



Pulmonary, gastrointestinal and urogenital pharmacology

Lovastatin-induced decrease of intracellular cholesterol level attenuates fibroblast-to-myofibroblast transition in bronchial fibroblasts derived from asthmatic patients



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ABSTRACT

Chronic inflammation of the airways and structural changes in the bronchial wall are basic hallmarks of asthma. Human bronchial fibroblasts derived from patients with diagnosed asthma display in vitro predestination towards TGF- β -induced fibroblast-to-myofibroblast transition (FMT), a key event in the bronchial wall remodelling. Statins inhibit 3-hydroxymethyl-3-glutaryl coenzyme A reductase, a key enzyme in the cholesterol synthesis pathway and are widely used as antilipidemic drugs. The pleiotropic anti-inflammatory effects of statins, independent of their cholesterol-lowering capacity, are also well established. Since commonly used anti-asthmatic drugs do not reverse the structural remodelling of the airways and statins have tentative anti-asthmatic activity, we have studied the effect of lovastatin on FMT in populations of human bronchial fibroblasts derived from asthmatic patients. We demonstrate that the intensity of FMT induced by TGF- β_1 was strongly and dose-dependently attenuated by lovastatin. Furthermore, we show that neither the suppression of prenylation of signalling proteins nor the effect on reactive oxygen species formation are important for lovastatin-induced inhibition of myofibroblast differentiation. On the other hand, we show that a squalene synthase inhibitor, zaragozic acid A, reduced the TGF- β_1 -induced FMT to an extent comparable to lovastatin effect. Additionally we demonstrate that in bronchial fibroblast populations, both inhibitors (lovastatin and zaragozic acid A) attenuate the TGF- β_1 -induced Smad2 nuclear translocation in a manner dependent on intracellular cholesterol level. Our data suggest that statins can directly, by decrease of intracellular cholesterol level, affect basic cell signalling events crucial for asthmatic processes and potentially prevent perilous bronchial wall remodelling associated with intensive myofibroblast formation.

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1. Introduction

Bronchial asthma is one of the most common chronic diseases throughout the world, characterised by a continuously increasing rate of prevalence over the last decades (Anandan et al., 2010). Recent studies indicate that a “vicious circle” of chronic inflammation of the airways and structural changes in the bronchial wall are crucial for the development of asthma (Durrani et al., 2011; Holgate, 2010). Airway remodelling is characterized by epithelial damage and subepithelial fibrosis including extracellular matrix deposition and the accumulation of myofibroblasts mainly as a result of fibroblast-to-myofibroblast transition (FMT)

(Al-Muhsen et al., 2011; Descalzi et al., 2007). The cellular and molecular mechanisms of bronchial wall remodelling are not yet fully understood. It is well documented that FMT is triggered by transforming growth factors β (TGF- β) and the pro-inflammatory cytokines released by cells of airway walls (Halwani et al., 2011; Hardie et al., 2009). TGF- β is a principal mediator of subepithelial fibrosis and is highly expressed in asthmatic bronchi (Batra et al., 2004; Redington et al., 1997). However, our previous research revealed that bronchial fibroblasts derived from asthmatic patients have some inherent features which facilitate their TGF- β -induced FMT (Michalik et al., 2009, 2012).

Commonly used anti-inflammatory drugs (corticosteroids, β_2 agonists and anticholinergic agents) are applied in asthma management since they have been shown to interfere with processes leading to the structural changes of the asthmatic airway wall. However, their ability to prevent or reverse airway remodelling during the natural history of this disease is still controversial

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(Barnes, 2006; Mauad et al., 2007; Riccioni et al., 2004). This has prompted research on the implementation of complementary forms of preventive and therapeutic anti-asthmatic strategies. Statins are lipid-lowering agents widely used to reduce hyperlipidemia (Evans et al., 2004; Rutishauser, 2011). Accumulating data demonstrate that apart of inhibition of 3-hydroxymethyl-3-glutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in the cholesterol synthesis pathway, statins have anti-inflammatory, anti-fibroproliferative and immunomodulatory effects that are independent of their lipid-lowering effect (Lahousse et al., 2012; Ridker et al., 2005). The pleiotropic effects of statins can be mediated by antioxidant mechanism, the inhibition of prenylation of small GTP-binding signalling molecules or activation of peroxisome proliferator-activated receptors (PPAR) (Copaja et al., 2012; Liao and Laufs, 2005; Marzilli, 2010). Accordingly, the tentative anti-asthmatic activity of statins suggested by epidemiological (Huang et al., 2011) and clinical studies (Braganza et al., 2011; Hothersall et al., 2008) was interpreted in terms of their interference with airway inflammation (Imamura et al., 2009; McKay et al., 2004).

On the other hand, the effect of statins on the responsiveness of constitutive bronchial cells to extrinsic cues has not been studied in the context of asthma. In particular, it is not known how statins influence the potential of human lung fibroblasts to adopt a TGF- β -induced myofibroblastic phenotype, a phenomenon previously described in human tenon's fibroblasts (Meyer-Ter-Vehn et al., 2008). In the current study, we aimed to elucidate the mechanisms of lovastatin effect on the efficiency of TGF- β -induced FMT in the populations of human bronchial fibroblasts (HBFs) derived from asthmatic patients.

2. Materials and methods

2.1. Cells and culture conditions

Primary human bronchial fibroblasts (HBFs) were isolated from bronchial biopsies obtained during bronchoscopy examination from 8 patients diagnosed with moderate asthma (according to GINA 2009). The average age in the group was: 42 ± 6.8 years; mean duration of asthma: 7 years; the mean value of forced expiratory volume in one second, per cent of predicted (FEV_1) 73 ± 8 ; female per cent 63. All asthmatics were atopic non-smokers and used inhaled β_2 -agonists on demand; two of them used inhaled corticosteroids. The patients were treated in the Department of Medicine of the Jagiellonian University and were in stable clinical condition. The study was approved by the Jagiellonian University's Ethics Committee (KBET/362/B/2003) and informed, written consent was obtained from all study participants. HBFs were cultured in DMEM with 10% foetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO₂ and used between 5 and 15 passages. Cells were plated at a density of 5000 cells/cm² and cultured in serum-free DMEM supplemented with 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) (see Michalik et al., 2009, 2011). When indicated human recombinant TGF- β_1 (BD Biosciences, Franklin Lakes, NJ, USA) was administered 24 h after cell seeding. In some experiments N-acetyl-cysteine (Sigma-Aldrich) or methyl- β -cyclodextrin (Sigma-Aldrich) were added to the culture medium (1 h before TGF- β_1 stimulation).

2.2. Administration of lovastatin

Lovastatin (Sigma-Aldrich) was dissolved in ethanol (Sigma-Aldrich). It was converted from its inactive lactone to its active dihydroxyl open acid form by the addition of 2 vol of lovastatin dissolved in ethanol to 1 vol 0.05 mM NaOH, incubated for 20 min at

37 °C, and subsequently neutralized with 0.05 mM HCl to pH 7.2. For experiments, lovastatin was diluted in the culture medium from stock solution (6.67 mM) to the working concentrations (from 2.5 up to 20 μ M) so that the final concentration of ethanol in the culture medium did not exceed 1.56%, i.e., a concentration below the limits defined by the effect of this solvent on cell viability, proliferation and TGF- β_1 -induced FMT (data not shown).

2.3. Proliferation and viability tests

For cell viability tests, HBFs were cultivated in DMEM with 10% FCS for 24 h, then the cultured medium was replaced with DMEM supplemented with 0.1% BSA and containing a range of lovastatin concentrations (from 0 to 20 μ M). Cell viability was determined by the fluorescein diacetate/ethidium bromide (EB) test using Leitz Orthoplan fluorescence microscopy system and expressed as percentage of fluorescein-positive/EB-negative cells.

For proliferation tests, cells were seeded at an initial density of 10×10^3 cells/cm², incubated for 24 h at 37 °C, then the culture medium was replaced with fresh DMEM supplemented with 0.1% BSA with or without lovastatin. The incubation time varied from 1 to 7 days; next the cells were harvested by trypsinization, and counted using Bürker's chamber.

2.4. Immunofluorescence staining

Myofibroblasts were identified by immunodetection of α -smooth muscle actin (α -SMA) as described previously (Michalik et al., 2012). In brief, cells growing on glass coverslips were fixed in 3.7% paraformaldehyde, permeabilised in 0.1% Triton X-100, blocked with 1% BSA and incubated with a mouse monoclonal antibody against human α -SMA (clone 1A4, Sigma-Aldrich), and Alexa Fluor 488 goat anti-mouse IgG (clone A11001, Sigma-Aldrich). Visualization of specimens mounted in polyvinyl alcohol (Mowiol; Sigma-Aldrich) was performed with a Leica DMIRE2 microscope. A similar protocol was used for immunodetection of p-Smad (with a rabbit polyclonal antibody against Smad2, phospho S467, Abcam) and compatible secondary antibodies conjugated with Alexa Fluor 546 (Invitrogen, Carlsbad, CA, USA). Nuclei were stained with Hoechst 33342 (Sigma-Aldrich) where indicated.

2.5. Isoprenoid supplementation studies

HBFs were incubated in DMEM with 10% FBS for 24 h, then the cultured medium was replaced with DMEM supplemented with 0.1% BSA, 5 ng/ml TGF- β_1 and lovastatin (5 or 10 μ M) and 10 μ M of either geranylgeranyl pyrophosphate (GGPP) or farnesyl pyrophosphate (FPP) (Sigma-Aldrich) was added to the lovastatin-containing medium. In some experiments mevalonate (250 or 500 μ M, Sigma-Aldrich) was added to the culture medium with or without lovastatin. Next, HBFs were cultured in supplemented medium for 7 days, and the cells were fixed and stained for α -SMA and monitored for reversal of lovastatin-induced morphological changes and α -SMA expression.

2.6. Preparation of protein extracts and immunoblotting

Cells were cultured in Petri dishes in control or experimental conditions until confluence for 7 days. Afterwards, total cell proteins were collected using the lysis buffer (0.1 mol/l Tris-HCl, 15% glycerol, 2 mM EDTA, 2% SDS, 10 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml aprotinin, pH 7.4). The protein concentration was determined using the Bradford method and aliquots containing 10 μ g of protein from whole cell lysates were separated by 15% SDS-polyacrylamide gel electrophoresis under reducing conditions and then transferred onto a polyvinylidene

difluoride membrane (Hybond-P, Amersham Pharmacia Biotech). After blocking in PBS-T (0.1% Tween 80 in PBS) containing 5% skimmed milk at room temperature, the membrane was incubated with mouse monoclonal anti- α -SMA antibody (Sigma-Aldrich, 1:1000), mouse monoclonal anti-GAPDH antibody (Sigma-Aldrich, 1:10000), goat polyclonal anti-Smad2 antibody (AbD Serotec, 1:500) or rabbit polyclonal antibody against p-Smad2 (1:1000, Abcam), respectively. After washing, the membrane was incubated with the relevant secondary antibody conjugated with horseradish peroxidase (1:3000, Invitrogen). The bands were detected with the chemiluminescent reagent Super Signal West Pico Substrate (Pierce, Rockford, IL) and chemiluminescence was recorded using a MicroChemi imager (ALAB).

2.7. Cholesterol content assay

HBFs were seeded at a density of $0.75 \times 10^4/\text{cm}^2$ in DMEM supplemented with 10% FBS, cultivated for 24 h. Then, the medium was aspirated and cells were incubated in DMEM supplemented with 0.1% BSA and 5 or 10 μM lovastatin or 50 μM zaragozic acid A for 7 days. Afterwards, the cellular lipid fraction was extracted in 0.5 ml of hexane-isopropanol (3:2)—30 min with shaking. After collection of the sample, a second round of lipid extraction was performed with 250 μl of hexane-isopropanol. The samples were pooled and evaporated under a gentle stream of nitrogen. Lipids were redissolved in 0.5 ml of buffer (0.1 M potassium phosphate, pH 7.4, 50 mM NaCl, 5 mM cholic acid, and 0.1% Triton X-100) for 5 h with shaking. The cholesterol content was determined by the fluorometric method using Amplex Red Cholesterol Assay Kit (Invitrogen) according to the manufacturer's directions and the Infinite M200PRO microplate reader (Tecan).

2.8. Measurement of intracellular reactive oxygen species

HBFs were seeded at a density of 10×10^3 cells/ cm^2 and cultured in DMEM containing 10% FBS. 16 h before reactive oxygen species level measurements, the medium was replaced with DMEM containing 0.1% BSA and vehicle (control) or TGF- β_1 in concentration 5 and 10 ng/ml. Cells treated with 50 μM H_2O_2 for 90 min directly before measurements served as a positive control. Intracellular reactive oxygen species level measurements were conducted using 2',7'-dichlorodihydrofluorescein-diacetate (DCFH-DA, Sigma) as described previously (Kurosawa et al., 2009) with minor modifications. Cells were loaded with 20 μM DCFH-DA in PBS with Ca^{2+} and Mg^{2+} containing 5.6 mM glucose and 1% FBS for 10 min in darkness and 37 °C. After staining, the cells were washed twice with PBS and immediately used for experiments. Images were captured with a wide field fluorescence microscope DMI6000B equipped with adequate filters (ex. BP 480/40, dichroic mirror 505 nm and em. BP 527/30). The time between the end of staining and observations was strictly controlled. All images were background subtracted and the fluorescence intensity of individual cells was analyzed with the LAS AF 2.6.0.7266 program.

3. Results

3.1. Lovastatin inhibits the proliferation and attenuates TGF- β_1 -induced FMT of asthmatic bronchial fibroblasts in a dose-dependent manner

Statins have been investigated as a potential cell growth inhibitor mainly in the context of rapidly dividing cancer cells. The antiproliferative activity of lovastatin was attributed to reduced levels of mevalonate and its downstream products (including cholesterol), which regulate membrane integrity, cell

signalling and cell cycle progression (Brusselmans et al., 2007; Chan et al., 2003; Depasquale and Wheatley, 2006). Therefore, we first evaluated the influence of lovastatin on HBF proliferation in serum free and serum containing medium, i.e., under conditions suppressing and promoting cell proliferation, respectively. Lovastatin dose-dependently inhibited HBF proliferation in all concentrations tested (2.5–20 μM). This effect was visible both in fast growing (in the presence of serum containing medium—Fig. 1A) and in slow growing cultures (in serum-free conditions—Fig. 1B). On the other hand, lovastatin did not profoundly reduce the percentage of viable cells in concentrations up to 20 μM even in serum free conditions during the first three days of culture. During longer incubation in lovastatin containing medium, for up to one week, the viability of cells slightly decreased in a dose-dependent manner (Fig. 1C).

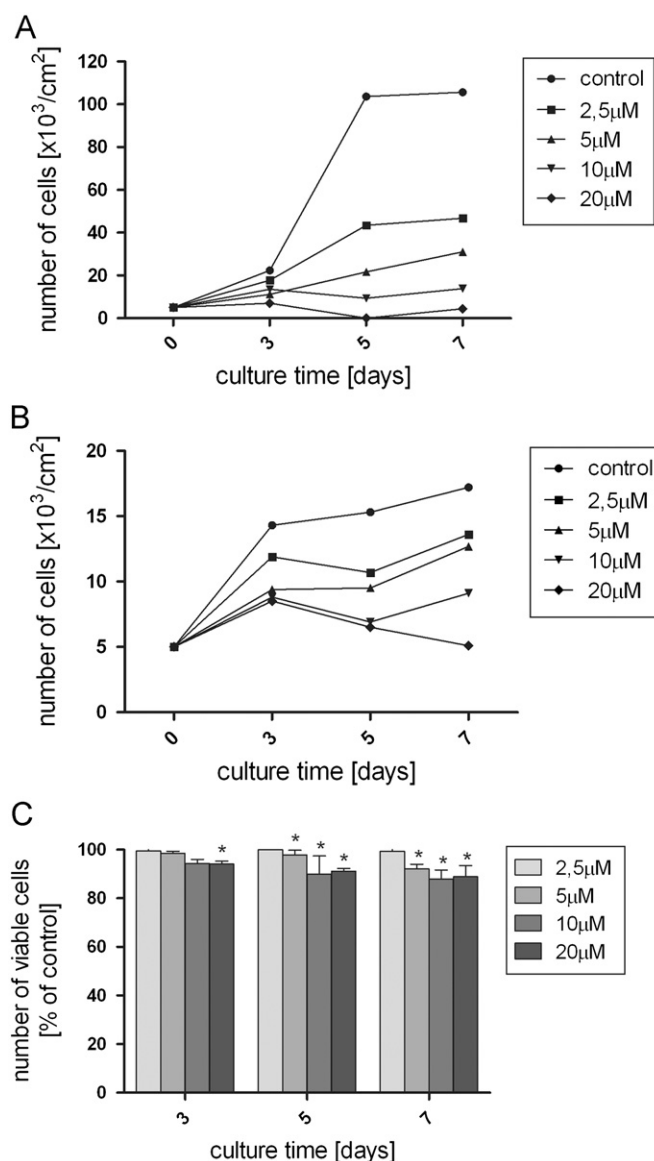


Fig. 1. Cell proliferation and viability in lovastatin treated HBF populations. HBFs derived from asthmatic patients were cultured in medium containing 10% FBS (A) or in serum free medium supplemented with 0.1% BSA ((B) and (C)) in the absence or in the presence of lovastatin in concentrations of 2.5, 5, 10 or 20 μM for 3, 5 or 7 days and then the total number of cells was counted ((A) and (B)) and the number of viable cells was detected using the fluorescein diacetate/ethidium bromide viability test. Values represent means $\pm$ SD of three independent experiments. The significance of differences between untreated (control) and lovastatin-treated cells was determined with a Kruskal–Wallis non-parametric ANOVA, (*) $P < 0.05$.

As transdifferentiation of HBFs towards a myofibroblastic phenotype is crucial for the process of bronchial wall remodelling in asthma and is interrelated with their proliferation, we checked the effect of lovastatin on TGF- β_1 -induced FMT. Fibroblasts derived from asthmatics were cultured in serum free medium supplemented with TGF- β_1 in the absence or in the presence of 2.5–20 μ M lovastatin. After one week of incubation, we quantified the cells positive for α -SMA (the typical marker of myofibroblasts) in HBF populations and found considerably smaller fractions of α -SMA expressing HBFs in the lovastatin containing media (Fig. 2). The inhibition of FMT was dose-dependent; in the presence of 20 μ M lovastatin, the number of myofibroblasts did not exceed 20%, i.e., the level of FMT efficiency observed upon TGF- β treatment in non-asthmatic HBF populations (Michalik et al. 2009).

3.2. Effects of GGPP, FPP and mevalonic acid on lovastatin-inhibited FMT in asthmatic HBFs

Statins are inhibitors of mevalonate synthesis from HMG-CoA—the first step in cholesterol synthesis. However, the inhibition of mevalonate synthesis also affects production of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate

(GGPP)—intermediate isoprenoids, which have been shown to affect posttranslational modification of a range of signalling molecules, including small G proteins (Liao, 2002). To investigate whether such modifications are relevant to the observed FMT inhibition, we attempted to reverse the action of lovastatin on TGF- β_1 -induced FMT by the addition of GGPP or FPP to the culture medium. Both of these isoprenoids turned out to be ineffective as demonstrated by counting the myofibroblasts (Fig. 3A) and confirmed by immunoblotting of α -SMA (Fig. 3B). In contrast, the lovastatin effect was partially reversed when mevalonate was added to the culture medium, suggesting that, indeed, the attenuation of FMT by lovastatin was caused by its inhibiting capacity against HMG-CoA reductase. These results indicate that the observed effects are not associated with the prenylation of signalling proteins. Instead, the inhibition of the cholesterol synthesis pathway might be essential for the statin-induced attenuation of HBF transdifferentiation.

3.3. TGF- β_1 induced FMT of asthmatic HBFs is not dependent on reactive oxygen species generation

Increased formation of reactive oxygen species caused by the excessive TGF- β has been observed in asthmatic airways (Brown

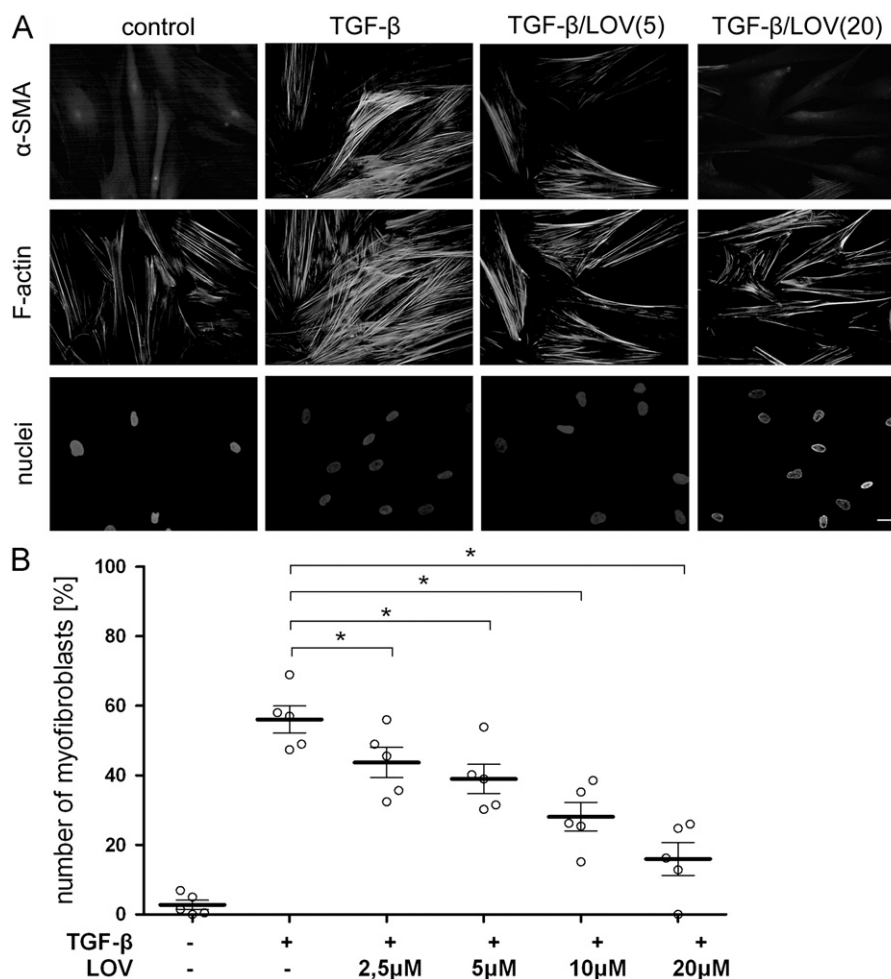


Fig. 2. Dose-dependent attenuation of TGF- β_1 -induced FMT in asthmatic HBFs in presence of lovastatin. HBFs derived from asthmatic patients ($n=5$) were cultured in DMEM with 10% FCS for 24 h, then with DMEM supplemented with 0.1% BSA (control), with TGF- β_1 (5 ng/ml) or TGF- β_1 and lovastatin (2.5, 5, 10 and 20 μ M) for 7 days. Next fibroblasts were immunostained for α -SMA and counterstained for DNA and the number of α -SMA positive cells (myofibroblasts) in the population of HBFs was evaluated. (A) Representative images of HBFs after 7-day culture. Immunofluorescent staining of α -SMA, F-actin cytoskeleton detection with TRITC-phalloidin and Hoechst staining of cell nuclei. Bar=50 μ m. (B) Graphs showing the percentage of myofibroblasts after 7 day culture of HBFs. Each point represents a single HBF culture (the means of at least three separate experiments for each culture are presented, in one experimental point at least 100 cells were counted; only HBFs with α -SMA incorporated into stress fibres were considered as myofibroblasts). Bars on graphs represent mean values. The significance of differences between untreated and lovastatin-treated HBFs was determined by Kruskal–Wallis non-parametric ANOVA, (*) $P < 0.05$.

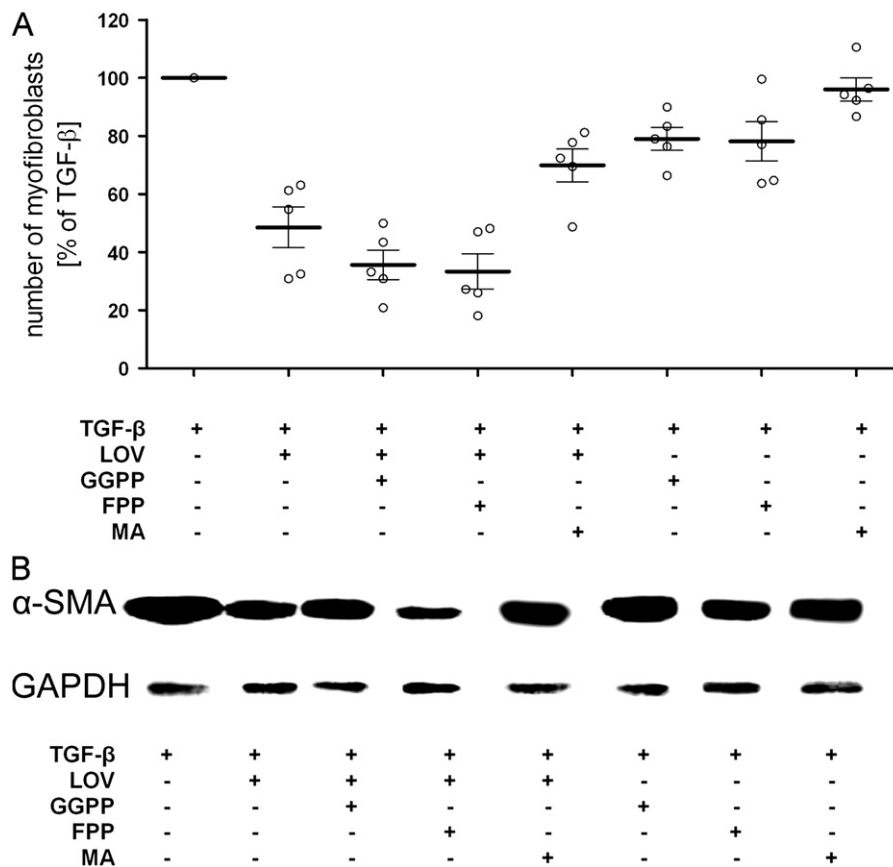


Fig. 3. Effect of GGPP, FPP and mevalonic acid on lovastatin-inhibited FMT in asthmatic HBFs. HBFs derived from asthmatic patients ($n=5$) were cultured in serum free medium with 5 ng/ml TGF- β_1 and in the same medium supplemented with 10 μ M lovastatin or lovastatin and 10 μ M geranylgeranyl pyrophosphate (GGPP), 10 μ M farnesyl pyrophosphate (FPP) or 250 μ M mevalonic acid (MA), respectively, for 7 days. (A) HBFs were immunostained for α -SMA and counterstained for DNA for evaluation of myofibroblast fraction in fibroblast populations [%]. Each point represents a single HBF culture (the means of at least three separate experiments for each culture; in one experimental point at least 100 cells were counted); horizontal bars represent mean values. (B) Protein extracts (10 μ g of protein per lane) were subjected to SDS-PAGE electrophoresis and α -SMA protein GAPDH was detected by immunoblotting. Representative immunoblots are shown under the graphs.

et al., 2012; Liu and Gaston Pravia, 2010; Sugiura et al., 2009). Matsuzawa et al. (2011) have recently hypothesized that lovastatin-induced attenuation of TGF- β_1 stimulated α -SMA expression in cultured human lung fibroblasts might be connected to the antioxidant effect of this drug. In an attempt to identify the mechanisms responsible for the inhibitory effect of lovastatin on FMT in asthmatic HBF populations, we measured the levels of intracellular reactive oxygen species in TGF- β_1 -treated and untreated cells. In the positive control we confirmed that the level of reactive oxygen species significantly increased after incubation of HBFs in 50 μ M of H_2O_2 for 90 min. The treatment of HBFs derived from four different asthmatic patients with 5 ng/ml TGF- β_1 did not affect reactive oxygen species levels, in contrast to the higher (10 ng/ml) concentration of this growth factor (Fig. 4). Because in our model, FMT was efficiently induced by TGF- β_1 at a concentration of 5 ng/ml, it seems less plausible that the inhibitory effect of lovastatin on FMT in asthmatic bronchial fibroblasts resulted from its antioxidant activity. Accordingly, administration of N-acetyl-cysteine (NAC) which is a well characterized antioxidant, did not reduce the number of myofibroblasts in TGF- β_1 -treated HBF populations (Fig. 4B). Thus, antioxidative effects of lovastatin (Patel et al., 2007) are not responsible for its inhibitory action on TGF- β_1 induced FMT in asthmatic HBFs.

3.4. Zaragozic acid A diminishes TGF- β_1 -induced FMT of asthmatic bronchial fibroblasts in a manner dependent on cholesterol

Neither the protein prenylation mechanism, nor reactive oxygen species generation, affected the efficacy of TGF- β_1 -dependent FMT in asthmatic HBFs. Instead, our data suggest that the attenuation of FMT occurs through the inhibitory effect of lovastatin on cholesterol biosynthesis leading to a decrease in cellular cholesterol content. To confirm this hypothesis we investigated the effect of a squalene synthase inhibitor—zaragozic acid A (Kourounakis et al., 2011) on the of FMT efficiency in HBFs. Zaragozic acid A did not influence the percentage of myofibroblasts in asthmatic HBF populations in the absence of TGF- β_1 (data not shown). However, it attenuated the TGF- β_1 -induced FMT to an extent comparable to the inhibitory effect of lovastatin and this activity was not prevented by mevalonate (Fig. 5). Moreover, methyl- β -cyclodextrin (M β CD) – known to reduce membrane cholesterol level in cultured cells (Kilsdonk et al., 1995) – efficiently diminished TGF- β_1 -induced FMT in asthmatic HBFs (Fig. 6A). These results strongly suggest that a decrease in the level of cholesterol may be critical for FMT inhibition in asthmatic HBFs. On the other hand, the process of FMT was investigated in serum-free cultures. In such conditions, due to constant amounts of cell membranes in quiescent HBF

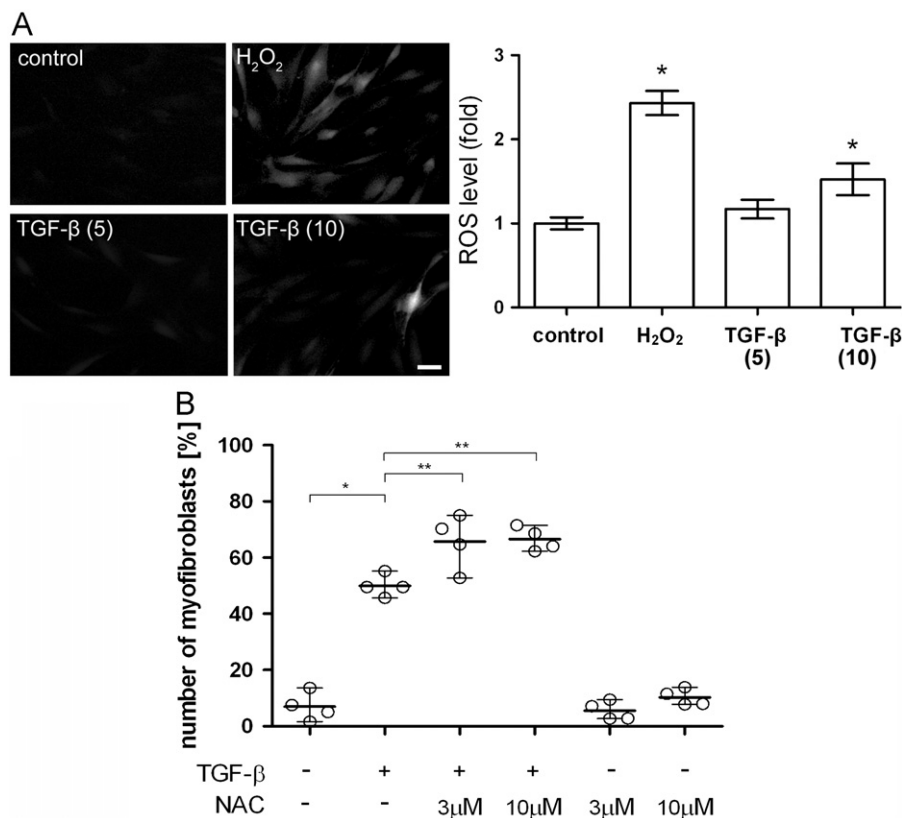


Fig. 4. TGF-β₁-induced ROS generation does not participate in FMT. (A) HBFs derived from asthmatic patients (*n*=4) were cultured in DMEM with 10% FCS for 24 h, and then stimulated with vehicle (control), 50 μM H₂O₂ (positive control—H₂O₂) for 90 min, or incubated with TGF-β₁ (5 and 10 ng/ml, respectively) for 16 h. Then, intracellular ROS levels were measured by DCF fluorescence assay. Quantitative data show relative ROS levels (in comparison to control=1) as means ± S.E.M. for 100 cells. * *P* < 0.05. (B) Cells were cultured in DMEM supplemented with 0.1% BSA with or without TGF-β₁ (5 ng/ml) and NAC (3 and 10 mM) for 7 days. Next, cells were immunostained for α-SMA and counterstained for DNA for evaluation of myofibroblast fraction in fibroblast populations [%]. Each point represents a single HBF culture; horizontal bars represent mean values. (*) Statistically significant versus control at *P* < 0.05; (**) statistically significant versus TGF-β₁ treated cells, *P* < 0.05.

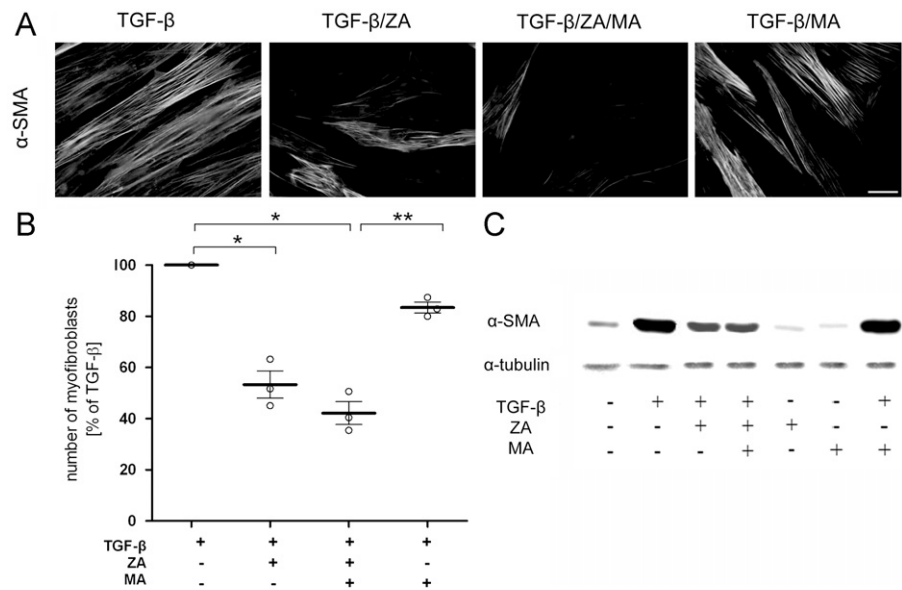


Fig. 5. Effect of zaragozic acid A on FMT in asthmatic HBFs. HBFs derived from asthmatic patients (*n*=3) were cultured in serum free medium with 5 ng/ml TGF-β₁ and in the same medium supplemented with 10 μM zaragozic acid A or ZA and 250 μM mevalonic acid, respectively, for 7 days. Next fibroblasts were immunostained for α-SMA and the number of myofibroblasts in the population of HBFs was evaluated ((A) and (B)) or protein extracts (10 μg of protein per lane) were subjected to SDS-PAGE electrophoresis and α-SMA protein/α-tubulin were detected by immunoblotting (C). Representative fluorescent images of HBFs after 7-day culture (immunofluorescent staining of α-SMA). Bar=50 μm (A). Graphs showing the percentage of myofibroblasts after 7 day culture of HBFs. Each point represents a single HBF culture (the means of at least three separate experiments for each culture are presented, in one experimental point at least 100 cells were counted). Bars on graphs represent mean values. The significance of differences between untreated and ZA-treated HBFs was determined by Kruskal–Wallis non-parametric ANOVA, (*) *P* < 0.05 (B). Representative immunoblots (C).

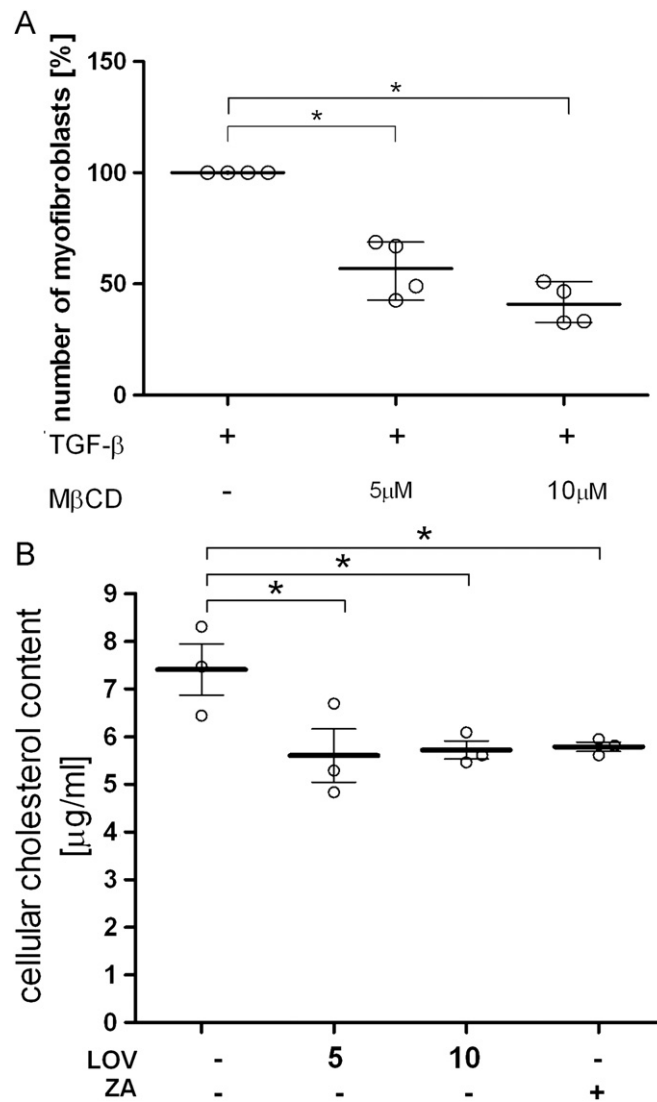


Fig. 6. Decreased cellular level of cholesterol is essential for FMT inhibition in asthmatic HBFs. (A) HBFs derived from asthmatics ($n=4$) were cultured in DMEM supplemented with 0.1% BSA (control), with or without TGF- β_1 (5 ng/ml) and M β CD (5 and 10 mM) for 7 days. Next, cells were immunostained for α -SMA and counterstained for DNA for evaluation of myofibroblast fraction in culture [%]. Each point represents a single HBF culture; horizontal bars represent mean values. (*) Statistically significant versus TGF- β_1 treated cells, $P < 0.05$. (B) HBFs were cultured in DMEM with 0.1% BSA (control) with or without 5 or 10 μ M lovastatin or 10 μ M zaragozic acid A, respectively, for 7 days. Next, cells were treated with hexane:isopropanol (3:2) and cholesterol amounts were measured using the Amplex Red Cholesterol Assay Kit, Invitrogen. Both agents significantly decrease cholesterol levels in asthmatic HBFs; (*) $P < 0.05$.

populations, de novo synthesis of cholesterol may not disturb the cellular metabolic balance. However, elucidation of the amount of cholesterol in quiescent HBFs cultured under lovastatin and zaragozic acid A stress showed significantly reduced cholesterol levels in HBF populations treated with both agents in all conditions tested (Fig. 6B).

3.5. Lovastatin and zaragozic acid A reduce TGF- β_1 -induced nuclear translocation of p-Smad

Phosphorylation and subsequent nuclear translocation of Smads proteins are the main cellular events, which occur during the activation of TGF- β_1 signalling. To further scrutinize the molecular mechanisms of lovastatin and zaragozic acid A action on TGF- β_1 -induced FMT, we followed the fate of Smad2 proteins in TGF- β_1 -stimulated HBFs in the presence of both agents. Cytoimmunofluorescence analyses of phosphorylated Smad2 revealed that both lovastatin and zaragozic acid A remarkably blocked the nuclear translocation of this protein (Fig. 7A and B). On the other hand, the analysis of immunoblots of total protein

extracts revealed that increased levels of Smad2 phosphorylation on S467 (C-terminal SXS motif), in TGF- β_1 stimulated cells, were neither affected by the lovastatin nor by zaragozic acid A (Fig. 7C). It should be noted that the antibody used for p-Smad2 detection generated two bands presumably corresponding to two isoforms of the Smad2 protein with very close molecular weights (MW). In contrast to the protein with lower MW, the amount of p-Smad2 protein with higher MW, which increased upon TGF- β_1 stimulation, was not diminished by the inhibitors of cholesterol synthesis.

4. Discussion

Whereas the clinical studies on asthmatics treated with statins have not yet provided conclusive results (Yuan et al., 2012), it has been suggested that statins could reduce asthma symptoms through their anti-inflammatory effect (Hothersall et al., 2006; Kim et al., 2007; Xu et al., 2012). Accordingly, the outcome of a large prospective population-based cohort study (the Rotterdam

Study) showed the lower risk of death in chronic obstructive pulmonary disease (COPD) patients using statins (Lahousse et al., 2012). Statins have been shown to reduce the numbers of cells associated with inflammatory response in the airways (McKay et al., 2004; Zeki et al., 2010) and to attenuate airway inflammation by suppressing antigen sensitisation, interleukine production and antigen presentation in the lung (Imamura et al., 2009). They could also enhance the clearance of apoptotic cells, a key modulator of the airway inflammatory response (Tan et al., 1999). The results of the present study demonstrate for the first time that lovastatin attenuates both TGF- β_1 -induced phenotypic transdifferentiation of asthmatic bronchial fibroblasts into myofibroblasts in culture and HBF proliferation. This raises the potential for employing statins for therapy of bronchial asthma and other diseases dependent on tissue remodelling. For example, COPD comprises strong fibrotic component and airway remodelling in this disease is related to FMT undergone by airway cells.

Previous studies have shown that statins are able to prevent the transdifferentiation of fibroblasts derived from heart (Copaja et al., 2012), intestine (Burke et al., 2009), tendon (Meyer-Ter-Vehn et al., 2008), nose (Park et al., 2012) and lung (Watts et al., 2005). Generally, it was suggested that statins influence FMT due to their pleiotropic biological effects via the mechanisms involving reactive oxygen species scavenging and the inhibition of Rho-dependent signalling rather than through the lowering of cellular cholesterol level (Meyer-Ter-Vehn et al., 2008; Porter et al., 2004; Watts et al., 2005). TGF- β -mediated fibrosis is regulated by reactive oxygen species formation (Liao and Laufs, 2005) and statins are also known to reduce oxidative stress (Bondi et al., 2010; Liu and Gaston Pravia, 2010). However, in contrast to previous report (Matsuzawa et al., 2011), our measurements demonstrated the lack of reactive oxygen species generation in the cells exposed to TGF- β_1 at the FMT-inducing concentration. Moreover, the number of myofibroblasts in TGF- β_1 -treated HBF populations were not reduced by NAC treatment. These indicate that lovastatin inhibited α -SMA expression in TGF- β -treated asthmatic bronchial fibroblasts in a manner independent of reactive oxygen species scavenging activity.

Furthermore, our results show that lovastatin-induced attenuation of FMT in asthmatic HBFs is not reversed by GGPP or FPP. Prenylation mechanisms regulate small Rho GTPases, which affect actin cytoskeleton organization (Chrzanowska-Wodnicka, Burridge, 1992; Porter and Turner, 2011; Rikitake and Liao, 2005). Because isoprenyl groups are derived from cholesterol precursors, the inhibition of HMG-CoA reductase by statins hampers Rho activation. It has been reported that statins induce actin cytoskeleton reorganization in mouse fibroblasts (Koch et al., 1997), epithelial cells (Maddala et al., 2001), corneal endothelium (Shivanna et al., 2010) and mammary carcinoma cells (Farina et al., 2002) by a cholesterol-independent effect connected with protein prenylation. Moreover, the production of extracellular matrix (ECM) proteins (characteristic for bronchial wall remodelling) by both airway fibroblasts and smooth muscle cells is abated by statins due to prenylation inhibitions and this process is reversed by GGPP (Schaafsma et al., 2011a, 2011b). In contrast, our results advocate that the suppression of prenylation of signalling proteins in HBFs is not critically important for statin-induced inhibition of myofibroblast formation. Instead, we postulate that the lovastatin-induced inhibition of FMT is directly connected with decreased cholesterol level in lovastatin-treated cells. The addition of mevalonate attenuated the effect of lovastatin on FMT, whereas M β CD and zaragozic acid A mimicked the action of lovastatin on FMT. Both lovastatin and zaragozic acid A reduced cholesterol levels in the quiescent HBFs. It should be stressed, that HBFs in our experimental model were cultured in serum free conditions, i.e., they were critically dependent on intrinsic cholesterol synthesis.

Recently, it was suggested that the signalling events at the plasma membrane are affected by cholesterol depletion due to disintegration of specific cholesterol-rich domains called “lipid rafts” (Brusselmans et al., 2007; Chen et al., 2008). It was also suggested that caveolae and caveolin-1 associated with lipid rafts play important role in establishing of bronchial myocytes contractile phenotype, characteristic of asthma (Gosens et al., 2011). Lipid rafts are thought to form platforms for the regulation and transduction of receptor-mediated signalling including the TGF- β

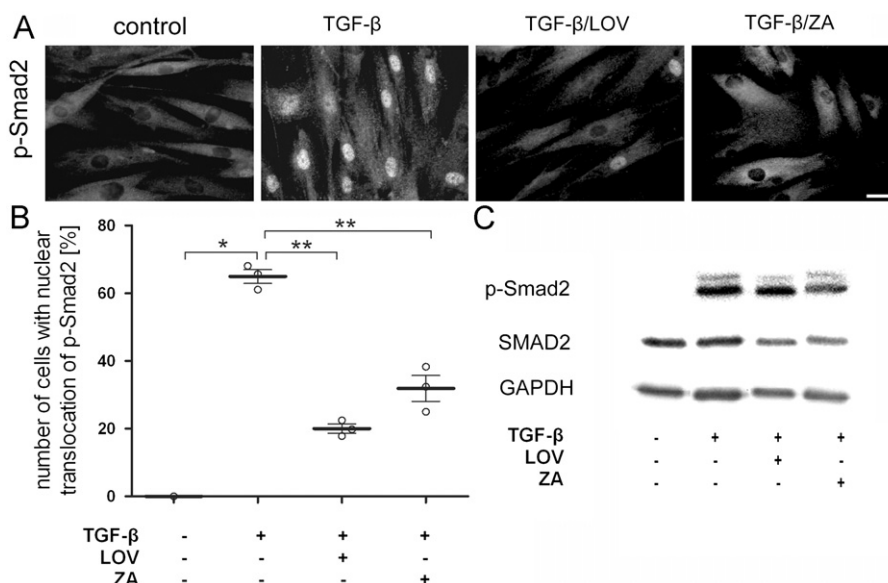


Fig. 7. TGF- β_1 -induced Smad2 phosphorylation and nuclear translocation in lovastatin-treated HBFs. HBFs from asthmatics ($n=4$) were cultured in DMEM supplemented with 0.1% BSA for 24 h and then with the same medium supplemented only with TGF- β_1 (5 ng/ml) or TGF- β_1 /lovastatin (10 mM) or TGF- β_1 /ZA for 1 h. Next cells were immunostained for p-Smad2. Bar=50 μ m (A). Quantitative data showing the number of fibroblasts with nuclear translocation of p-Smad2 proteins. (*) Statistically significant versus control at $P < 0.05$; (**) significant decrease compared with TGF- β_1 treated cells, $P < 0.05$ (B). Effect of lovastatin and zaragozic acid A on TGF- β_1 -induced Smad2 phosphorylation in HBFs. Protein extracts (10 μ g of protein per lane) were subjected to SDS-PAGE electrophoresis and p-Smad2 and Smad2/GAPDH were detected by immunoblotting. Representative immunoblots are shown under the graphs (C).

pathway (Chen et al., 2007; Matarazzo et al., 2012). In agreement with this concept, the present study reveals that lovastatin attenuates the TGF- β_1 -induced Smad2 nuclear translocation in HBF populations. Similar results were obtained by Burke et al. (2009) who showed that simvastatin impaired Smad3 phosphorylation upon TGF- β_1 treatment in intestinal fibroblasts. In our experiments the level of Smad2 phosphorylation increased significantly upon TGF- β_1 stimulation but this effect was not attenuated by the lovastatin or zaragozic acid A treatments. However, it is worth pointing out that the anti-p-Smad2 (Ser 467) antibody detected two bands in HBF protein extracts and the applied inhibitors of cholesterol biosynthesis specifically lowered the intensity of the upper band. It could be suggested that this band corresponds to the amount of Smad2 phosphorylated on more than one residue of the protein. The TGF- β -activated nuclear translocation of Smad complexes depends not only on Smad2 phosphorylation on the C-terminus (on serines 465 and 467) by the TGF- β_1 receptor, but also on Smad2 N-terminus phosphorylation (on threonine 8) driven by extracellular signal-regulated kinases (ERK/MAPK) (Funaba et al., 2002). It was recently shown that TGF- β_1 treatment of serum starved human airway fibroblasts was able to activate ERK phosphorylation (Zhou et al., 2012) and the activation of MAPK by TGF- β requires localization of TGF- β receptors in lipid rafts (Zuo and Chen, 2009). Interestingly, statins are known to inhibit the ERK pathway in normal and cancer cells (He et al., 2008; Kamigaki et al., 2011). Thus, attenuated levels of cholesterol may influence the effectiveness of TGF- β signalling through lipid-raft dependent ERK activation.

Previous studies have suggested several beneficial effects of statins both independent and dependent on their action against hypercholesterolemia, including anti-inflammatory properties. With our in vitro model, we demonstrate that statins can directly affect basic transdifferentiation events crucial for asthmatic processes. The identification of functional links between the cellular cholesterol level and FMT requires further studies. However, the results presented in this and the other recently published in vitro studies (Ghavami et al., 2011; Schaafsma et al., 2011a, 2011b) point at airway remodelling in asthma as a new possible target of statins.

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References

- Al-Muhsen, S., Johnson, J.R., Hamid, Q., 2011. Remodeling in asthma. *J. Allergy Clin. Immunol.* 128, 451–462.
- Anandan, C., Nurmatov, U., van Schayck, O.C., Sheikh, A., 2010. Is the prevalence of asthma declining? Systematic review of epidemiological studies. *Allergy* 65, 152–167.
- Barnes, P.J., 2006. Corticosteroids: the drugs to beat. *Eur. J. Pharmacol.* 533, 2–14.
- Batra, V., Musani, A.L., Hastie, A.T., Khurana, S., Carpenter, K.A., Zangrilli, J.G., et al., 2004. Bronchoalveolar lavage fluid concentrations of transforming growth factor (TGF)- β_1 , TGF- β_2 , interleukin (IL)-4 and IL-13 after segmental allergen challenge and their effects on alpha-smooth muscle actin and collagen III synthesis by primary human lung fibroblasts. *Clin. Exp. Allergy* 34, 437–444.
- Bondi, C.D., Manickam, N., Lee, D.Y., Block, K., Gorin, Y., Abboud, H.E., et al., 2010. NAD(P)H oxidase mediates TGF- β_1 -induced activation of kidney myofibroblasts. *J. Am. Soc. Nephrol.* 21, 93–102.
- Braganza, G., Chaudhuri, R., McSharry, C., Weir, C.J., Donnelly, I., Jolly, L., et al., 2011. Effects of short-term treatment with atorvastatin in smokers with asthma—a randomized controlled trial. *BMC Pulm. Med.* 11, 16.
- Brown, S.D., Baxter, K.M., Stephenson, S.T., Esper, A.M., Brown, L.A., Fitzpatrick, A.M., 2012. Airway TGF- β_1 and oxidant stress in children with severe asthma: association with airflow limitation. *J. Allergy Clin. Immunol.* 129, 388–396.
- Brusselmans, K., Timmermans, L., Van de Sande, T., Van Veldhoven, P.P., Guan, G., Shechter, I., et al., 2007. Squalene synthase, a determinant of raft-associated cholesterol and modulator of cancer cell proliferation. *J. Biol. Chem.* 282, 18777–18785.
- Burke, J.P., Watson, R.W., Murphy, M., Docherty, N.G., Coffey, J.C., O'Connell, P.R., 2009. Simvastatin impairs smad-3 phosphorylation and modulates transforming growth factor beta1-mediated activation of intestinal fibroblasts. *Br. J. Surg.* 96, 541–551.
- Chan, K.K., Oza, A.M., Siu, L.L., 2003. The statins as anticancer agents. *Clin. Cancer Res.* 9, 10–19.
- Chen, C.L., Liu, I.H., Fliesler, S.J., Han, X., Huang, S.S., Huang, J.S., 2007. Cholesterol suppresses cellular TGF- β responsiveness: implications in atherogenesis. *J. Cell Sci.* 120, 3509–3521.
- Chen, C.L., Huang, S.S., Huang, J.S., 2008. Cholesterol modulates cellular TGF- β responsiveness by altering TGF- β binding to TGF- β receptors. *J. Cell Physiol.* 215, 223–233.
- Chrzanowska-Wodnicka, M., Burridge, K., 1992. Rho, rac and the actin cytoskeleton. *Bioessays* 14, 777–778.
- Copaja, M., Venegas, D., Aranguiz, P., Canales, J., Vivar, R., Avalos, Y., et al., 2012. Simvastatin disrupts cytoskeleton and decreases cardiac fibroblast adhesion, migration and viability. *Toxicology* 294, 42–49.
- Depasquale, I., Wheatley, D.N., 2006. Action of lovastatin (Mevinolin) on an in vitro model of angiogenesis and its co-culture with malignant melanoma cell lines. *Cancer Cell. Int.* 30, 6–9.
- Descalzi, D., Folli, C., Scordamaglia, F., Riccio, A.M., Gamalero, C., Canonica, G.W., 2007. Importance of fibroblasts-myofibroblasts in asthma-induced airway remodeling. *Recent Pat. Inflamm. Allergy Drug Discov.* 1, 237–241.
- Durrani, S.R., Viswanathan, R.K., Busse, W.W., 2011. What effect does asthma treatment have on airway remodeling? Current perspectives. *J. Allergy Clin. Immunol.* 128, 439–448.
- Evans, M., Roberts, A., Davies, S., Rees, A., 2004. Medical lipid-regulating therapy: current evidence, ongoing trials and future developments. *Drugs* 64, 1181–1196.
- Farina, H.G., Bublik, D.R., Alonso, D.F., Gomez, D.E., 2002. Lovastatin alters cytoskeleton organization and inhibits experimental metastasis of mammary carcinoma cells. *Clin. Exp. Metastasis* 19, 551–559.
- Funaba, M., Zimmerman, C.M., Mathews, L.S., 2002. Modulation of Smad2-mediated signaling by extracellular signal-regulated kinase. *J. Biol. Chem.* 277, 41361–41368.
- Ghavami, S., Mutawe, M.M., Sharma, P., Yeganeh, B., McNeill, K.D., Klonisch, T., et al., 2011. Mevalonate cascade regulation of airway mesenchymal cell autophagy and apoptosis: a dual role for p53. *PLoS One* 6, e16523, <http://dx.doi.org/10.1371/journal.pone.0016523>.
- Gosens, R., Stelmack, G.L., Bos, S.T., Dueck, G., Mutawe, M.M., Schaafsma, D., et al., 2011. Caveolin-1 is required for contractile phenotype expression by airway smooth muscle cells. *J. Cell. Mol. Med.* 15, 2430–2442.
- Halwani, R., Al-Muhsen, S., Al-Jahdali, H., Hamid, Q., 2011. Role of transforming growth factor- β in airway remodeling in asthma. *Am. J. Respir. Cell Mol. Biol.* 44, 127–133.
- Hardie, W.D., Glasser, S.W., Hagood, J.S., 2009. Emerging concepts in the pathogenesis of lung fibrosis. *Am. J. Pathol.* 175, 3–16.
- He, Y.P., Zhao, L.Y., Zheng, Q.S., Liu, S.W., Zhao, X.Y., Lu, X.L., et al., 2008. Involvement of ERK and AKT signaling in the growth effect of arginine vasopressin on adult rat cardiac fibroblast and the modulation by simvastatin. *Mol. Cell Biochem.* 317, 33–41.
- Holgate, S.T., 2010. A look at the pathogenesis of asthma: the need for a change in direction. *Discov. Med.* 9, 439–447.
- Hothersall, E., McSharry, C., Thomson, N.C., 2006. Potential therapeutic role for statins in respiratory disease. *Thorax* 61, 729–734.
- Hothersall, E., Chaudhuri, R., McSharry, C., Donnelly, I., Lafferty, J., McMahon, A.D., et al., 2008. Effects of atorvastatin added to inhaled corticosteroids on lung function and sputum cell counts in atopic asthma. *Thorax* 63, 1070–1075.
- Huang, C.C., Chan, W.L., Chen, Y.C., Chen, T.J., Chou, K.T., Lin, S.J., et al., 2011. Statin use in patients with asthma: a nationwide population-based study. *Eur. J. Clin. Invest.* 41, 507–512.
- Imamura, M., Okunishi, K., Ohtsu, H., Nakagome, K., Harada, H., Tanaka, R., et al., 2009. Pravastatin attenuates allergic airway inflammation by suppressing antigen sensitization, interleukin 17 production and antigen presentation in the lung. *Thorax* 64, 44–49.
- Kamigaki, M., Sasaki, T., Serikawa, M., Inoue, M., Kobayashi, K., Itsuki, H., et al., 2011. Statins induce apoptosis and inhibit proliferation in cholangiocarcinoma cells. *Int. J. Oncol.* 39, 561–568.
- Kilsdonk, E.P., Yancey, P.G., Stoudt, G.W., Bangerter, F.W., Johnson, W.J., Phillips, M.C., et al., 1995. Cellular cholesterol efflux mediated by cyclodextrins. *J. Biol. Chem.* 270, 17250–17256.

- Kim, D.Y., Ryu, S.Y., Lim, J.E., Lee, Y.S., Ro, J.Y., 2007. Anti-inflammatory mechanism of simvastatin in mouse allergic asthma model. *Eur. J. Pharmacol.* 557, 76–86.
- Koch, G., Benz, C., Schmidt, G., Olenik, C., Aktories, K., 1997. Role of Rho protein in lovastatin-induced breakdown of actin cytoskeleton. *J. Pharmacol. Exp. Ther.* 283, 901–909.
- Kourounakis, A.P., Katselou, M.G., Matralis, A.N., Ladopoulou, E.M., Bavavea, E., 2011. Squalene synthase inhibitors: an update on the search for new antihyperlipidemic and antiatherosclerotic agents. *Curr. Med. Chem.* 18, 4418–4439.
- Kurosawa, T., Nakamura, H., Yamaura, E., Fujino, H., Matsuzawa, Y., Kawashima, T., et al., 2009. Cytotoxicity induced by inhibition of thioredoxin reductases via multiple signaling pathways: role of cytosolic phospholipase A(2) α -dependent and -independent release of arachidonic acid. *J. Cell Physiol.* 219, 606–616.
- Lahousse, L., Loth, D.W., Joos, G.F., Hofman, A., Leufkens, H.G., Brusselle, G.G., et al., 2012. Statins, systemic inflammation and risk of death in COPD: the Rotterdam study. *Pulm. Pharmacol. Ther.*, <http://dx.doi.org/10.1016/j.pupt.2012.10.008>, pii: S1094-5539(12)00166-6.
- Liao, J.K., 2002. Isoprenoids as mediators of the biological effects of statins. *J. Clin. Invest.* 110, 285–288.
- Liao, J.K., Laufs, U., 2005. Pleiotropic effects of statins. *Annu. Rev. Pharmacol. Toxicol.* 45, 89–118.
- Liu, R.M., Gaston Pravia, K.A., 2010. Oxidative stress and glutathione in TGF- β -mediated fibrogenesis. *Free Radic. Biol. Med.* 48, 1–15.
- Maddala, R.L., Reddy, V.N., Rao, P.V., 2001. Lovastatin-induced cytoskeletal reorganization in lens epithelial cells: role of Rho GTPases. *Invest. Ophthalmol. Vis. Sci.* 42, 2610–2615.
- Marzilli, M., 2010. Pleiotropic effects of statins: evidence for benefits beyond LDL-cholesterol lowering. *Am. J. Cardiovasc. Drugs* 10 (Suppl 1), 3–9.
- Matarazzo, S., Quitadamo, M.C., Mango, R., Ciccone, S., Novelli, G., Biocca, S., 2012. Cholesterol-lowering drugs inhibit LOX-1 receptor function by membrane raft disruption. *Mol. Pharmacol.* 82, 246–254.
- Matsuzawa, Y., Kawashima, T., Yamazaki, R., Yamaura, E., Makiyama, T., Fujino, H., et al., 2011. Inhibitory effects of clinical reagents having anti-oxidative activity on transforming growth factor- β 1-induced expression of α -smooth muscle actin in human fetal lung fibroblasts. *Toxicol. Sci.* 36, 733–740.
- Mauad, T., Bel, E.H., Sterk, P.J., 2007. Asthma therapy and airway remodeling. *J. Allergy Clin. Immunol.* 120, 997–1009.
- McKay, A., Leung, B.P., McInnes, I.B., Thomson, N.C., Liew, F.Y., 2004. A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. *J. Immunol.* 172, 2903–2908.
- Meyer-Ter-Vehn, T., Katzenberger, B., Han, H., Grehn, F., Schlunck, G., 2008. Lovastatin inhibits TGF- β -induced myofibroblast transdifferentiation in human tenon fibroblasts. *Invest. Ophthalmol. Vis. Sci.* 49, 3955–3960.
- Michalik, M., Pierzchalska, M., Legutko, A., Ura, M., Ostaszewska, A., Soja, J., et al., 2009. Asthmatic bronchial fibroblasts demonstrate enhanced potential to differentiate into myofibroblasts in culture. *Med. Sci. Monit.* 15, BR194–B201.
- Michalik, M., Pierzchalska, M., Włodarczyk, A., Wójcik, K.A., Czyż, J., Sanak, M., et al., 2011. Transition of asthmatic bronchial fibroblasts to myofibroblasts is inhibited by cell-cell contacts. *Respir. Med.* 105, 1467–1475.
- Michalik, M., Wójcik, K.A., Jakieta, B., Szpak, K., Pierzchalska, M., Sanak, M., et al., 2012. Lithium attenuates TGF- β 1-induced fibroblasts to myofibroblasts transition in bronchial fibroblasts derived from asthmatic patients. *J. Allergy (Cairo)*, <http://dx.doi.org/10.1155/2012/206109>.
- Park, I.H., Park, S.J., Cho, J.S., Moon, Y.M., Moon, J.H., Kim, T.H., et al., 2012. Effect of simvastatin on transforming growth factor beta-1-induced myofibroblast differentiation and collagen production in nasal polyp-derived fibroblasts. *Am. J. Rhinol. Allergy* 26, 7–11.
- Patel, T.N., Shishehbor, M.H., Bhatt, D.L., 2007. A review of high-dose statin therapy: targeting cholesterol and inflammation in atherosclerosis. *Eur. Heart J.* 28, 664–672.
- Porter, K.E., Turner, N.A., 2011. Statins and myocardial remodelling: cell and molecular pathways. *Expert Rev. Mol. Med.* 13, e22.
- Porter, K.E., Turner, N.A., O'Regan, D.J., Balmforth, A.J., Ball, S.G., 2004. Simvastatin reduces human atrial myofibroblast proliferation independently of cholesterol lowering via inhibition of RhoA. *Cardiovasc. Res.* 61, 745–755.
- Redington, A.E., Madden, J., Frew, A.J., Djukanovic, R., Roche, W.R., Holgate, S.T., et al., 1997. Transforming growth factor- β 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am. J. Respir. Crit. Care Med.* 156, 642–647.
- Riccioni, G., Di Ilio, C., D'Orazio, N., 2004. Review: pharmacological treatment of airway remodeling: inhaled corticosteroids or antileukotrienes? *Ann. Clin. Lab. Sci.* 34, 138–142.
- Ridker, P.M., Cannon, C.P., Morrow, D., Rifai, N., Rose, L.M., McCabe, C.H., et al., 2005. C-reactive protein levels and outcomes after statin therapy. *N. Engl. J. Med.* 352, 20–28.
- Rikitake, Y., Liao, J.K., 2005. Rho GTPases, statins, and nitric oxide. *Circ. Res.* 97, 1232–1235.
- Rutishauser, J., 2011. Statins in clinical medicine. *Swiss Med. Wkly*, <http://dx.doi.org/10.4414/smw.2011.13310>.
- Schaafsma, D., Dueck, G., Ghavami, S., Kroeker, A., Mutawe, M.M., Hauff, K., et al., 2011a. The mevalonate cascade as a target to suppress extracellular matrix synthesis by human airway smooth muscle. *Am. J. Respir. Cell. Mol. Biol.* 44, 394–403.
- Schaafsma, D., McNeill, K.D., Mutawe, M.M., Ghavami, S., Unruh, H., Jacques, E., et al., 2011b. Simvastatin inhibits TGF β 1-induced fibronectin in human airway fibroblasts. *Respir. Res.* 12, 113, <http://dx.doi.org/10.1186/1465-9921-12-113>.
- Shivanna, M., Jalimarada, S.S., Srinivas, S.P., 2010. Lovastatin inhibits the thrombin-induced loss of barrier integrity in bovine corneal endothelium. *J. Ocul. Pharmacol. Ther.* 26, 1–10.
- Sugiura, H., Ichikawa, T., Liu, X., Kobayashi, T., Wang, X.Q., Kawasaki, S., et al., 2009. N-acetyl-L-cysteine inhibits TGF- β 1-induced profibrotic responses in fibroblasts. *Pulm. Pharmacol. Ther.* 22, 487–491.
- Tan, A., Levrey, H., Dahm, C., Polunovsky, V.A., Rubins, J., Bitterman, P.B., 1999. Lovastatin induces fibroblast apoptosis in vitro and in vivo. A possible therapy for fibroproliferative disorders. *Am. J. Respir. Crit. Care Med.* 159, 220–227.
- Watts, K.L., Sampson, E.M., Schultz, G.S., Spiteri, M.A., 2005. Simvastatin inhibits growth factor expression and modulates profibrogenic markers in lung fibroblasts. *Am. J. Respir. Cell Mol. Biol.* 32, 290–300.
- Xu, L., Dong, X.W., Shen, L.L., Li, F.F., Jiang, J.X., Cao, R., et al., 2012. Simvastatin delivery via inhalation attenuates airway inflammation in a murine model of asthma. *Int. Immunopharmacol.* 12, 556–564.
- Yuan, C., Zhou, L., Cheng, J., Zhang, J., Teng, Y., Huang, M., et al., 2012. Statins as potential therapeutic drug for asthma? *Respir. Res.* 13, 108, <http://dx.doi.org/10.1186/1465-9921-13-108>.
- Zeki, A.A., Bratt, J.M., Rabowsky, M., Last, J.A., Kenyon, N.J., 2010. Simvastatin inhibits goblet cell hyperplasia and lung arginase in a mouse model of allergic asthma: a novel treatment for airway remodeling? *Transl. Res.* 156, 335–349.
- Zhou, X., Hu, H., Balzar, S., Trudeau, J.B., Wenzel, S.E., 2012. MAPK regulation of IL-4/IL-13 receptors contributes to the synergistic increase in CCL11/eotaxin-1 in response to TGF- β 1 and IL-13 in human airway fibroblasts. *J. Immunol.* 188, 6046–6054.
- Zuo, W., Chen, Y.G., 2009. Specific activation of mitogen-activated protein kinase by transforming growth factor-beta receptors in lipid rafts is required for epithelial cell plasticity. *Mol. Biol. Cell.* 20, 1020–1029.