Appleman (1979) with some modifications (Bauer and Schwabe, 1980). The assay mixture (final volume 200 μl) contained: Tris–HCl 30 mM; pH 7.4, $MgCl_2$ 5 mM, 0.5 μM of either cyclic AMP or cyclic GMP as substrate including [$^3$H]cAMP or [$^3$H]cGMP (about 30,000 c.p.m. per well), 100 mM EGTA, PDE isoenzyme-specific activators and inhibitors as described and cellular lysates. Incubations were performed for 30 min at 37 °C in 96-well plates. Thereafter, the reactions were terminated by adding 50 ml 0.2 M HCl per well. Assays were left on ice for 10 min and then 25 μg 5′-nucleotidase (Crotalus atrox) was added. Following an incubation for 10 min at 37 °C assay mixtures were loaded onto QAE-Sephadex A25 columns (1 ml bed volume). Columns were eluted with 2 ml 30 mM ammonium formiate (pH 6.0). Thereafter radioactivity in the eluate was measured. Results were corrected for blank values (measured in the presence of denatured protein) that were below 2% of total radioactivity. Cyclic AMP degradation did not exceed 25% of the amount of substrate added. The final DMSO concentration was 0.3% (v/v) in all assays. Selective inhibitors and activators of PDE isoenzymes were used to determine activities of PDE families as described previously (Rabe et al., 1993) with modifications. Briefly, PDE4 was calculated as the difference of PDE activities at 0.5 μM cyclic AMP in the presence and absence of 1 μM piclamilast. The difference between piclamilast-inhibited cyclic AMP hydrolysis in the presence and absence of 10 μM motapizone was defined as PDE3. The fraction of cyclic GMP (0.5 μM) hydrolysis in the presence of 10 μM motapizone that was inhibited by 100 nM sildenafil reflected PDE5. At the concentrations used in the assay piclamilast (RP, 1 μM), motapizone (10 μM) and sildenafil (100 nM) completely blocked PDE4, PDE3 and PDE5 activities without interfering with activities from other PDE families. PDE1 was defined as the increment of cyclic AMP hydrolysis (in the presence of 1 μM piclamilast and 10 μM motapizone) or cyclic GMP hydrolysis induced by 1 mM $Ca^{2+}$ and 100 nM calmodulin. The increase of cyclic AMP (0.5 μM) degrading activity (in the presence of 1 mM piclamilast and 10 mM motapizone) induced by 5 mM cyclic GMP represented PDE2. The PDE2 inhibitor PDP (100 nM) completely inhibited this cyclic GMP-induced activity increment further verifying that this activity is PDE2.

### 2.4. Treatment of human lung fibroblasts with TGF-β

$3\times10^5$ human lung fibroblasts were seeded into 6-well cell culture plates and cultured for 24 h. Thereafter, cells were washed with PBS, original medium was replaced with medium containing no fetal calf serum and cells were cultured for further 24 h until treatment with TGF-β. In the case of inhibitor studies the inhibitors were applied 30 min before TGF-β treatment. The final DMSO concentration in cell culture did not exceed 0.1% (v/v). 48 h after TGF-β treatment cells were harvested for subsequent western blot analysis by trypsinization, washed in PBS, and frozen as a cell pellet in liquid nitrogen.

### 2.5. Preparation of cell extracts

Frozen cell pellets were resuspended in sonification buffer (20 mM Tris–HCl pH 8.5, 1 mM EDTA, 5% (v/v) glycerin, 1 mM dithiothreitol, 0.5 mM phenylmethyl-sulfonyl fluoride) and sonicated (Bandelin sonicator, Sonopuls HD2070 plus cup booster BR30, 3×15 s). The resulting suspension was centrifuged at 20,000 ×g for 10 min at 4 °C to pellet cellular detritus. Supernatants were collected and protein concentration was determined by Bradford assay before western blotting.

### 2.6. Western blot analysis

Proteins (5 μg) of the samples were separated on a 7.5–12% SDS polyacrylamide gel. Thereafter, proteins were blotted onto a nitrocellulose transfer membrane (Protran nitrocellulose transfer membrane, Schleicher & Schuell Bioscience GmbH, Dassel, Germany) for 2–3 h. Membranes were blocked for 2 h in 5% (w/v) milk powder in PBS containing 0.1% Tween 20 (PBT), incubated for 2 h with the anti-alpha-smooth muscle actin antibody (1:2000) or anti-CTGF-antibody (1:1000), washed three times in PBT, and incubated for 1 h with a horseradish peroxidase-coupled secondary antibody (1:20,000). After final washing with PBT (3 times for 10 min each) blots were developed by using a chemiluminescence detection system (Lumi Light Plus, Roche GmbH, Mannheim, Germany) and a luminescent image analyzer (Fujifilm LAS1000 Pro). To control equal protein loading, blots were additionally hybridized with a primary anti-ERK2 antibody (1:3000) and proceeded further on. In some of the performed experiments the anti-ERK2 antibody detected two bands in western blotting representing the phosphorylated and unphosphorylated form of ERK2 as typically present in starved cells. A densitometric analysis of the blots was performed by using the AIDA image analyzer software. During the analysis the luminometric signal of interest (e.g. alpha-smooth muscle actin or PDE5) was normalized to the luminescence signal of ERK2.

### 2.7. Immunohistochemistry

$1\times10^5$ human lung fibroblasts were seeded in chamber slides. 24 h later original medium was replaced with medium containing no fetal calf serum and the cells were allowed to grow for another 24 h after treatment with TGF-β1, $PGE_2$ and inhibitors (RP). After 48 h the cells were fixed in ice-cold methanol/acetone (1:1) for 3 min, rehydrated for 30 min in TBS-buffer (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris–Base, pH 7.6) and incubated for 2 h with the monoclonal anti-alpha-smooth muscle actin antibody diluted 1:50 (v/v) in TBS-buffer. After three times washing with TBT-buffer the slides were incubated for 30 min with the Alexa Fluor 488 goat anti-mouse antibody

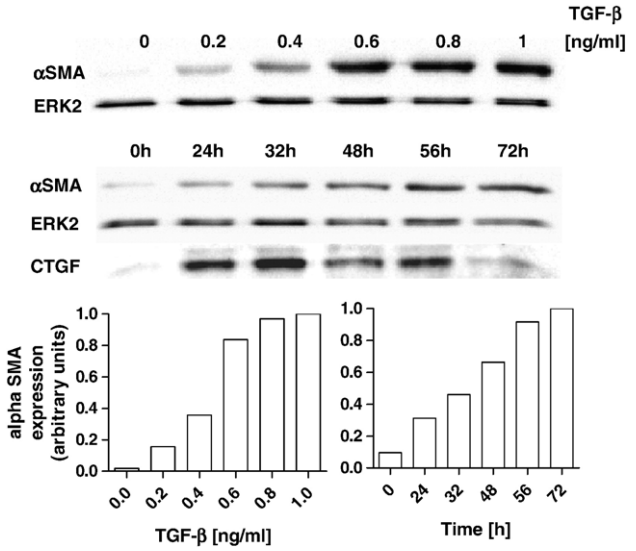


Fig. 1. Concentration- and time-dependent induction of alpha-smooth muscle actin and CTGF by TGF-β1 in HFL-1 lung fibroblasts. HFL-1 cells cultured for 24 h without fetal calf serum were treated with increasing concentrations of TGF-β1 and harvested for alpha-smooth muscle actin expression analysis after 48 h (upper panel). Otherwise, cells were incubated with 10 ng/ml TGF-β1 and harvested after 24, 32, 48, 56 and 72 h for analysis of alpha-smooth muscle actin and CTGF expression (lower panel). To confirm equal protein loading each blot was additionally hybridized with an anti-ERK2 antibody. A densitometric analysis of alpha-smooth muscle actin expression normalized to ERK2 was performed (see column graphs). The maximum alpha-smooth muscle actin signal was set to 1. αSMA, alpha-smooth muscle actin.

(1:100 (v/v)), washed again three times, covered with an anti-fading reagent and a glass slide and then analyzed by fluorescence microscopy. For a standardized comparison of samples photographs were taken under identical exposure times.

### 2.8. cAMP measurements

Human lung fibroblasts ($7.5 \times 10^5$ cells in 200 μl) were preincubated for 5 min with 100 nM piclamilast (or vehicle control) at 37 °C in 96-well plates. Thereafter, $PGE_2$ was added at different concentrations (1, 10 and 100 nM) as indicated. After an incubation of 30 min the reaction was stopped and cells were lysed by adding HCl to a final concentration of 0.3 M. Thereafter, the cellular pellet was removed by centrifugation at 1000 ×g for 5 min. Supernatants were stored at −20 °C until measurement. For this purpose samples were thawed, diluted 1:2 (v/v) with 0.1 M HCl and subsequently prepared as described by the manufacturer for the indirect cAMP ELISAs.

## 3. Results

For our studies we used various human lung fibroblast cell lines (HFL-1, GM06114, IMR-90) and primary normal human lung fibroblasts (NHFL) in order to consider a different genetic background of the cells and therefore avoiding generalized interpretation of results which might be simply due to cell-specific differences. In the inhibitor studies the TGF-β1 used to induce differentiation to myofibroblasts was varied between a suboptimal (0.4 ng/ml) and a supraoptimal (10 ng/ml) concentration since the local physiological concentrations of TGF-β in areas of fibrosis are not known and the effect of inhibitors might depend on them.

As shown in Fig. 1 for HFL-1 cells and in Fig. 2 for primary human lung fibroblasts, treatment with TGF-β1 dose- and time-dependently increased the expression of alpha-smooth muscle actin, a well-accepted marker of fibroblast to myofibroblast conversion (Gabbiani and Badonnel, 1976, Sousa et al., 2007). The expression was induced by culturing the cells with at least 0.2 ng/ml TGF-β1, and started about 24 h after stimulation. Higher concentrations than 1 ng/ml TGF-β1 did not further increase alpha-smooth muscle actin expression (data not shown). Interestingly the time course of induction somehow differed between the different cells analyzed (data not shown) indicating a genetic variability between these human lung fibroblast cell lines for this particular response. To verify the induction of differentiation to myofibroblasts by another marker, we analyzed the expression of CTGF which has been described to be induced by TGF-β as a down-stream signal, thereby forcing the differentiation to non-proliferating myofibroblasts by an interaction with insulin-like growth factor-2 (Grotendorst et al., 2004; Leask and Abraham, 2003). In contrast to alpha-smooth muscle actin its intracellular expression was rather transient after TGF-β treatment, which might hint to a secretion of CTGF out of the cells at later points in time after stimulation (Figs. 1 and 2).

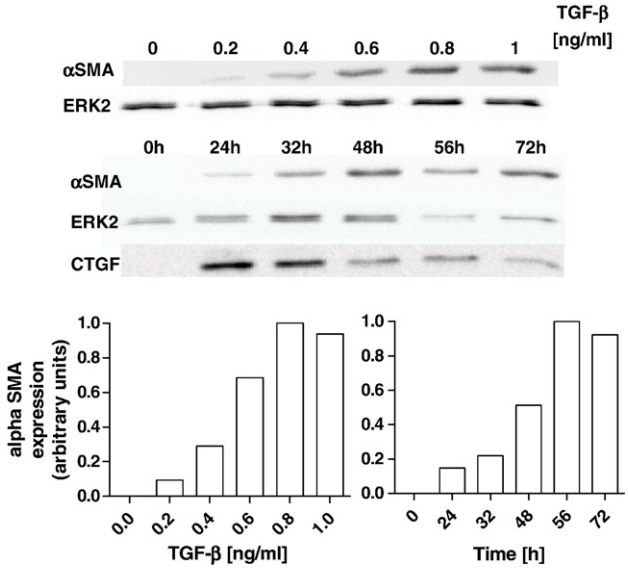


Fig. 2. Time- and concentration-dependent induction of alpha-smooth muscle actin and CTGF by TGF-β1 in normal human lung fibroblasts (NHFL). Normal human lung fibroblasts cultured for 24 h without fetal calf serum were treated with increasing concentrations of TGF-β1 and harvested for alpha-smooth muscle actin expression analysis after 48 h (upper panel). Otherwise, cells were incubated with 10 ng/ml TGF-β1 and harvested after 24, 32, 48, 56 and 72 h for analysis of alpha-smooth muscle actin and CTGF expression (lower panel). To confirm equal protein loading each blot was additionally hybridized with an anti-ERK2 antibody. A densitometric analysis of alpha-smooth muscle actin expression normalized to ERK2 was performed. The maximum alpha-smooth muscle actin signal was set to 1. αSMA, alpha-smooth muscle actin.

We investigated the activities of cyclic nucleotide phosphodiesterase family members in HFL-1 and IMR-90 before and 48 h after TGF-β1-induced differentiation to myofibroblasts. As shown in Fig. 3A and B the main PDE activities in these cells are PDE5 (hydrolysis of cGMP), PDE1 (hydrolysis of cAMP and cGMP), which was lower in HFL-1 cells than in IMR-90 cells and, to a really small extent, PDE4 (hydrolysis of cAMP). The expression of PDE4 and PDE5 protein and mRNA in human lung fibroblasts was confirmed by western blotting and Taq-Man-PCR (data not shown). We found no PDE2 and PDE3 activities. The same PDE activity pattern was observed for primary human lung fibroblasts (Fig. 3C). For comparison of fibroblasts emanating from different tissues we also analyzed the phosphodiesterase activities in human dermal fibroblasts (Table 1) and detected no PDE5 activity indicating that the

Table 1
Expression of family-specific phosphodiesterase activities in human dermal fibroblasts

| PDE subtype | Cyclic nucleotide hydrolysis (pmol/min/1 × $10^8$ cells +/− SD) |
|---|---|
| PDE1 (cAMP) | 479.0 +/− 22.0 |
| PDE1 (cGMP) | 1016.1 +/− 100.1 |
| PDE2 | 3.0 +/− 9.1 |
| PDE3 | 124.9 +/− 52.0 |
| PDE4 | 198.5 +/− 200.9 |
| PDE5 | 38.2 +/− 69.9 |

Human dermal fibroblasts were cultured up to passage three and subsequently harvested for analysis of PDE1- (cA and cG), PDE2-, PDE3-, PDE4- and PDE5-hydrolysis activity. The mean values and standard deviations (SD) of three independent experiments are shown.cA, cAMP; cG, cGMP.

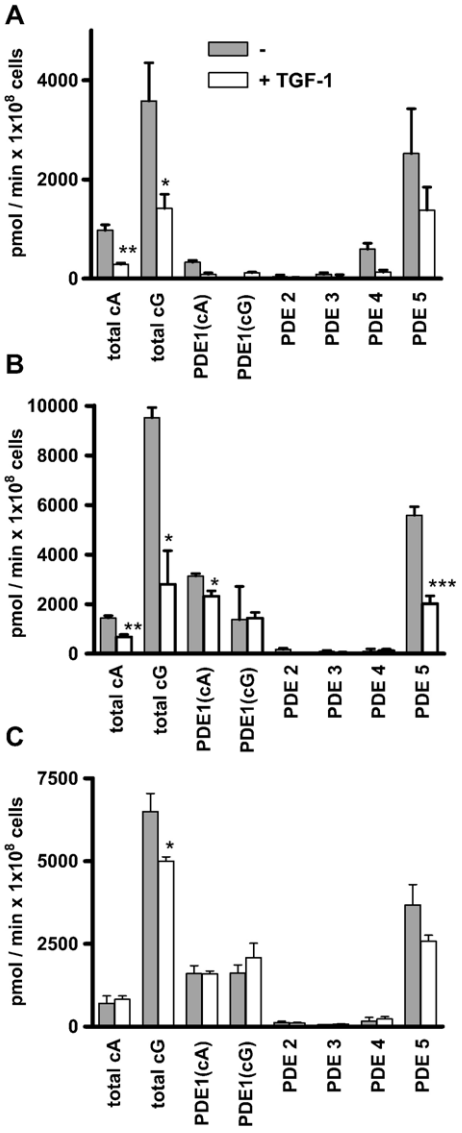


Fig. 3. Expression of family-specific phosphodiesterase activities in human lung fibroblasts. HFL-1 (A), IMR-90 (B) lung fibroblasts and primary human lung fibroblasts (C) cultured for 48 h with or without TGF-β1 (10 ng/ml) were analyzed for total cAMP-(total cA), total cGMP-(total cG), PDE1-(cA and cG), PDE2-, PDE3-, PDE4- and PDE5-hydrolysis activity. The mean values and standard deviations of three independent experiments are shown. Statistical analysis on the TGF-β1 induced effects was performed by use of the student's *t*-test. cA, cAMP; cG, cGMP; *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

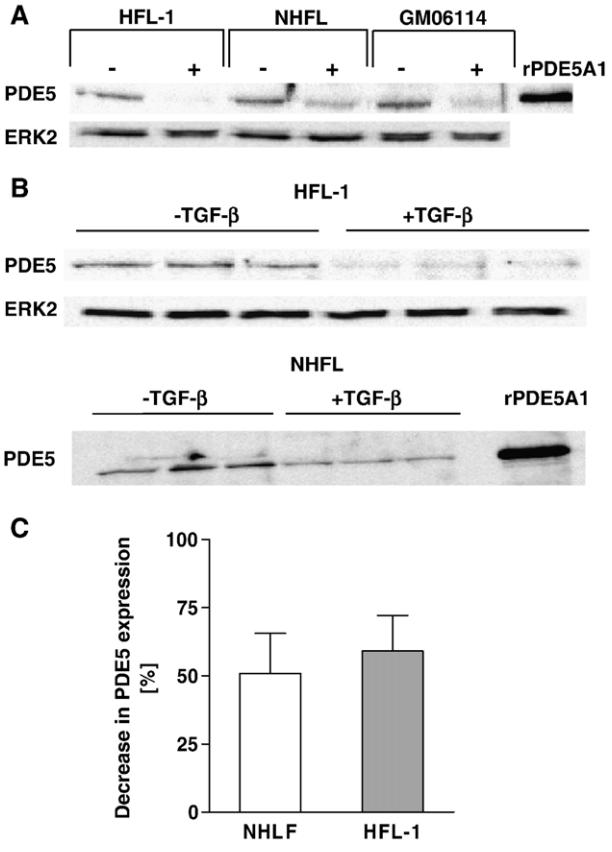


Fig. 4. Phosphodiesterase 5 protein becomes downregulated in human lung fibroblasts after TGF-β1-induced differentiation to myofibroblasts. A) HFL-1, GM06114 cells and normal human lung fibroblasts (NHFL) were cultured for 24 h without fetal calf serum and were subsequently treated with TGF-β1 for 48 h or were left untreated. Thereafter, cells were harvested for PDE5 protein expression analysis. B) HFL-1 and NHFL cells were treated with TGF-β1 for 48 h or left untreated. Three independent experiments were performed and the PDE5 protein expression of the cells is shown in one representative western blot for each cell type. C) Densitometric analysis of the western blots shown in B) was performed and the reduction of PDE5 protein expression due to TGF-β1 treatment was calculated (+/−standard deviation). To confirm equal protein loading blots were additionally hybridized with an anti-ERK2 antibody. Human recombinant PDE5A1 protein (rPDE5A1) was used as a control.

strong PDE5 activity is characteristic for fibroblasts of the lung. Interestingly, in human lung fibroblasts the cGMP-hydrolyzing activity per cell decreased significantly by about 50% after TGF-β1 treatment (Fig. 3A, B and C; total cG), mainly due to down-regulation of PDE5 activity. The overall cAMP hydrolysis per cell was reduced in HFL-1 and IMR90 cells but not in primary human lung fibroblasts and GM06114 cells (data not shown). Therefore, this response to TGF-β1 seems to be more variable and to depend on the genetic background of the cells analyzed. We ruled out that the reduced cGMP-phosphodiesterase activity per cell observed after TGF-β1 treatment was not simply due to a reduced amount of protein per cell by showing that protein does not change significantly by treatment with TGF-β1. Thus, for $1\times10^5$ HFL-1 cells the protein content was 10.9+/−1.0 mg before and 13.9+/−1.9 mg after TGF-β1 treatment, for NHFL 13.4+/−0.4 and 17.5+/−2.5 mg, respectively. Instead, western blotting for human PDE5A protein revealed a significant and reproducible down-regulation of PDE5 protein by about half in all TGF-β-treated human lung fibroblasts investigated (Fig. 4), indicating that down-regulation of the amount of protein is the reason for reduced PDE5 activity.

Since the major fraction of PDE activity in human lung fibroblasts seems to be PDE5, we speculated whether inhibition of this enzyme might be a favourable approach to prevent the TGF-β1-induced differentiation from fibroblasts to myofibroblasts. Therefore, we treated HFL-1 cells, GM06114 cells and primary human lung fibroblasts with sildenafil 30 min prior to

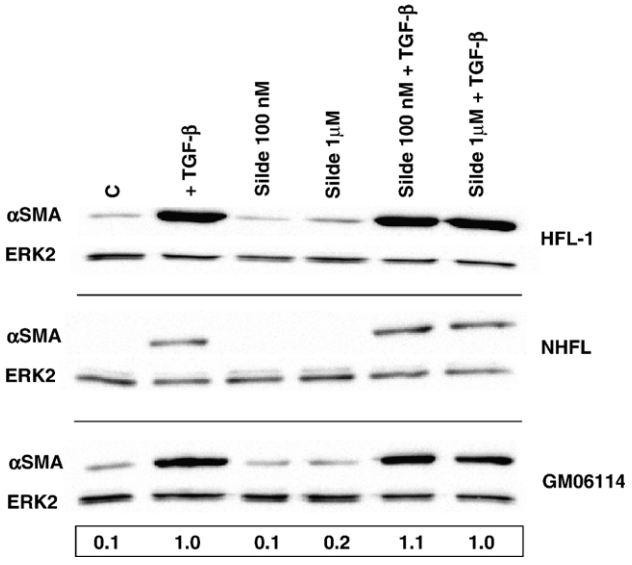


Fig. 5. Treatment with sildenafil does not affect TGF-β1-induced alpha-smooth muscle actin expression. HFL-1, GM06114 cells and normal human lung fibroblasts (NHFL) were cultured for 24 h without fetal calf serum and were subsequently treated with/without TGF-β1 (10 ng/ml) in the presence or absence of sildenafil (100 nM and 1 μM) for 48 h. Thereafter, cells were harvested and analyzed for alpha-smooth muscle actin expression by western blotting. To confirm equal protein loading each blot was additionally hybridized with an anti-ERK2 antibody. The mean values of a densitometric analysis of alpha-smooth muscle actin expression normalized to ERK2 of three independent experiments with human lung fibroblasts are shown in figures below the blots. The alpha-smooth muscle actin signal induced by TGF-β1 treatment was set to 1. Silde, sildenafil; αSMA, alpha-smooth muscle actin.

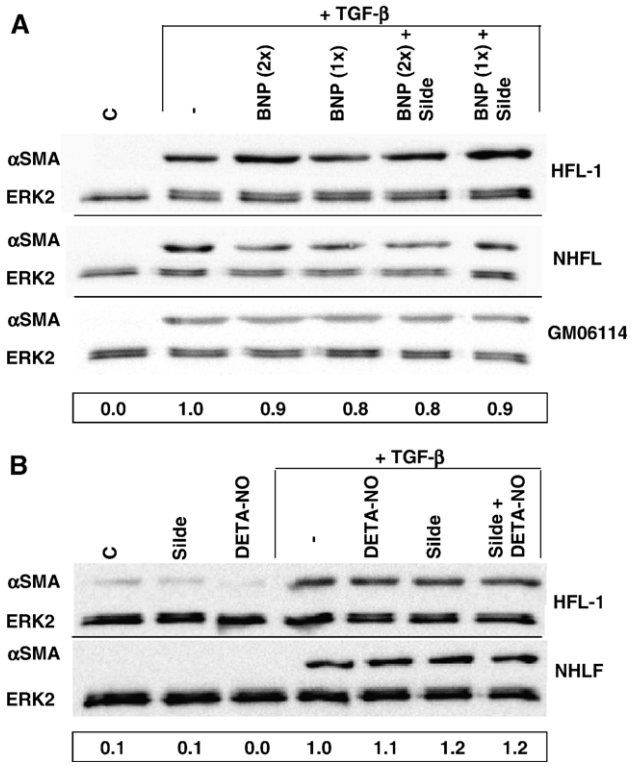


Fig. 6. Combined treatment with sildenafil and activators of particular or soluble guanylyl cyclase (brain natriuretic peptide/BNP and DETA-NONOate) does not affect TGF-β1-induced alpha-smooth muscle actin expression. A) HFL-1, GM06114 cells and normal human lung fibroblasts (NHFL) were cultured for 24 h without fetal calf serum and were subsequently treated with/without TGF-β1 (10 ng/ml) for 48 h in the presence or absence of sildenafil (1 μM) and of BNP (100 nM), which was applied once (1×) or twice (2×) per day. Thereafter, cells were harvested and analyzed for alpha-smooth muscle actin expression by western blotting. B) In another series of experiments TGF-β (0.4 ng/ml) treatment was combined with sildenafil (1 μM) and DETA-NONOate (100 nM). To confirm equal protein loading each blot was additionally hybridized with an anti-ERK2 antibody. A densitometric analysis of alpha-smooth muscle actin expression normalized to ERK2 was performed. The alpha-smooth muscle actin signal induced by TGF-β1 treatment was set to 1. The mean values of three independent experiments with human lung fibroblasts are shown as figures below the blots in A and B. Silde, sildenafil; DETA-NO, (*Z*)-1-[2-(2-Aminoethyl)-*N*-(2-ammonioethyl)-amino]-diazen-1-ium-1,2-diolate (DETA-NONOate); αSMA, alpha-smooth muscle actin.

TGF-β1 treatment (10 ng/ml) and measured the expression of alpha-smooth muscle actin after 48 h. As shown in Fig. 5 treatment with neither 100 nM sildenafil, which is selective for PDE5, nor with 1 mM sildenafil, which is an unselective concentration of this compound because PDE1 also becomes inhibited, did impair the induction of alpha-smooth muscle actin as well as its basal expression. The same is also true for the combination of sildenafil with brain natriuretic peptide (BNP) in order to activate the membrane-associated particulate guanylyl cyclase, or with the NO-donor DETA-NONOate which activates the soluble guanylyl cyclase (Fig. 6A and B). For these experiments BNP was added to the cells once or twice a day for 48 h because of the reported short half-life of this peptide (Kapoun et al., 2004). Since a possible inhibitory effect of sildenafil might depend on the strength of the signal evoked by TGF-β treatment (as discussed later) TGF-β1 was applied in

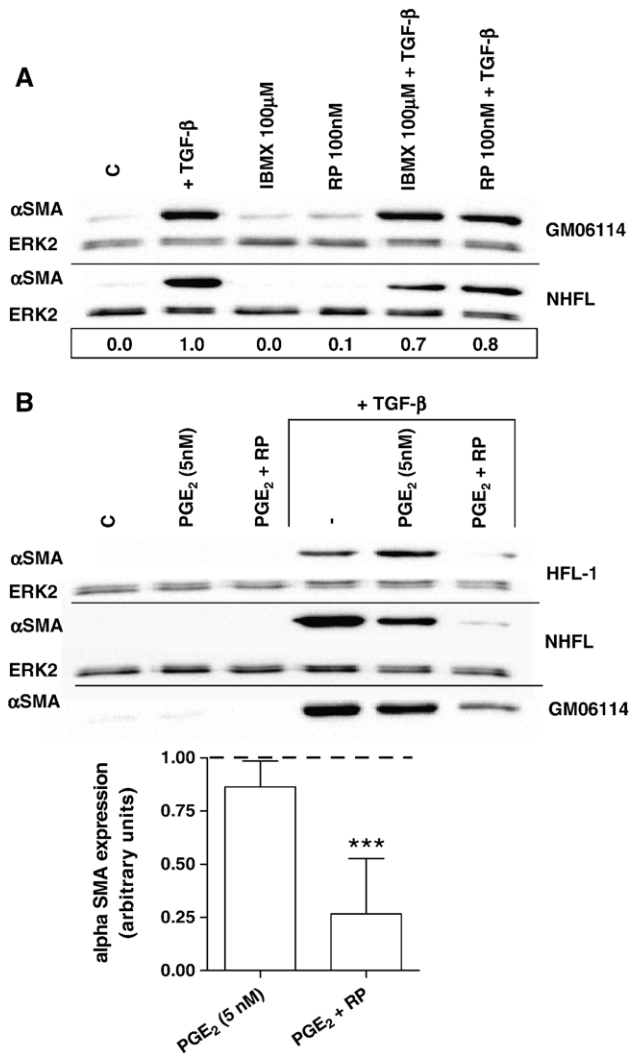


Fig. 7. The effects of treatment with the PDE4 inhibitor piclamilast, IBMX, and piclamilast plus $PGE_2$ on TGF-β1-induced alpha-smooth muscle actin expression. A) GM06114 cells and normal human lung fibroblasts (NHFL) were cultured for 24 h without fetal calf serum and were subsequently treated with/without TGF-β1 (10 ng/ml) for 48 h in the presence or absence of IBMX (100 μM) or the PDE4 inhibitor piclamilast (RP, 100 nM). Thereafter, cells were harvested and analyzed for alpha-smooth muscle actin expression by western blotting. The figures below the blots indicate the mean expression of alpha-smooth muscle actin resulting from a densitometric analysis. The alpha-smooth muscle actin signal induced by TGF-β1 treatment was set to 1. B) HFL-1 cells, normal human lung fibroblasts (NHFL) and GM06114-cells were cultured for 24 h without fetal calf serum and were subsequently treated with/without TGF-β1 (10 ng/ml) for 48 h in the presence or absence of the PDE4 inhibitor piclamilast (RP, 100 nM), $PGE_2$ (5 nM) or both. Afterwards, cells were harvested and analyzed for alpha-smooth muscle actin expression by western blotting. To confirm equal protein loading each blot was additionally hybridized with an anti-ERK2 antibody. A densitometric analysis of the alpha-smooth muscle actin signal normalized to the ERK2 signal was performed. The column graph shows the significant (***, $p<0.005$, *t*-test) reduction of alpha-smooth muscle actin expression by $PGE_2$ plus piclamilast treatment. The dashed line indicates the expression of alpha-smooth muscle actin evoked by TGF-β1 treatment, which was set to 1. αSMA, alpha-smooth muscle actin.

two concentrations, 10 ng/ml and 0.4 ng/ml. These approaches also failed to affect alpha-smooth muscle actin expression.

In addition to PDE5 inhibition, treatment with the unspecific PDE inhibitor IBMX (3-isobutyl-1-methylxanthine) and the highly PDE4-selective inhibitor piclamilast (Hatzelmann and Schudt, 2001) alone did also only slightly influence alpha-smooth muscle actin expression induced by treatment with 10 ng/ml TGF-β1, as shown for GM06114 and NHFL cells (Fig. 7A). However, combining piclamilast with $PGE_2$ as an adenylate cyclase (AC) activator synergistically suppressed alpha-smooth muscle actin expression significantly ($p<0.005$, *t*-test) and thus fibroblast to myofibroblast transition as shown both by western blotting (Figs. 7B and 8A) and immunhistochemistry (Fig. 8B). The latter also demonstrates the filamentous staining of alpha-smooth muscle actin, which proves the specificity of the antibody used. Accordingly, under conditions of $PGE_2$ plus piclamilast treatment intracellular cAMP concentrations increased in a synergistic manner

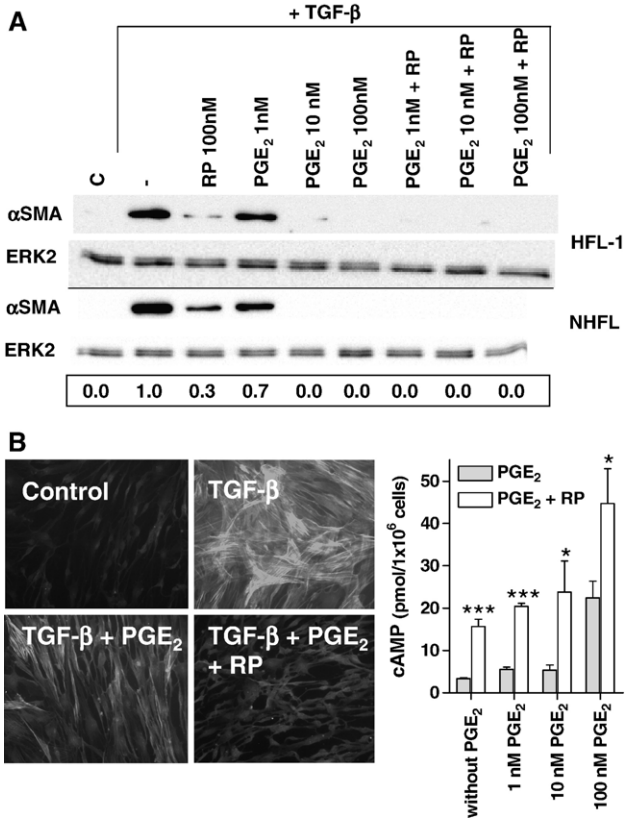


Fig. 8. Combined treatment with a PDE4 inhibitor and $PGE_2$ synergistically inhibits TGF-β1 induced alpha-smooth muscle actin expression in human lung fibroblasts by increasing cAMP. A) GM06114-cells and normal human lung fibroblasts (NHFL) were cultured for 24 h without fetal calf serum and were subsequently treated with/without TGF-β1 (0.4 ng/ml) for 48 h in the presence or absence of piclamilast (RP, 100 nM) plus/minus $PGE_2$ (1, 10, 100 nM). Thereafter, cells were harvested and analyzed for alpha-smooth muscle actin expression by western blotting. To confirm equal protein loading each blot was additionally hybridized with an anti-ERK2 antibody. B) Pictures on the left: Immunohistochemistry for alpha-smooth muscle actin on NHFL cells treated with/without TGF-β1 (0.4 ng/ml) for 48 h in the presence of $PGE_2$ (1 nM) plus/minus piclamilast (RP, 100 nM). Pictures were taken by applying identical exposure times. Right panel: Intracellular cAMP concentrations in lung fibroblasts after treatment with piclamilast (RP, 100 nM) plus/minus $PGE_2$ (1, 10, 100 nM). The mean values plus/minus standard deviations of three experiments are shown. A statistical analysis (*t*-test) was performed for comparison of $PGE_2$ versus $PGE_2$ plus piclamilast treatment. *, $p<0.05$; ***, $p<0.005$; αSMA, alpha-smooth muscle actin.

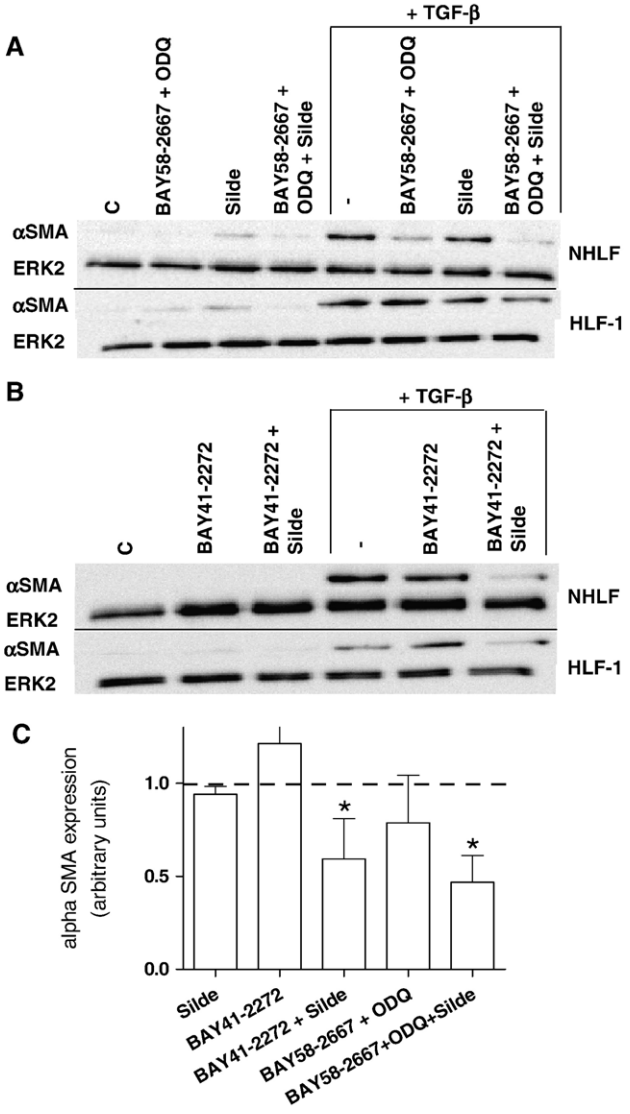


Fig. 9. Combined treatment with BAY58-2667, ODQ and sildenafil or BAY41-2272 plus sildenafil suppresses induction of alpha-smooth muscle actin by TGF-β1 in human lung fibroblasts. Normal human lung fibroblasts (NHFL) and HFL-1 cells were cultured for 24 h without fetal calf serum and were subsequently treated with/without TGF-β1 (0.4 ng/ml) for 48 h in the presence or absence of sildenafil (1 μM) with BAY58-2667 (10 μM) plus ODQ (10 μM; A) or BAY41-2272 (10 μM; B). Thereafter, cells were harvested and analyzed for alpha-smooth muscle actin expression by western blotting. To confirm equal protein loading each blot was additionally hybridized with an anti-ERK2 antibody. Silde, sildenafil; ODQ, (1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one). A densitometric analysis of the alpha-smooth muscle actin signal normalized to the ERK2 signal was performed. The corresponding column graph shows the significant reduction of alpha-smooth muscle actin expression caused by the different treatment conditions. A statistical analysis (t-test) was performed, which proves the down-regulation of alpha-smooth muscle actin by BAY41-2272 plus sildenafil (*, $p<0.05$) and BAY58-2667/ODQ/sildenafil treatment ($p<0.05$). The dashed line indicates the expression of alpha-smooth muscle actin evoked by TGF-β1 treatment, which was set to 1. αSMA, alpha-smooth muscle actin.

(Fig. 8B, right panel). Whereas treatment of lung fibroblasts with 5 nM $PGE_2$ only had a minor effect, the combination with a PDE4 inhibitor strongly blocked alpha-smooth muscle actin expression (Fig. 7B). Interestingly, the PDE4 inhibitor by itself also suppressed alpha-smooth muscle actin expression when cells were stimulated with submaximal 0.4 ng/ml TGF-β1 (Fig. 8A, third lane), whereas it was ineffective when cells were treated with a supramaximal concentration of 10 ng/ml TGF-β1 (Fig. 7A, last lane). This observation might reflect a distinct balance between the fibrosis-promoting TGF-β1 signaling intensity and the blocking effect(s) of increased intracellular cAMP pool(s).

To see whether activation of the sGC might also affect lung fibroblast to myofibroblast differentiation we treated NHFL and HFL-1 cells with BAY58-2667 and ODQ and combined these treatments with sildenafil. ODQ, which is by itself an inhibitor of sGC, has been published to potentiate the effect of BAY58-2667 on sGC activation by increasing Vmax (Schmidt et al., 2003) and was thus included in this experimental setup. Fig. 9A and C shows that BAY58-2667 (10 μM) combined with ODQ (10 μM) only partially suppressed the induction of alpha-smooth muscle actin by TGF-β1; however, the combination with sildenafil, strongly reduced alpha-smooth muscle actin expression indicative for a synergistic action. This finding was confirmed using another GC activator (BAY41-2272), which by itself inactive suppressed TGF-β1 induced alpha-smooth muscle actin expression if combined with sildenafil (Fig. 9B and C).

## 4. Discussion

Increasing intracellular cyclic nucleotide concentrations by means of selective inhibitors of phosphodiesterases or activators of soluble guanylyl cyclase is a therapeutic approach under clinical investigation and clinical use for various lung diseases such as asthma, COPD and pulmonary hypertension (Evgenov et al., 2004; Lee et al., 2005; Lipworth, 2005). Until now the therapeutic potential of this approach for fibrotic lung diseases has not been fully investigated. Within this study we analyzed the expression of the PDE family members 1–5 and showed that besides PDE1, PDE5 is the predominant cGMP-hydrolyzing enzyme in human lung fibroblasts and plays the major role in cGMP hydrolysis in these cells. cAMP hydrolysis, which is minor in comparison to cGMP, is mediated by PDE1 and PDE4.

It should be pointed out that in dermal fibroblasts no PDE5 activity could be detected. This clearly shows that a distinct cell type might be characterized by its inherent PDE activity due to a specific tissue localisation.

The detection of PDE4 and PDE5 activity in different human lung fibroblasts adds to the work of Kraft et al. (2004) who analyzed PDE activities in rat lung fibroblasts. Thus, we can presume that no major differences between rat and human lung fibroblasts exist which are of crucial importance for the interpretation of results arising from PDE inhibitor testing in animal (rat) models of fibrosis in ongoing work. Furthermore, Martin-Chouly et al. (2004) and Kohyama et al. (2002) described inhibitory effects of PDE4 inhibitors like cilomilast on cytokine secretion or matrix metalloproteinase production by human lung fibroblasts, further supporting the expression and crucial function of PDE4 in these cells.

Interestingly, TGF-β1 induced differentiation to myofibroblasts was accompanied by a reduction in cGMP hydrolysis,

PDE5-activity and PDE5 protein expression. However, PDE5 still remained the prominent PDE activity. These observations are in line with Kraft et al. (2004) who reported reduced PDE5-activity with increasing passage numbers of rat lung fibroblasts which might be due to myofibroblast formation. One might speculate that down-regulation of cGMP hydrolysis is associated with a reduced impact of cGMP signaling on myofibroblast function.

Despite the high expression and activity of PDE5 in human lung fibroblasts no functional correlate of PDE5 inhibition has been published yet. However, recent publications revealed that sildenafil and vardenafil reduce the synthesis of collagen and the expression of alpha-smooth muscle actin of myofibroblasts derived from the fibrotic tissue of patients with Peyronie's disease (Valente et al., 2003; Ferrini et al., 2006). In addition, in a rat model of Peyronie's disease sildenafil prevented fibrotic plaque formation by up to 90% indicating that PDE5 inhibitors might be useful to counteract fibrosis under certain circumstances. Thus, it is obvious to ask whether inhibition of PDE5 might inhibit lung fibroblast to myofibroblast differentiation induced by TGF-β1 or reduce alpha-smooth muscle actin expression of existing myofibroblasts. By using alpha-smooth muscle actin as a marker for TGF-β1-induced fibroblast to myofibroblast differentiation we could not observe any effect of sildenafil on differentiation independent of whether fibroblasts were stimulated with sub- or supraoptimal concentrations of TGF-β1. Also by combining a particular guanylyl cyclase activator like the vasoactive hormone BNP with sildenafil no effects could be shown. The same is true for the basal expression of alpha-smooth muscle actin by preexisting myofibroblasts observed in our culture, which was also not affected by these treatments. We should point out that treatment with BNP by itself was also ineffective in suppression of TGF-β1-induced alpha-smooth muscle actin. This might be cell type-specific since a previous publication by Kapoun et al. (2004) demonstrated reduced expression of alpha-smooth muscle actin mRNA in TGF-β-treated cardiac fibroblasts upon BNP treatment by DNA chip analysis and real time PCR. However, in that study alpha-smooth muscle actin protein expression was not investigated. Incubation of TGF-β1-treated lung fibroblasts with the NO-donor DETA-NONOate, a stimulator of soluble guanylyl cyclase, alone or in combination with sildenafil did not affect alpha-smooth muscle actin expression. However, the combined treatment of sildenafil with BAY58-2667 and ODQ inhibited alpha-smooth muscle actin expression strongly whereas BAY58-2667, ODQ and its combination were only partly effective. BAY58-2667 has been described as a potent activator of sGC. While it activates this enzyme by itself its activity is much higher in the presence of ODQ (Stasch et al., 2001). In addition, combined treatment of sildenafil with another NO-independent activator of the sGC (BAY41-2272) was also effective in preventing TGF-β1-induced alpha-smooth muscle actin expression.

Since DETA-NONOate, BAY58-2667 and BAY41-2272 are all activators of the soluble guanylyl cyclase this data seems contradictory, but might be explained by the fact that DETA-NONOate as a NO-donor is consumed in the cell culture and thus might be active only transiently and more moderately compared to ODQ plus BAY58-2667 or BAY41-2272. Thus, our data indicate that under conditions of high and persistent guanylyl cyclase activation (as induced by BAY58-2667 and ODQ or BAY41-2272 but probably not by DETA-NONOate treatment) PDE5 plays a role in controlling TGF-β1-induced alpha-smooth muscle actin expression. Therefore, PDE5 might be a therapeutic target for blocking one of the key steps in progression of lung fibrosis, namely the differentiation of fibroblasts to myofibroblasts.

This also holds true for PDE4. Despite its low activity, which in lung fibroblasts as well as in TGF-β1-induced lung myofibroblasts was highly reproducible, we could demonstrate that the PDE4 inhibitor piclamilast synergistically enhances the anti-fibrotic effect of the lipid mediator $PGE_2$ even at concentrations as low as 100 nM. Whereas treatment with low concentrations of $PGE_2$ only partially inhibited TGF-β1-induced alpha-smooth muscle actin expression the combination with piclamilast completely blocked alpha-smooth muscle actin expression as shown by western blotting and immunohistochemistry without inducing any cytotoxicity (measured by lactat dehydrogenase release; data not shown). $PGE_2$ by itself concentration-dependently suppressed alpha-smooth muscle actin expression. The inhibitory effect of $PGE_2$ on fibroblast to myofibroblast transition has already been reported and depends on prostaglandin E receptor 2 signaling and increased cAMP levels (Kolodsick et al., 2003). Our own results show that the localized subcellular cAMP pool controlled by PDE4, which might be small based on the overall minor activity of PDE4 measured in the whole cell lysate, distinctly modulates the TGF-β-induced signaling leading to the phenotypic switch from lung fibroblast to myofibroblast.

It is interesting to note that piclamilast itself in the absence of $PGE_2$ suppressed alpha-smooth muscle actin expression induced by a low concentration of TGF-β (0.4 ng/ml), whereas it was unable to do so at the higher concentration of 10 ng/ml TGF-β (as IBMX was also unable to do so). This means that the gating effect of a PDE4 inhibitor on TGF-β-induced signaling depends on the signal intensity elicited by the stimulus TGF-β. We can only speculate whether PDE4 inhibitors will inhibit lung fibrosis in-vivo. Obviously, this will depend on the balance between local TGF-β and $PGE_2$ concentrations in the fibrotic and adjacent area and the tissue concentration and potency of the PDE4-inhibiting drug.

Of further impact on the therapeutic use of PDE4 inhibitors in lung fibrosis is the observation that PDE4 inhibitors do not suppress the basal expression of alpha-smooth muscle actin in lung fibroblast cultures attributed to the presence of a low number of myofibroblasts (e.g. induced by a weak autocrine TGF-β release; Marshall et al., 2004; Kang et al., 2004; Skutek et al., 2001). This implies that the expression of the alpha-smooth muscle actin by differentiated myofibroblasts and/or the survival of existing myofibroblasts cannot be affected by PDE4-controlled cAMP signaling, whereas the differentiation step induced by TGF-β signaling is target of PDE4-controlled intracellular cAMP pools. The same mechanism of action has

been reported for interferon gamma (IFN-γ) which blocks TGF-β1-induced differentiation to myofibroblasts but does not affect differentiated cells (Gu et al., 2004).

How might cyclic nucleotides inhibit TGF-β induced myofibroblast formation? The identification of the target(s) of cAMP within the TGF-β-induced signal transduction pathway controlling fibroblast to myofibroblast differentiation is rather unclear. A recent publication by Schiller et al. (2003) provided evidence that cAMP-elevating agents via activation of protein kinase A (PKA) inhibit TGF-β2 signaling by preventing the interaction of the TGF-β2-induced SMAD (mothers against decapentaplegic homolog) 3/4 complex with the transcription co-activators cAMP response element binding (CREB)-protein and p300. However, these studies were done using HaCat keratinocytes and it remains unclear whether this mechanism would also transfer to lung fibroblasts. Since it has been reported that TGF-β signaling is only a transient prerequisite for the induction of fibroblast to myofibroblast differentiation and that this process is sustained by a subsequent secretion and autocrine action of CTGF in concert with insulin-like growth factor-2 induced by TGF-β (Grotendorst et al., 2004), it is also possible that signaling evoked by these factors is a target of PDE4-controlled cAMP. Thus Stratton et al. (2002) provided first evidence that activation of the Ras/MEK/ERK pathway is required for TGF-β-induced CTGF expression and that this pathway is inhibited by prostacyclin derivatives in a PKA-dependent manner. In cardiac fibroblasts cAMP-elevating agents inhibit the profibrotic effects of TGF-β largely through inhibiting ERK1/2 phosphorylation but also by reducing Smad-mediated recruitment of transcriptional co-activators (Liu et al., 2006). Also cGMP has recently been reported to inhibit TGF-β1 induced signaling by inhibiting Smad2 and Smad3 nuclear translocation but not phosphorylation in a protein kinase G-dependent manner (Li et al., 2007; Saura et al., 2005).

In summary, our data provide evidence of a crucial role of PDE4 and PDE5 in the control of TGF-β induced phenotypic switch from fibroblast to myofibroblasts. This extends the role of PDE4 in fibroblasts beyond secretion of cytokines (Kohyama et al., 2004), production of matrix metalloproteinases (Martin-Chouly et al., 2004), contraction and chemotaxis (Kohyama et al., 2002). Modulation of intracellular cAMP in human lung fibroblasts by inhibition of PDE4 as well as interference with the cGMP signaling by activation of soluble guanylyl cyclase and/or inhibition of PDE5 might be a therapeutic approach to mitigate progression of lung fibrosis, although probably not to resolve fibrosis. Whether inhibitors of PDE5 or PDE4 will be more effective in-vivo remains open, but may depend on the basal activities of adenylate and guanylyl cyclases in the fibrotic tissue.

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