Pentoxifylline and its active metabolite lisofylline attenuate transforming growth factor β₁-induced asthmatic bronchial fibroblast-to-myofibroblast transition*

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Bronchial asthma is characterized by persistent airway inflammation and airway wall remodeling. Among many different cells and growth factors triggering changes in bronchi structure, transforming growth factor β₁-induced fibroblast to myofibroblast transition is believed to be very important. The aim of this study was to evaluate whether theophylline (used in asthma therapy) and two other methylxanthines (pentoxifylline and its active metabolite lisofylline), may affect transforming growth factor β₁-induced fibroblast to myofibroblast transition in bronchial fibroblasts derived from asthmatic patients. We show here for the first time that selected methylxanthines effectively reduce transforming growth factor β₁-induced fibroblast transformation in asthmatic bronchial fibroblast populations. PTX was found to be the most effective methylxanthine. The number of differentiated myofibroblasts after PTX, LSF and THEO administration was reduced at least twofold. Studies on the use of methylxanthines opens a new perspective in the development of novel strategies in asthma therapy through their two-pronged, anti-inflammatory and anti-fibrotic action. In the future they can be considered as promising anti-fibrotic drugs.

Key words: theophylline, pentoxifylline, lisofylline, transforming growth factor type β₁, fibroblast-to-myofibroblast transition, asthma

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INTRODUCTION

Bronchial asthma is one of the most common chronic airway diseases, developing rapidly in recent years (GINA 2015). Asthma is considered to be an immune-mediated disorder due to its pathophysiological mechanism. It is commonly believed that irreversible changes in the structure of the bronchial tree, i.e. bronchial wall remodeling, are the consequences of chronic and protracted inflammation. However, not only immune cells and the variety of inflammatory mediators that they secrete are involved in triggering and prolongation of inflammation, but also different bronchial structural cells. There are some explicit indications that fibrotic lesions in the bronchi may appear before the first asthma symptoms (Baldwin & Roche, 2002; Beckett & Howarth, 2003). The process of bronchial wall remodeling underlines onerous and dangerous symptoms such as wheezing, coughing, shortness of breath and finally bronchial obstruction (Shifren et al., 2012). Bronchial wall remodeling includes defective epithelium regeneration, mucous hypersecretion, smooth muscle cell hypertrophy and hyperplasia, increased deposition of extracellular matrix (ECM) proteins, and significant increase in myofibroblast populations (Al-Muhсен et al., 2011). Myofibroblasts can originate from different sources but predominantly are formed by fibroblast-to-myofibroblast transition (FMT) from tissue-specific fibroblasts (Bergeron et al., 2010). Myofibroblasts, due to α-SMA expression, contractile properties and enhanced ECM synthesis, display a phenotype intermediate between fibroblasts and smooth muscle cells and are principally responsible for bronchial wall stiffening and fibrosis (Phan, 2008). A wide variety of cytokines and growth factors cause FMT in vitro but the most common and potent FMT inducer is transforming growth factor type β₁ (TGF-β₁) (Batra et al., 2004, Michalik et al., 2009, 2011). An increased level of this growth factor has been described in the airways of asthmatics (Makinde et al., 2007). Our previous studies indicate that TGF-β₁ is responsible for increased FMT in asthmatics (Michalik et al., 2009). Moreover, the two isoforms of this growth factor, TGF-β₁ and TGF-β₂, are equally responsible for increased HBFs predisposition to FMT in asthmatics (Michalik et al., 2009). TGF-β₁ is a pleiotropic factor and can activate a number of different signalling pathways among which the most important is the canonical, Smad-dependent pathway. The primary TGF-β effector is a substrate of TGF-β receptor serine-threonine kinase, R-Smad protein (Smad-2 and 3). The phosphorylated R-Smads interact with Smad-4 (2:1) and translocate to the nucleus where they affect target gene transcription (including profibrotic genes) (Dijke & Hill, 2004). TGF-β₁ signalling activity can be modulated by other signalling systems (Makinde et al., 2007; Michalik et al., 2012).

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Abbreviations: α-SMA, α-smooth muscle actin; AS, asthmatic; BSA, bovine serum albumin; CTGF, connective tissue growth factor; Cytokeratin; ECM, extracellular matrix; FDA/EB, fluoresceine diacetate/ethidium bromide; FMT, fibroblasts to myofibroblasts transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBFs, human bronchial fibroblasts; LSF, lisofylline; PIC, protease inhibitor cocktail; PTX, pentoxifylline; TGF-β₁, transforming growth factor type β₁; THEO, theophylline.
Bronchial asthma pharmacotherapy is based mainly on anti-inflammatory drugs. Most commonly, inhaled or systemic glucocorticosteroids are used. Typical treatment also involves β2-agonists, Cromones, methylxanthines, antileukotrienes and antihistamine drugs (GINA 2015). All these drugs affect inflammation extinction, but only marginally influence remodeling changes (Mauad et al., 2007). Due to a smaller number of reports dealing with remodeling as one of the causes of asthma, challenges in obtaining airway tissue and lack of non-invasive markers, there is still no available therapy aimed directly against the fibrotic response of bronchi.

Methylxanthines are bronchodilating drugs sometimes used in asthma pharmacotherapy, but currently only theophylline (THEO) is used in clinical practice (GINA 2015). Because of THEO side effects, there have been attempts to synthesize analogues which could be safer for patients. Methylxanthines are successfully used in the treatment of difficult cases related to pediatric lung diseases (Onatibia-Astibia et al., 2016). They are reported to exhibit anti-inflammatory properties (Fredholm, 1985; Ito et al., 2002; Tilley, 2011). The mechanism of action of methylxanthines has not been fully elucidated, but it is presumably based on their ability to inhibit cellular phosphodiesterases, enzymes responsible for the cAMP degradation. Consequently this second messenger is accumulated inside the cell.

There are reports showing that some anti-inflammatory drugs may decrease subepithelial reticular basement membrane thickening and subepithelial fibrosis (Ward et al., 2002, Kasahara et al., 2002). One possible explanation of these observations may be a direct inhibitory effect on bronchial wall remodeling. Following proceedings and reports indicating that remodeling may develop much earlier than the first asthma symptoms, we investigated the effects of selected methylxanthines on human bronchial fibroblasts (HBFs) isolated from asthmatic (AS) patients. Our study was aimed to assess THEO currently used in asthma therapy, and two other methylxanthines – pentoxifylline (PTX) and its active metabolite – lisofylline (LSF) on TGF-β1-induced FMT in HBFs cultures derived from asthmatics. We investigated the effect of selected methylxanthines on the TGF-β1-induced α-SMA level and myofibroblasts number. We also checked the influence of the studied compounds on the canonical TGF-β1/Smad signalling pathway, in particular TGF-β1-induced Smad-2 phosphorylation and translocation to the nucleus.

MATERIALS AND METHODS

Cell culture. The study was performed on human bronchial fibroblasts (HBFs) derived from asthmatic patients (n=5). All patients were treated in the Department of Medicine of Jagiellonian University. The study was approved by the University’s Ethics Committee (KBET 122.6120.69.2015). Informed, written consent was obtained from all study participants. HBFs were isolated from patients diagnosed with bronchial asthma (2 males, 3 females; average age: 43.4±8.7 years; mean FEV1%: 79.5±12.1% of predicted). All patients were under inhaled glucocorticosteroid therapy, and samples were collected in remission of disease. The protocol of HBFs isolation were described previously (Pierzchalska et al., 2003; Michalik et al., 2009). HBFs were cultured between 5–20 passages in DMEM supplemented with glucose (4.5 g/L), 10% fetal bovine serum (FBS), antimycotics and antibiotics in standard culture conditions (3% CO2, 37°C, 95% humidity). Apart from viability and proliferation tests, all experiments were carried out in serum-free culture medium supplemented with 0.1% bovine serum albumin (BSA).

Compound preparation. HBFs were exposed to TGF-β1 (5 ng/ml), PTX, LSF and THEO (5–100 μM). TGF-β1 was obtained from BD Bioscience, THEO and PTX (Fig. 1) were obtained from Sigma Aldrich. LSF (PTXM1(R)) was prepared by the biotransformation method (Fig. 1). Details are described in Pękala and coworkers (2007). In brief, stereoreduction of PTX to LSF was conducted using whole cells of Lactobacillus kefiri strain DSM 20587 which is a natural source of R-specific alcohol dehydrogenase. Biotransformation was performed with regeneration of the cofactor NADPH, in 0.2 M potassium phosphate buffer, pH 6.5 with 5 mM MgCl2, and with glucose as a co-substrate. PTX and LSF identification was determined by HPLC analysis and optical rotation of the LSF was confirmed by polarimetric analysis. TGF-β1, working solution was prepared according to manufacturer’s protocol in 1mg/ml of BSA. THEO, PTX and LSF were dissolved in water to 1mg/ml stock solution (logS =−0.78, −1.18, −1.69 respectively). No compound aggregation or precipitation was observed. Cells were preincubated with THEO, PTX and LSF for 3h and then exposed to TGF-β1.

Cell viability and proliferation assays. For viability assay, cells were seeded at an initial density of 5×10^4 cells/cm², in standard medium. After 24 hrs of culture, cells were incubated with selected methylxanthines and cultured for a further 24 or 48 hrs. Cell viability was determined by fluoresceine diacetate/ethidium bromide (FDA/EtBr) staining using a Leitz Orthoplan fluorescence microscope and expressed as percent of fluoresceine-positive/EtBr-negative cells. Cells were counted in different, representative fields of view (10–20). At least 500 cells were counted in triplicates.

For proliferation assay, cells were seeded at an initial density of 5×10^4 cells/cm², in standard medium. After 24 hrs of culture cells were incubated with selected methylxanthines and cultured for the next 1, 3, 5 or 7 days. After incubation, cells were fixed with 3.7% formaldehyde and then stained with 0.05% crystal violet (CV; in methanol/water (1:4)) solution. Then CV solution excess was removed and cells were carefully rinsed with distilled water. CV was eluted with 1.33% citric acid/1.09% sodium citrate in methanol/water (1:1). Absorbance was read at 540 nm.

Immunocytochemical staining. Visualization of α-SMA positive cells was made by immunocytochemical staining. HBFs were fixed with 3.7% paraformaldehyde, permea-
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RESULTS

To investigate the potential effects of theophylline, pentoxifylline and theophylline on bronchial wall remodeling, we first evaluated the effect of selected methylxanthines (administered at concentrations which can be achieved in vivo in patient serum, i.e. 5–100 μM and which are acceptable in therapy), on HBFs viability and proliferation rate. The results of the experiments revealed that the investigated methylxanthines did not exhibit a significant cytotoxic effect on HBFs in culture (Fig. 2A). Furthermore, we show that fibroblast proliferation was not greatly affected by THEO, PTX and LSF (Fig. 2B). These observations suggest that methylxanthines do not have any harmful, cytotoxic or cytostatic effects at the cellular level.

We next investigated selected methylxanthines impact on TGF-β1-induced FMT in our experimental model. As illustrated in Fig. 3A, the percentage of myofibroblasts in TGF-β1-treated HBFs populations was lower after THEO, PTX and LSF treatment. The number of myofibroblasts (cells with α-SMA incorporated into F-actin stress fibers) in HBFs populations was gradually reduced by increasing concentrations (10–100 μM) of all tested compounds in the culture medium (Fig. 3B). PTX proved to be the most effective methylxanthine. All compounds administered alone did not affect the FMT potential of HBFs (Fig. 3A, B). Subsequently we investigated whether the observed reduced number of myofibroblasts after methylxanthine exposure is reflected in the amount of α-SMA protein level. As measured by ELISA and Western Blot assays, the TGF-β1-induced α-SMA level in HBFs decreased moderately but significantly after PTX and LSF exposure when compared to TGF-β1 administered alone (Fig. 4A, B respectively).

In order to confirm whether the observed differences in FMT efficiency (number of myofibroblasts) and the level of α-SMA in TGF-β1-induced HBFs were associated with activation of the TGF-β1/Smad pathway, we measured the level of Smad-2 phosphorylation and p-Smad translocation to the nucleus (Fig. 5). As illustrated in Fig. 5C none of the investigated methylxanthines reduced TGF-β1-induced Smad-2 phosphorylation. However all of them caused inhibition of p-Smad-2 translocation to the nucleus (Fig 5A, B). Among all studied methylxanthines, again the most potent compound was PTX.

DISCUSSION

Methylxanthines are bronchodilatory drugs that are not usually used as first-line drugs in asthma and chronic ob-
Structural pulmonary disease (Tilley, 2011; Margay et al., 2015). Although the first reports of beneficial methylxanthine effects in asthma come from the nineteenth century when intake of strong coffee was recommended in order to prevent asthmatic symptoms (Persson, 1985), their mechanism of action has been still not fully elucidated. However, it is known that by inhibiting phosphodiesterase activity, accumulates cAMP inside the cells (Tilley, 2011). Theophylline used at low doses has been shown to exert immunomodulatory function by activation of histone deacetylases (Ito et al., 2002). Recently, increasing data have shown that some methylxanthines have anti-inflammatory, neuroprotective and cardioprotective effects (Ohta & Sitkovsky, 2011; Gu & Lambert, 2013; Saglani & Lloyd, 2015) and may have great potential in prevention and therapy of different diseases, including asthma (Oñatibia-Astibia et al., 2016).

The majority of drugs currently available in bronchial asthma therapy were designed to extinguish inflammation. However, they do not affect (or affect only marginally) the process of remodelling (Berair & Brightling, 2014). Despite intensive research on the process of remodelling in asthma, no drug can directly inhibit fibrotic changes in the bronchial tree. The anti-inflammatory drugs used in asthma (glucocorticosteroids, β2-adrenergic receptor agonists, leukotriene modifiers et al.) can potentially affect bronchial wall remodelling (sub-epithelial fibrosis, ECM protein deposition, ASM hyperplasia and proliferation) indirectly by their action on inflammatory cells and mediators that are known as effectors in the remodelling process (Berair & Brightling, 2014). Methylxanthines have also been shown to reduce eosinophils and improve lung function in asthma (Sullivan et al., 1994; Wang et al., 2011) but their direct effect on airway remodelling is unknown. With this in mind, we veri-

Figure 3. THEO, PTX and LSF limit TGF-β1-induced FMT in asthmatic HBFs.
HBFs (n=4) were pre-incubated for 3h with THEO, PTX and LSF (10–100 μM) and then exposed to TGF-β1 (5 ng/ml) for 7 days. (A) Representative images of F-actin cytoskeleton in HBFs cultures are presented on the top panels. α-SMA positive cells in HBFs cultures are shown on the bottom panels. Bar=100 μm (B) Number of myofibroblasts in HBFs cultures. At least 200 cells were counted in triplicates. Values represented as means with S.E., *p<0.05. THEO, theophylline; PTX, pentoxifylline; LSF, lisofylline; HBFs, human bronchial fibroblasts; TGF-β1, transforming growth factor type β1; α-SMA, α-smooth muscle actin.

Figure 4. THEO, PTX and LSF slightly reduce TGF-β1-induced α-SMA expression in asthmatic HBFs.
HBFs (n=4) were pre-incubated for 3 h with THEO, PTX and LSF (50 μM) and then exposed to TGF-β1 (5 ng/ml) for 7 days. Relative α-SMA protein level was measured by ELISA (A) and Western Blot (B) analysis. (A) Values represented as means with S.E., *p<0.05. Experiments were run in quadruplicates. (B) Representative immunoblot of α-SMA and GAPDH level. THEO, theophylline; PTX, pentoxifylline; LSF, lisofylline; TGF-β1, transforming growth factor type β1; α-SMA, α-smooth muscle actin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
fied whether the selected methylxanthines affect the FMT process, an important remodelling component in asthma pathogenesis. We show here that THEO, PTX and LSF significantly decrease TGF-β-1-induced FMT in HBF cultures derived from bronchial asthma patients. Our results strongly indicate that among all tested compounds the most potent was PTX. This methylxanthine reduced to the greatest extent the number of myofibroblasts in HBF populations. Interestingly, activity comparable to the parent compound was shown by its active metabolite, LSF. PTX is relatively stable after administration into the body. However it is worth noting that the product of its metabolism also affects the FMT process. This could be critical in terms of possible PTX dosage. It should be emphasized that PTX is applied in therapy in a border range of concentrations compared to THEO. There are reports showing that PTX may possess more potent anti-inflammatory effects in asthma than THEO (Entzian et al., 1998). Moreover, PTX has been proven to have anti-fibrotic properties for liver, cardiac and submucosal fibrosis (Prabhu et al., 2015; Khalifa & Nemenqani, 2014; Zhang et al., 2016). LSF was earlier reported to decrease ECM protein synthesis (such as collagen IV and laminin) by mesangial cells (Bolick et al., 2003).

To our knowledge this is the first report showing the anti-fibrotic effects of THEO, PTX and LSF on HBFs derived from asthmatics. Corresponding results were obtained by Yano et al. who revealed that THEO may reduce profibrotic gene (α-SMA and COL1) expression in human lung fibroblasts (Yano et al., 2006). The authors demonstrated that THEO was able to exert anti-fibrotic effects through a cAMP-PKA dependent pathway. Our results do not confirm this observation. The observed reduction of the α-SMA protein level by the cAMP analogue in TGF-β-1-simulated HBFs (unpublished data) along with a lack of a significant reduction of TGF-β-1-induced α-SMA level by THEO, indicates that cAMP-accumulation by methylxanthines, although reducing FMT in HBF populations, is not the most important effect. It cannot be excluded that methylxanthines affect other signalling pathways in HBFs derived from asthmatics but not in normal lung fibroblasts. We have previously shown that HBFs isolated from asthmatic patients display some inherent features which facilitate their TGF-β-1-induced FMT to fibroblasts derived from non-asthmatic donors (Michalk et al., 2009; 2012; 2013; Sarna et al., 2015).

Our finding of an inhibitory effect of PXF and LSF on TGF-β-1-induced FMT in asthmatic HBF populations place these drugs among interesting candidates for anti-fibrotic therapy in asthma, but the mechanism of their action needs to be clarified. Our results revealed that PTX and LSF significantly attenuate the TGF-β-1-induced FMT by inhibition of p-Smad-2 translocation to the nucleus, which is a key event during the activation of TGF-β-1 signalling. However, the evaluated methylxanthines did not significantly affect Smad-2 phosphorylation. These observations suggest that the tested methylxanthines may interact with cytoplasmic Smad proteins by inhibiting their nuclear accumulation and TGF-β-1-specific signal transduction, resulting in the induction of profibrotic gene expression (including α-SMA) in HBF isolated from asthmatic patients. This is consistent with the study of Lin et al. who reported that PTX is able to block Smad3/4 activated transcription in tubulointerstitial fibrosis (Lin et al., 2005). Furthermore they demonstrated that PTX may be a potent CTGF inhibitor. Our earlier studies on HBFs revealed that CTGF is crucial for TGF-β-1-induced FMT, moreover under the TGF-β-1 HBFs derived from asthmatics are able to synthesize a large amount of this growth factor (Wójcik et al., 2012). Therefore, the most powerful PTX effect can be explained by the potential impact of this compound on CTGF secretion in HBFs. However, this hypothesis needs to be confirmed by further research.

Our results concern the effect of methylxanthines on FMT, but there are other processes associated with remodeling. PTX is used in therapy because of its spasmylytic properties against vascular smooth muscle cells but it has also been shown to inhibit the proliferation of airway smooth muscle cells (Chiou et al., 2006; Sang et al., 2015). ASM hyperplasia is one of the causes of airway narrowing in asthma. Admittedly this requires further research, but it cannot be excluded that PTX, besides reducing TGF-β-1-induced FMT, may also affect the smooth muscle layer.

It can be concluded that both PTX and LSF are promising compounds in the context of searching for new therapeutic strategies in asthma, acting directly on bronchial wall remodeling. The group of methylxanthines and their derivatives may be a fruitful direction in research on new anti-fibrotic agents.

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Conflict of interest

Authors declare no conflict of interest.

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