The effect of statins and the synthetic LXR agonist T0901317 on expression of ABCA1 transporter protein in human lung epithelial cell lines *in vitro*

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Running title: Effect of statins and an LXR agonist on ABCA1

Abstract

Background: The pathogenesis of chronic obstructive pulmonary disease (COPD) is associated with dyslipidemia, an established co-morbidity. Statins treat hypercholesterolemia, but more recently have been trailed in the setting of COPD for their potential anti-inflammatory benefits. The outcomes of prospective trials however have been inconsistent. Thus, we hypothesize that the variation in results may have been due to statin-induced downregulation of ATP-binding cassette transporter A1 (ABCA1), thereby reducing cholesterol export. This study aims to elucidate whether statin treatment in a cellular model of COPD leads to a decrease in ABCA1 protein expression.

Methods: To mimic the inflammatory environment of COPD, two commonly used lung epithelial cell lines (BEAS-2B and A549) were treated with tumor necrosis factor (TNF), and co-treated with cholesterol/25-hydroxycholesterol (25-OH) to mimic dyslipidemia. ABCA1 protein was detected by Western Blotting.

Results: We unexpectedly showed that statins did not affect ABCA1 expression. However, the LXR agonist T0901317 significantly increased ABCA1 expression in both cell lines, while TNF, cholesterol or 25-OH induced ABCA1 protein upregulation in BEAS-2B cells, indicating cell line differences in response. There was also evidence of synergistic impacts of combined treatments on ABCA1 upregulation in BEAS-2B cells.

Conclusion: statins did not have an impact on ABCA1 expression in lung epithelial cell lines, disproving our original hypothesis. However, we showed for the first time, the effect of the inflammatory cytokine TNF, cholesterol/25-OH, statins and the LXR agonist T0901317 on expression of ABCA1 transporter protein in human lung epithelial cell lines *in vitro.* We hope

that these *in vitro* studies may prove beneficial for addressing dyslipidemia in COPD in the future.

Keywords: simvastatin, atorvastatin, ABCA1, A549, BEAS-2B, LXR agonists

Introduction

The current therapies for chronic obstructive pulmonary disease (COPD) address symptomatic control to improve quality of life for people living with COPD. Yet, declining lung function continues somewhat unabated due to the fact that inflammation in COPD is relatively refractory to corticosteroids. In recent years, dyslipidemia has been recognised as a co-morbidity of COPD and a contributor to disease pathogenesis in COPD [1]. Elevated levels of cholesterol [2, 3], or oxidized derivatives, such as the oxysterol 25-hydroxycholesterol [4], have been linked to inflammation and other pathologies in COPD. Statins are the conventional treatment for hypercholesterolemia and have also been the subject of extensive research to exploit the potential pleiotropic beneficial effects in COPD, reduction of inflammation in particular, beyond their lipid-lowering impact (reviewed in [5, 6]). Cumulative *in vitro* evidence and multiple retrospective clinical studies provided a therapeutic rationale behind the Prospective Randomized Placebo-Controlled Trial of Simvastatin in the Prevention of COPD Exacerbations (STATCOPE) [7]. However, the outcomes from STATCOPE were discordant with earlier observational studies that demonstrated significant reduction in exacerbations (reviewed in [8]).

Although there are a number of reasons for the failure of statins to live up to their promise in STATCOPE, including patient selection and aspects of study design [8] or oral delivery route [5], in this study we propose an alternative hypothesis. We propose that the disparity may be due to statin-induced downregulation of one of the major transporter proteins responsible for removing intracellular cholesterol (a process known as cholesterol efflux) namely the ABCA1 (ATP-binding cassette A1) transporter. Statins are known to reduce ABCA1 in the cardiovascular context [9-11], but whether statins have a similar repressive impact on ABCA1 in COPD is unknown at present. The importance of ABCA1, and the critical role it plays in

cholesterol efflux and homeostasis, has emerged recently in chronic respiratory disease (reviewed in [12]). Knocking-out ABCA1 in mice results in excessive pulmonary inflammation and abnormalities [13], while conversely, mice overexpressing ABCA1 have attenuated neutrophilic airway inflammation [14]. More recently, cigarette smoke, a major risk factor in COPD, has been shown in *in vivo* and *in vitro* studies [15-17] to significantly decrease ABCA1 expression resulting in impaired cholesterol efflux, exaggerated inflammatory phenotype and hallmark features of COPD. Moreover, studies are now emerging that explore the therapeutic potential of agonists directed towards Liver X Receptors (LXR) [18] to upregulate ABCA1 in an attempt to counter cigarette smoke-induced impact in the context of COPD [19].

Taken together, these studies place ABCA1 as a critical regulator of lipid homeostasis and underscore the importance of research that links dyslipidemia and inflammation in COPD. We model this herein, by utilizing commonly-used lung epithelial cell lines (BEAS-2B and A549) treated with tumor necrosis factor (TNF) to simulate the inflammatory environment in COPD, and cholesterol and 25-hydroxycholesterol to model dyslipidemia. In this study, we use our *in vitro* model to examine the hypothesis that: 1) statins will decrease ABCA1 expression in lung epithelial cells; and 2) that synthetic LXR agonist T0901317 will reverse the statin-induced ABCA1 decrease.

Materials and Methods

Chemicals

TNF was purchased from R&D Systems (Minneapolis, MN). Atorvastatin calcium salt trihydrate, simvastatin (lactone), cholesterol, methyl-betacyclodextrin and T0901317 were from Sigma Aldrich (St. Louis, MO). Unless otherwise specified, all other chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MO). 25-hydroxycholesterol (25-OH) was purchased from Avanti Polar Lipids. Cholesterol and 25-OH were complexed to cyclodextrin as described in Yang and Gelissen [20].

Epithelial cell culture

The immortalized bronchial epithelial cell line BEAS-2B and the adenocarcinoma epithelial cell line A549 were purchased from the American Type Culture Collection (ATCC) and cultured in feeding media composed of Dulbecco's Modified Eagle's medium-low glucose, supplemented with streptomycin (10,000 µg/mL), penicillin (10,000 units/mL), L-glutamine (200 mM) and heat-inactivated foetal bovine serum (10%), under conditions of 37˚C incubation in a 5% CO_2 atmosphere.

Cell treatments, cell lysis and protein equilibration

Cells were seeded into 12-well plates at density of 10^5 cell/ml. After 48 h, treatments were added according to experimental conditions for a further 24 h. After treatment, 12-well plates were placed on ice, in preparation for cell lysis. The cells were washed twice with cold PBS, before being lysed with cell lysis buffer composed of IGEPAL buffer (1% IGEPAL detergent, 50 mM Tris, 150 mM NaCl, pH 7.8) and protease inhibitor cocktail (5 µg/mL). Cells were collected by

scraping, lysates collected and cellular DNA sheared by repeated drawing up and down through a 23-gauge syringe. The cell lysate was then centrifuged at 14,000 r.p.m at 4˚C for 5 minutes and total protein concentration of cell lysates was measured using the Bicinchoninic Acid (BCA) Assay kit (Sigma Aldrich) and the proteins equilibrated prior to SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting

ABCA1 was detected by Western blotting after 6% SDS-PAGE gels and anti-ABCA1 antibody ((1/2500, mouse monoclonal IgG_1, clone AB.H10: Merck, Darmstadt, Germany). HSP-70 was used as a loading control (1/5000, mouse monoclonal IgG_1, clone BRM-22: Sigma-Aldrich). Primary antibodies were detected with goat anti-mouse HRP-conjugated secondary antibodies (1/2000, Cell Signaling Technology, Danvers, MA) and visualized by enhanced chemiluminescence (PerkinElmer, Wellesley, MA). Western blots depicted in the figures are representative results all run on the same gel.

Statistical analysis

Statistical analysis was performed using the one-way ANOVA then Bonferroni's multiple comparison test. p values < 0.05 were sufficient to reject the null hypothesis for all analyses.

Results

Effect of TNF or cholesterol/25-OH, alone and in combination, on ABCA1 protein expression in BEAS-2B and A549 cells: establishing the *in vitro* model of COPD and dyslipidemia

In our *in vitro* model, the two lung epithelial cell lines (BEAS-2B and A549) are treated with TNF to model inflammatory conditions occurring in COPD, while cholesterol or 25-OH stimulation mimicked the condition of dyslipidemia. The results shown in Figure 1 serve to establish our *in vitro* model of COPD and dyslipidemia. Firstly, we show that the low level of basal ABCA1 protein expression in BEAS-2B cells can be significantly upregulated with TNF (2.5±0.1-fold), cholesterol (4.4±0.3-fold), or 25-OH (5.2±0.3-fold), compared to untreated control cells (Figure 1A: $p<0.05$). Secondly, to model the impact of dyslipidemia on the inflamed epithelium, BEAS-2B cells were stimulated with cholesterol/25-OH in the presence of TNF. As shown in Figure 1A, the simultaneous treatment of BEAS-2B with TNF + cholesterol or TNF + 25-OH increased ABCA1 protein expression by 6.3±0.5-fold or 8.2±0.9-fold, respectively, compared to untreated cells ($p<0.05$). The effect of combined treatments on ABCA1 protein upregulation in BEAS-2B was additive. In contrast, the pattern of ABCA1 upregulation in A549 cells (Figure 1B) differed from BEAS-2B (Figure 1A): that is, A549 cells expressed no significant changes in ABCA1 expression from basal levels when stimulated with TNF, cholesterol and 25-OH. However, there was a synergistic upregulation when A549 cells were stimulated with cholesterol or 25-OH in the presence of TNF; the combined stimuli resulted in significant increases in the ABCA1 expression (TNF + cholesterol 8.2±0.6-fold: TNF + 25-OH 8.6±0.8-fold) (Figure 1B: $p<0.05$).

Effect of the LXR agonist T0901317, and the statins simvastatin and atorvastatin, on ABCA1 expression in BEAS-2B and A549 cells

We then treated BEAS-2B and A549 cells with increasing concentrations (0.5 – 10 μM) of the synthetic LXR agonist T0901317 and examined the effect on ABCA1 protein levels (Figure 2). As shown in Figure 2A, T0901317 significantly induced ABCA1 production at all concentrations tested ($p<0.05$). A similar pattern of ABCA1 protein upregulation was observed after treatment of A549 cells with T0901317 (Figure 2B: $p<0.05$).

While it was expected that LXR agonists would increase ABCA1 expression (1 μM was chosen for future experiments), it was unknown whether statins modulate basal levels of ABCA1 protein. To address this, we conducted a pilot study whereby BEAS-2B and A549 were treated with increasing concentrations (0.5 – 10 μM) of two statins, simvastatin and atorvastatin, for 24 h before ABCA1 protein levels were measured by Western blotting. Negative controls were untreated cells, while positive controls were cells treated with TNF. In confirmation of earlier results, TNF induced ABCA1 protein upregulation in BEAS-2B (Figures 3A and 3B), but not A549 cells (Figures 3C and 3D). Notably, neither simvastatin nor atorvastatin resulted in a visible effect on basal ABCA1 expression in both BEAS-2B (Figures 3A and 3B) and A549 (Figures 3C and 3D) cell lines; thus, future experimentation will utilise 10 μM.

Statins have no effect on ABCA1 protein expression in an *in vitro* model of COPD and dyslipidemia

Our experiments thus far have established the *in vitro* model of COPD and dyslipidemia; BEAS-2B are the preferred epithelial cell line given significant ABCA1 upregulation under both inflamed (TNF) and/or dyslipidaemic conditions (cholesterol/25-OH). We also have determined

the relevant concentrations of LXR agonist and statins to use to address our study hypothesis that statins will decrease ABCA1 expression in lung epithelial cells and T0901317 will ameliorate this decrease.

Counter to our hypothesis however, simvastatin (10 μM) did not induce a significant decrease in ABCA1 protein level under any conditions in our *in vitro* COPD and dyslipidemia model (Figure 4). Specifically, as shown in Figure 4A, TNF stimulation significantly increased ABCA1 protein upregulation by 2.2±0.2-fold ($p<0.05$), but simvastatin did not repress TNF-induced ABCA1 protein levels. Cholesterol and 25-OH stimulation (Figures 4B and 4C) also significantly increased ABCA1 protein (3.2±0.4-fold and 4.1±0.3-fold, respectively: $p<0.05$), but again, simvastatin was without effect. Finally, there was no impact of simvastatin on ABCA1 protein produced by simultaneous treatment of BEAS-2B cells with TNF + cholesterol (Figure 4D) or TNF + 25-OH (Figure 4E). Taken together, these data show that simvastatin treatment does not decrease ABCA1 protein expression in BEAS-2B cells when stimulated with stimuli treated individually or in combination. The same experiments were performed with atorvastatin (10 μM) and, as shown in Figure 5, atorvastatin had no effect on ABCA1 protein expression in an *in vitro* model of COPD and dyslipidemia.

Combined effect of T0901317, statins, and TNF, alone and in combination, on ABCA1 protein expression in BEAS-2B cells

Since we have disproved the first part of our study hypothesis by showing that statins do not decrease ABCA1 expression, whether synthetic LXR agonist T0901317 will reverse the statin-induced ABCA1 decrease is now a moot point. However, given the interest in LXR agonists as potential therapeutics in chronic lung disease, we conducted a final experiment to examine the

effect of the LXR agonist T0901317, the statins simvastatin (Figure 6A) and atorvastatin (Figure 6B), and the inflammatory cytokine TNF, alone and in combination, on ABCA1 protein upregulation in BEAS-2B cells. We were interested in whether additive or synergistic upregulation of ABCA1 protein expression would be achieved with treatments added in combination. As demonstrated in Figure 6A, and confirmation of earlier experiments, while T0901317 and TNF an increase in ABCA1 protein levels, simvastatin was without effect. When treatments were combined, the effect on ABCA1 upregulation was greater than additive, i.e. TNF (2.4±0.1-fold) T0901317 + simvastatin (5.6±0.3-fold); TNF + T0901317 + simvastatin (12.6±0.3-fold) (Figure 6A: $p<0.05$). The same pattern was observed in Figure 6B. These data show that treatments that induce ABCA1 expression individually (i.e. LXR agonists and TNF, but not statins) can co-contribute to enhance ABCA1 upregulation in a synergistic manner in BEAS-2B cells.

Discussion

In this study, we examined ABCA1 protein upregulation in BEAS-2B and A549, two human cell lines commonly used to perform *in vitro* investigation of lung epithelial responses in chronic respiratory disease. We have shown that ABCA1 protein can be significantly upregulated in BEAS-2B cells after treatment with inflammatory cytokine TNF, cholesterol, and its oxidized derivative, 25-cholesterol, and the LXR agonist T0901317. Apart from TNF, these treatments also induced ABCA1 upregulation in A549 cells. There was evidence of additive and synergistic effects of these treatments in both cell types. The statins, simvastatin and atorvastatin, had no effect on ABCA1, alone or in combination with other treatments.

Although the rationale for the current study was to model COPD and dyslipidemia *in vitro* and test the hypothesis that statins repress ABCA1 (and alternative hypothesis for the failure of STATCOPE), a hypothesis that was disproved by our experiments, our study has yielded new findings. Firstly, it shows for the first time that TNF upregulates ABCA1 protein in BEAS-2B, but not A549 cells. The impact of TNF on ABCA1 has previously been shown to be cell type specific [21] and may be related to TNF receptor expression [22]. Secondly, it demonstrates the utility of BEAS-2B cell lines for future studies of novel modulators and mechanisms of ABCA1 expression. A549 cells have already been used to study ABCA1 in the past [23-25]. Although of course, use of primary epithelial cells are optimal, and this is a limitation of our study, we have confirmed the usefulness of epithelial cell lines as validatory tool. The need for these tools was recently exemplified in a seminal report by Aguiar *et al*. [17] that performed bioinformatic analysis of the gene expression pattern for the family of ABC transporters (48 in total) in airway epithelial cells from people with COPD or asthma. A lung epithelial cell line (Calu-3) was used to further interrogate ABC gene expression, the impact of cigarette smoke, and provide an

enabling platform to discover the underlying molecular mechanism of ABC transporter expression.

Validated *in vitro* models are imperative because the need to understand regulation of ABC transporters, especially ABCA1, has come to the forefront recently through a renewed recognition of the importance of lipid homeostasis in respiratory disease and its co-morbidities. Airway cells, especially alveolar macrophages and type II pneumocytes, process considerable amounts of lipids to maintain a healthy equilibrium [12]. Thus, ABC lipid transporters have a critical protective role in the lung. However, this homeostasis can be perturbed with significant impact on disease pathogenesis. This was recently demonstrated by Sonett *et al*. [16] whereby cigarette smoke was shown to adversely affects cholesterol transport via an ABCA1-dependent mechanism in macrophages. Notably, downregulation of ABC transporters, particularly ABCA1, was observed in the lungs of patients diagnosed with COPD [16].

ABCA1 expression therefore represents a putative, and modifiable, link between co-morbidities, given that atherosclerosis is more common in smokers with airway limitation, than in those with normal lung function [26]. Thus, it follows that upregulating ABCA1 has the potential to block cigarette smoke-induced impact on the lung. The LXR agonist T0901317 is a potent inducer of ABC transporters [27]; accordingly, Sonett *et al*. [16] performed *in vitro* experiments in macrophages, and *in vivo* murine models that mimic hallmark features of COPD, to show that LXR activation significantly alters lung function and structure after chronic cigarette smoke exposure in a manner linked to increased ABCA1 activity.

Hence, pharmacological strategies that enhance ABCA1 activity may offer a novel therapeutic approach to reverse the damaging effects of cigarette smoke and protection from the development of COPD. In our study, we have shown that LXR agonist T0901317 robustly

increases ABCA1 protein upregulation in lung epithelial cell lines. However, further development and investigation of safe and specific LXR agonists are urgently required. This was underscored by the recent review of LXR agonists as potential therapeutics in inflammatory diseases [18] and the significant challenges encountered, including significant liver toxicity [18]. These challenges may be overcome through the development of LXRβ-selective agonists (there are two isoforms of LXR (LXRα and LXRβ) and LXRα activation has been associated with liver toxicity [18]) or tissue-selective agonism. It is important to note that T0901317 has affinity for both LXRα and LXRβ and newer, selective LXRβ agonists could be assessed in our *in vitro* model in lung epithelial cell lines. Finally, a recent *in vivo* study [19] pharmacological activation of LXR with T0901317 in mice exposed to cigarette smoke resulted in adverse effects related to perturbed pulmonary surfactant homeostasis. Given that surfactants are regulated by ABC transporters, selective pharmacological targeting of ABCA1 may have merit.

A limitation of the current study is that the influence of statins, LXR agonist and other treatments on ABCA1 expression in lung epithelial cell lines was limited only to one type of analysis (Western blotting). Further investigations are warranted and could include investigation of transcriptional and post-transcriptional regulation of ABCA1 mRNA expression as well as delineation of the cellular signaling pathways responsible.

In summary, our study disproved our original hypothesis, but overall provided useful data to show the effect of statins and the synthetic LXR agonist T0901317 on expression of ABCA1 transporter protein in human lung epithelial cell lines *in vitro*.

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Author contributions

Conceived, designed and performed the experiments: PH, ICG, AJA. Intellectual input and *in vitro* model development: AS. Analysis and interpretation: PH, AJA. Wrote the paper: AJA.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. Effect of TNF or cholesterol/25-OH, alone and in combination, on ABCA1 protein expression in BEAS-2B and A549 cells: establishing the *in vitro* model. (A) BEAS-2B and (B) A549 cells were left untreated or treated with TNF (10 ng/mL), cholesterol (5 μg/mL), 25-OH (5 μg/mL), TNF + cholesterol, or TNF + 25-OH, for 24 h. Western blotting for ABCA1 was performed (compared to HSP-70 as loading control). Results are representative Western blots or densitometric analysis (normalized to HSP-70 and expressed as ABCA1 protein upregulation (fold change compared to untreated control cells)). Statistical analysis was performed using one-way ANOVA then Bonferroni's multiple comparison test (where * denotes a significant increase compared to untreated cells (p<0.05)). Data are mean+SEM values from n=3 independent experiments.

Figure 2. Effect of the LXR agonist T0901317 on ABCA1 expression in BEAS-2B and A549 cells. (A) BEAS-2B and (B) A549 cell lines were left untreated or treated with increasing concentrations of LXR agonist T0901317 (0.5 – 10 μM) for 24 h. Western blotting for ABCA1 was performed (compared to HSP-70 as loading control). Results are representative Western blots or densitometric analysis (normalized to HSP-70 and expressed as ABCA1 protein upregulation (fold change compared to untreated control cells)). Statistical analysis was performed using one-way ANOVA then Bonferroni's multiple comparison test (where * denotes a significant increase compared to untreated cells (p<0.05)). Data are mean+SEM values from n=3 independent experiments.

Figure 3. Effect of simvastatin or atorvastatin on ABCA1 expression in BEAS-2B and A549 cells. (A, B) BEAS-2B and (C, D) A549 cell lines were either: left untreated, treated with TNF (10 ng/mL), or treated with decreasing concentrations (10 – 0.5 μM) of (A, C) simvastatin or (B, D) atorvastatin for 24 h. Western blotting for ABCA1 was performed (compared to HSP-70 as loading control). Results are Western blots from a pilot study (n=1).

Figure 4. Simvastatin had no effect on ABCA1 protein expression in an *in vitro* model of COPD and dyslipidemia. BEAS-2B cells were treated with vehicle or simvastatin (10 μM) and then ABCA1 protein expression stimulated with (A) TNF (10 ng/mL), (B) cholesterol (5 μg/mL), (C) 25-OH (5 μg/mL), (D) TNF + cholesterol, or (E) TNF + 25-OH, for 24 h, compared to untreated control cells. Western blotting for ABCA1 was performed (compared to HSP-70 as loading control). Results are representative Western blots or densitometric analysis (normalized to HSP-70 and expressed as ABCA1 protein upregulation (fold change compared to untreated control cells)). Statistical analysis was performed using one-way ANOVA then Bonferroni's multiple comparison test (where * denotes a significant increase compared to untreated cells (p<0.05)). Data are mean+SEM values from n=3 independent experiments.

Figure 5. Atorvastatin had no effect on ABCA1 protein expression in an *in vitro* model of COPD and dyslipidemia. BEAS-2B cells were treated with vehicle or atorvastatin (10 μM) and then ABCA1 protein expression stimulated with (A) TNF (10 ng/mL), (B) cholesterol (5 μg/mL), (C) 25-OH (5 μg/mL), (D) TNF + cholesterol, or (E) TNF + 25-OH, for 24 h, compared to untreated control cells. Western blotting for ABCA1 was performed (compared to HSP-70 as loading control). Results are representative Western blots or densitometric analysis (normalized

to HSP-70 and expressed as ABCA1 protein upregulation (fold change compared to untreated control cells)). Statistical analysis was performed using one-way ANOVA then Bonferroni's multiple comparison test (where * denotes a significant increase compared to untreated cells (p<0.05)). Data are mean+SEM values from n=3 independent experiments.

Figure 6. Combined effect of T0901317, statins, and TNF, alone and in combination, on ABCA1 protein expression in BEAS-2B cells. BEAS-2B cells were treated with: (A) T0901317 (10 μM), simvastatin (10 μM), TNF (10 ng/mL), T0901317 + simvastatin, TNF + T0901317 + simvastatin; or (B) T0901317 (10 μM), atorvastatin (10 μM), TNF (10 ng/mL), T0901317 + simvastatin, TNF + T0901317 + atorvastatin, for 24 h, compared to untreated control cells. Western blotting for ABCA1 was performed (compared to HSP-70 as loading control). Results are representative Western blots or densitometric analysis (normalized to HSP-70 and expressed as ABCA1 protein upregulation (fold change compared to untreated control cells)). Statistical analysis was performed using one-way ANOVA then Bonferroni's multiple comparison test (where * denotes a significant increase (p<0.05)). Data are mean+SEM values from n=3 independent experiments.