Crucial role of fatty acid oxidation in asthmatic bronchial smooth muscle remodelling

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Abstract

Background Bronchial smooth muscle (BSM) remodelling in asthma is related to an increased mitochondrial biogenesis and enhanced BSM cell proliferation in asthma. Since mitochondria produce the highest levels of cellular energy and fatty acid β-oxidation is the most powerful way to produce ATP, we hypothesised that, in asthmatic BSM cells, energetic metabolism is shifted towards the β-oxidation of fatty acids.

Objectives We aimed to characterise BSM cell metabolism in asthma both *in vitro* and *ex vivo* to identify a novel target for reducing BSM cell proliferation.

Methods 21 asthmatic and 31 non-asthmatic patients were enrolled. We used metabolomic and proteomic approaches to study BSM cells. Oxidative stress, ATP synthesis, fatty acid endocytosis, metabolite production, metabolic capabilities, mitochondrial networks, cell proliferation and apoptosis were assessed on BSM cells. Fatty acid content was assessed *in vivo* using matrix-assisted laser desorption/ionisation spectrometry imaging.

Results Asthmatic BSM cells were characterised by an increased rate of mitochondrial respiration with a stimulated ATP production and mitochondrial β-oxidation. Fatty acid consumption was increased in asthmatic BSM both *in vitro* and *ex vivo*. Proteome remodelling of asthmatic BSM occurred *via* two canonical mitochondrial pathways. The levels of carnitine palmitoyl transferase (CPT)2 and low-density lipoprotein (LDL) receptor, which internalise fatty acids through mitochondrial and cell membranes, respectively, were both increased in asthmatic BSM cells. Blocking CPT2 or LDL receptor drastically and specifically reduced asthmatic BSM cell proliferation.

Conclusion This study demonstrates a metabolic switch towards mitochondrial β-oxidation in asthmatic BSM and identifies fatty acid metabolism as a new key target to reduce BSM remodelling in asthma.

Introduction

Asthma is the most common chronic airway disease and causes a substantial health and economic burden worldwide. According to the Global Initiative for Asthma (GINA), asthma is a heterogeneous disease defined by a history of respiratory symptoms, which vary over time and in intensity, together with variable expiratory airflow limitations [1].

Asthma pathophysiology involves chronic inflammation of both large and small airways, as well as bronchial remodelling [2]. The most crucial feature of bronchial remodelling appears to be bronchial smooth muscle (BSM) increased mass, associated with a reduced lung function [3, 4] and an increased exacerbation rate [5], which are poor prognostic indicators in asthma. Moreover, BSM remodelling can begin very early in life, often at preschool age, and predicts the persistence of asthma in school-aged children [6]. Thus, BSM remodelling plays a crucial role in asthma pathophysiology, and there is an unmet need to pharmacologically target BSM remodelling [7].

BSM hyperplasia appears to be the primary feature of BSM remodelling related to an increase in cell proliferation in asthmatic BSM both *in vitro* [8–10] and *ex vivo* [4] compared to non-asthmatic subjects. We previously demonstrated that this increased proliferation of BSM cells from patients with severe asthma was associated with increased mitochondrial biogenesis and mass [8]. However, although gallopamil, a calcium channel blocker, normalised mitochondrial biogenesis and cell proliferation *in vitro* [8], it only minimally reduced BSM mass *in vivo* [11], suggesting that other pathways are involved in this phenomenon. Additionally, we observed that BSM remodelling was associated with an increased mitochondrial mass in patients with nonsevere asthma, as patients with a high BSM mass presented a higher mitochondrial mass within their BSM [5]. Finally, we previously demonstrated that the increased BSM cell proliferation was more related to mitochondrial respiration than to anaerobic glycolysis in severe asthma [8]. Among the variety of sources of mitochondrial respiration, fatty acid β-oxidation is the most powerful way to produce ATP in various cell types [12]. For instance, a link between fatty acid metabolism and cell proliferation has been demonstrated previously in hepatocytes [13] and cancer cells [14]. Moreover, the blood concentration of low-density lipoprotein (LDL), a source of fatty acids in various cell types after interaction with its receptor [15–17] is negatively correlated with forced expiratory volume in 1 s (FEV_1) in asthma [18].

Therefore, we hypothesised that BSM metabolism was shifted, in asthmatic BSM cells, towards the β-oxidation of fatty acids, which may represent a potential target to reduce BSM cell proliferation in asthmatic patients.

Methods

A complete description of all methods used in this study is supplied in the supplementary material.

Study population

Patients with asthma and non-asthmatic subjects were prospectively recruited from the Centre Hospitalier Universitaire (Bordeaux, France), according to the GINA criteria [1]. Asthmatic patients were recruited from the Cohort of Bronchial Obstruction and Asthma (COBRA; ethics committee number 2008-A00294–51/1). Non-asthmatic subjects were recruited after surgical resection. This study received approval from the local and national ethics committees. Bronchial specimens from all subjects were obtained by either fibreoptic bronchoscopy or lobectomy in macroscopically normal areas, as described previously [19].

Cell culture

BSM cells were obtained from patient biopsies and bronchi dissected out from lobectomy, as described previously [8]. BSM cells were then used to assess cell proliferation (by manual counting, bromodeoxyuridine (BrdU) assay and flow cytometry), cell apoptosis (by luminescent assay), oxidative stress (using OxyBlot), oxygen consumption rate (using Oroboros), cellular and mitochondrial ATP synthesis (by bioluminescence) and various protein contents (by Western blot). The mitochondrial network was visualised by immunostaining (TOMM20).

BSM cell metabolites were quantified using 25 mM of uniformly labelled carbon-13 [U-^{13}C]-glucose and mass spectrometry. Fatty acid endocytosis was assessed by means of fluorescent BODIPY-ceramide. The metabolic capabilities of BSM cells were characterised *via* redox reactions associated with cellular respiration. carnitine palmitoyl transferase (CPT)1 and/or CPT2 were inhibited using either pharmacological (using etomoxir or perhexilin) or genetic silencing (using dedicated lentivirus) methods. The LDL receptor was inhibited by using a blocking antibody.

Proteomic analysis

Cells and tissue lysates were processed using radioimmunoprecipitation assay buffer. Each lysate was centrifuged and supernatants were used for the proteomic analysis at the mass spectrometry facility of Bordeaux University, as described previously [20]. Briefly, proteomic analysis was performed using an Ultimate 3000 RSLC Nano-UPHLC system (ThermoFisher Scientific, Waltham, MA, USA) coupled to a nanospray Orbitrap Fusion Lumos Tribrid Mass Spectrometer (ThermoFisher Scientific).

Matrix-assisted laser desorption/ionisation–mass spectrometry imaging analysis

Patient biopsies were frozen at −80°C and subsequently embedded in a gel of 5% carboxymethyl cellulose. Serial cryo-sections (12 µm thick) were cut from bronchial biopsies at −20°C using a NX70 Star cryostat (ThermoFisher Scientific) and thaw-mounted onto standard glass microscope slides for matrix-assisted laser desorption/ionisation (MALDI)–mass spectrometry imaging. These acquisitions were performed using a high-performance atmospheric pressure imaging ion source called AP-SMALDI 5 AF (TransMIT) connected to an orbital trapping mass spectrometer (QExactive Orbitrap, ThermoFisher Scientific). After MALDI–mass spectrometry imaging, we performed an α-smooth muscle actin immunostaining to localise the BSM area. We then estimated the relative lipid content within the BSM on the MALDI images. Fatty acid annotation was performed using the Human Metabolome Database and METASPACE software.

Results

Patients' characteristics

We recruited 21 patients with asthma and 31 non-asthmatic subjects, whose characteristics are presented in table 1. Unsurprisingly, asthmatic patients exhibited reduced FEV_1 compared to that observed in the non-asthmatic subjects.

Cell proliferation and mitochondrial mass are increased in asthmatic BSM

We initially confirmed that asthmatic BSM cell proliferation was increased compared to that of non-asthmatic subjects through manual cell counting, BrdU and flow cytometry assays (figure 1a–c). As demonstrated previously [8], no change in apoptosis was observed between the two groups (figure 1d). Furthermore, cellular oxidative damage, such as protein carbonylation, was not different between non-asthmatic and asthmatic BSM cells, indicating that oxidative stress was not implicated in the proliferative processes (figure 1e). Quantification of TOMM20 immunostaining exhibited an increase in the mitochondrial network in asthmatic BSM cells (figure 1f). In addition, we demonstrated that mitochondrial mass was significantly increased in asthmatic BSM cells using three complementary markers (*i.e.* porin, citrate synthase and TOMM20; figure 1g–i).

Mitochondrial bioenergetic function is enhanced in asthmatic BSM

Functional evaluation of the mitochondrial bioenergetics of BSM cells revealed an increased rate of mitochondrial respiration along with a higher maximal capacity in asthmatic BSM cells compared to that

TABLE 1 Clinical characteristics of all subjects

	Non-asthmatic subjects	Asthmatic patients	p-value
Subjects	31	21	NS
Age years	65±8	55±16	NS
Male/female	17/14	6/15	NS
BMI kg·m^{-2}	24.9±5.0	25.9±5.4	NS
Smoking history			
Pack-years	36.3±21.8	1.9±2.9	0.0001
Current smokers n/N	13/31	0/21	0.0006
Ex-smokers n/N	12/31	8/21	NS
Treatments			
ICS yes/no	0/31	21/0	<0.0001
LABA yes/no	0/31	20/1	<0.0001
OCS yes/no	0/31	3/18	NS
FEV_1 L	2.47±0.85	1.97±0.68	0.03
FEV_1 % predicted	89.3±22.2	75.8±22.7	0.03
FEV_1/FVC ratio	0.74±0.12	0.71±0.10	NS
FVC L	3.37±1.15	2.78±0.77	0.04
FVC % predicted	99.2±19.8	89.9±20.4	NS
F_{eNO} ppb	ND	32.8±26	

Data are presented as n or mean±SD, unless otherwise stated. Comparisons of continuous variables was made using either Mann–Whitney or t-tests; categorical variables were analysed using Fisher's exact tests. BMI: body mass index; ICS: inhaled corticosteroid; LABA: long-acting β_2-agonist; OCS: oral corticosteroid; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; F_{eNO}: fractional exhaled nitric oxide; NS: nonsignificant; ND: not determined.

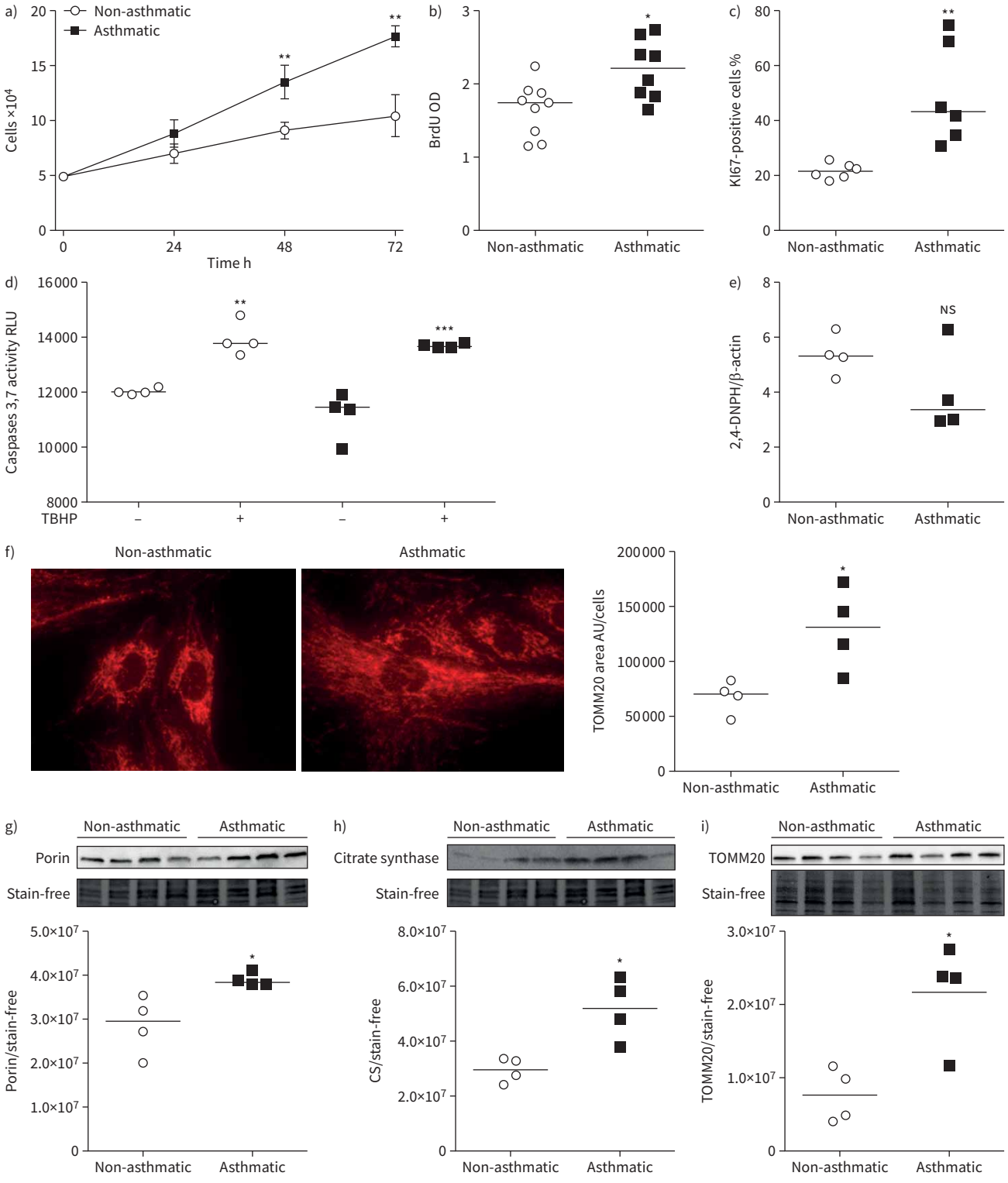

FIGURE 1 Increased asthmatic bronchial smooth muscle (BSM) cell proliferation is associated with mitochondrial mass. a) Growth curves of non-asthmatic (n=12) and asthmatic (n=10) BSM cells were obtained by performing cell counting at 72 h; **: p<0.01 using two-way ANOVA. Cell proliferation was assessed by b) bromodeoxyuridine (BrdU) incorporation assay (n=9) or c) Ki67 staining and flow cytometry (n=6), using non-asthmatic and asthmatic BSM cells; **: p<0.01 using Mann–Whitney test. d) Levels of basal apoptosis (–) and induced apoptosis (+) using 100 µM tert-butyl-hydroxide (TBHP) for 1 h were analysed by measuring caspase 3 and 7 activities and bioluminescent assays; **: p<0.01, ***: p<0.001 using one-way ANOVA test. e) Immunoblot detection of 2,4-dinitrophenylhydrazine (DNPH) carbonyl groups using whole-cell lysates of non-asthmatic (n=4) and asthmatic (n=4) BSM cells normalised by β-actin content; *: p<0.05 using Mann–Whitney test. f) Representative

mitochondrial networks and quantification were obtained by immunofluorescence imaging of TOMM20 in non-asthmatic (n=4) and asthmatic BSM cells (n=4). Immunoblot analysis of mitochondrial mass markers, including g) porin (n=4), h) citrate synthase (n=4) and i) TOMM20 (n=4) protein expression levels in non-asthmatic and asthmatic BSM cells. *: p<0.05, using Mann–Whitney test. Stain-Free gel technology was used for loading control expression quantification. Data are presented as a) mean±SEM or b–i) dot plots with the median. OD: optical density; NS: nonsignificant.

observed in the non-asthmatic subjects (figure 2a). The steady-state ATP content was significantly increased in asthmatic BSM cells compared to BSM cells from non-asthmatic subjects (figure 2b). Interestingly, half of this ATP production was sensitive to the inhibition of oxidative phosphorylation (OXPHOS) by rotenone, antimycin and potassium cyanide (figure 2c).

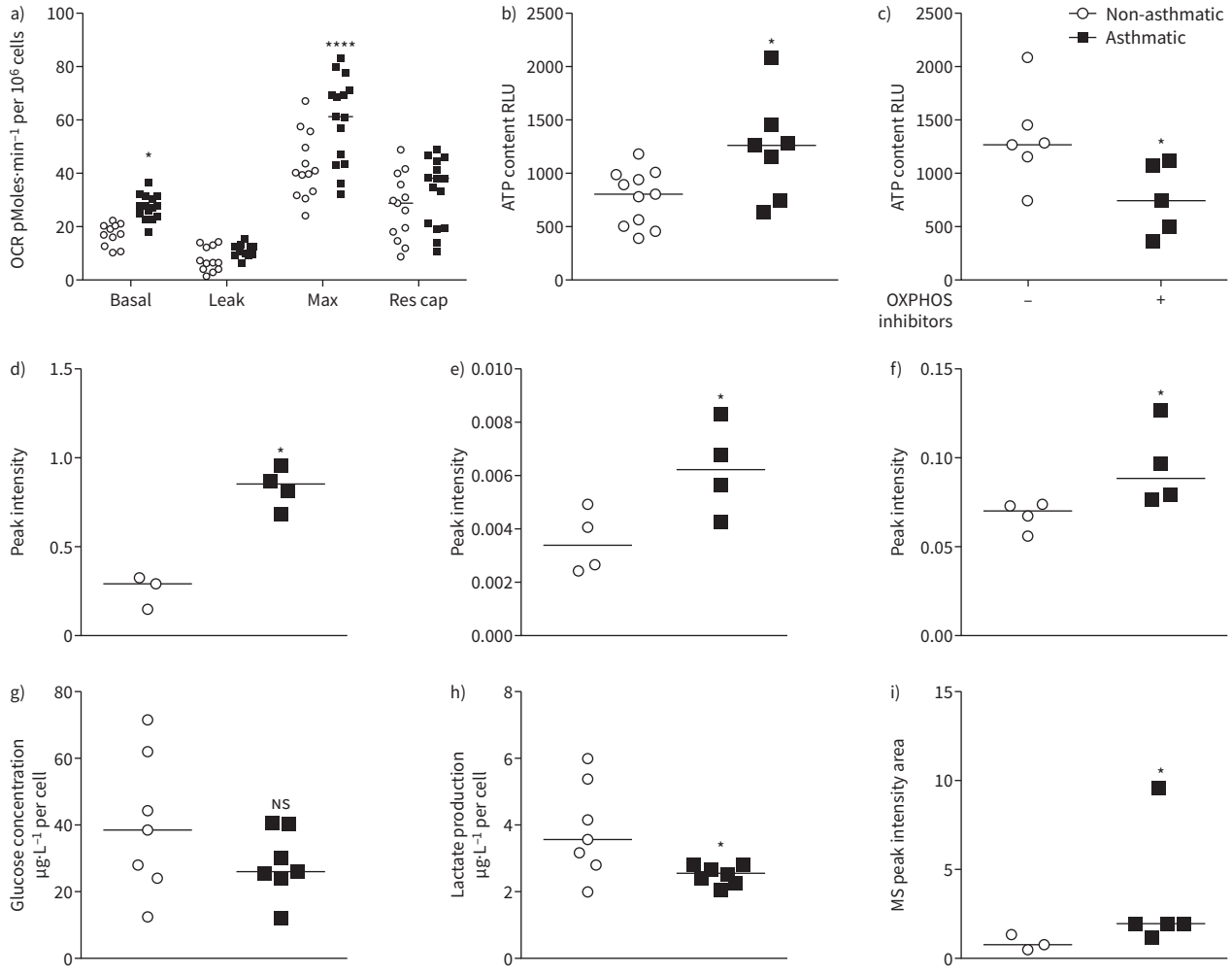

FIGURE 2 Increased energy metabolism is observed in asthmatic bronchial smooth muscle (BSM) cells. a) Oxygen consumption rate (OCR) was measured in different respiratory states: basal (DMEM 5 mM glucose), leak (30 µM oligomycin) and maximal (max, 5 µM carbonyl cyanide m-chlorophenylhydrazone), in non-asthmatic (n=16) and asthmatic (n=15) BSM cells. Reserve capacity (res cap) was calculated as the difference between the maximal and basal respiratory rates. The different rates were corrected for non-mitochondrial respiration determined after the addition of 1 mM potassium cyanide. *: p<0.05, ****: p<0.0001 using two-way ANOVA. b) Steady-state ATP content was measured in non-asthmatic (n=12) and asthmatic (n=8) BSM cells using a luminescence assay. c) The portion of ATP produced by mitochondria in asthmatic BSM cells was inhibited using a cocktail of oxidative phosphorylation (OXPHOS) inhibitors (see methods section) (n=8). Metabolomics was performed using carbon-13 (^{13}C) mass spectrometry (MS) analysis of d) pyruvate, e) succinate and f) malate peak intensity in non-asthmatic (n=5) and asthmatic (n=5) BSM cells. g) Resting glucose and h) lactate production concentrations were assessed in the culture medium of non-asthmatic (n=12) and asthmatic (n=10) BSM cells. i) Steady-state ATP content in non-asthmatic (n=5) and asthmatic (n=5) BSM cells using ^{13}C-mass spectrometry. Data are presented as dot plots with the median. *: p<0.05, using Mann–Whitney test. RLU: relative light units; NS: nonsignificant.

Glucose oxidative catabolism is stimulated in asthmatic BSM cells

As glucose metabolism is a major pathway in cellular bioenergetics, we investigated the fate of glucose using tracing methods. Levels of ^{13}C-pyruvate (figure 2d), ^{13}C-succinate (figure 2e) and ^{13}C-malate (figure 2f) were significantly increased in asthmatic BSM cells compared to those of non-asthmatic subjects. However, no difference in glucose uptake was observed between the asthmatic and non-asthmatic BSM cells (figure 2g), whereas lactate production was decreased in asthmatic BSM cells (figure 2h), suggesting a stimulation of oxidative catabolism. Accordingly, a significant increase in ^{13}C-ATP was also observed in asthmatic BSM cells (figure 2i). These results strongly confirmed the increase in tricarboxylic acid (TCA) cycle activity in asthmatic BSM cells. Since the TCA cycle can be fuelled by numerous anaplerotic sources in addition to glucose, we performed an unbiased proteomics investigation to identify additional changes in cell metabolism.

Proteome remodelling reveals a stimulation of the fatty acid oxidation system in asthmatic BSM

Raw data from the label-free proteomics approach were shared through the Proteomics Identifications Database (under accession number PXD015566) and revealed significant modifications in the expression of 213 proteins ($p<0.05$). Using Ingenuity Pathway Analysis, we demonstrated significant alterations in various canonical pathways including "mitochondrial dysfunction" and "oxidative phosphorylation" (figure 3a), in complete agreement with the stimulation of mitochondrial bioenergetics in asthmatic BSM cells described earlier. Moreover, after analysing the specific upregulated and downregulated proteins in asthmatic BSM cells, we focused our attention on CPT2, since it was the most significantly upregulated protein (3.063-fold increase; $p=0.016$) among the mitochondrial dysfunction annotation box in asthmatic BSM cells compared to that observed in the non-asthmatic BSM cells (figure 3b and supplementary table S1). CPT2 is a mitochondrial fatty acid transporter allowing that allows fatty acids to follow the β-oxidation pathway for ATP production. CPT2 expression was significantly increased in asthmatic BSM cells *in vitro* compared to that in BSM cells from non-asthmatic subjects (figure 3c).

Fatty acid consumption is specifically strengthened in asthmatic BSM

To be metabolised, fatty acid molecules first need to enter BSM cells through LDL-R, one of the primary cell surface receptors for triglyceride-enriched lipoproteins. We observed a significant increase in LDL-R protein expression *in vitro* in asthmatic BSM compared to that observed in the non-asthmatic subjects (figure 4a). Next, we assessed the short-term (*i.e.* 20 min) fatty acid endocytosis by asthmatic and non-asthmatic BSM cells using BODIPY lipid ceramide, a fluorescent tool for the analysis of fatty acid endocytosis (figure 4b). We observed significantly increased fluorescence within asthmatic BSM cells, indicating increased absorption into asthmatic BSM cells (figure 4c). BODIPY fluorescent lipid ceramide uptake by BSM cells was also quantified using a fluorimeter, with the results showing a significant increase in absorption by asthmatic BSM cells compared to BSM cells from non-asthmatic subjects (figure 4d). Moreover, to confirm that fatty acid consumption by asthmatic BSM cells was increased in asthmatic BSM cells, we measured the resting concentrations of fatty acids in the culture medium of asthmatic and non-asthmatic BSM cells. As expected, we observed a significantly lower resting concentration of fatty acids in asthmatic BSM cell medium compared to that the in non-asthmatic cell medium (figure 4e). To elucidate the capacity of BSM cells to perform OXPHOS associated with fatty acid oxidation, we used a Biolog MitoPlate assay, which measures the reduction of cytochrome c [21], an intermediate of the respiratory chain. Using palmitoyl-DL-carnitine as a unique energetic substrate for BSM cells, we observed a significant increase in the dye reduction by asthmatic BSM cells, suggesting a higher level of OXPHOS associated with fatty acid oxidation (figure 4f).

To further assess fatty acid metabolism *in vivo*, we performed MALDI–mass spectrometry imaging of asthmatic and non-asthmatic bronchial biopsy slices. Using BSM immunostaining following mass spectrometry imaging, we estimated the relative lipid content within BSM (figure 5 and supplementary figure S1). Among the most representative fatty acids, we observed a significant decrease in arachidonic (figure 5a), stearic (figure 5b) and linoleic (figure 5c) acid contents in asthmatic BSM compared to those observed in the non-asthmatic subjects. However, the difference in the palmitic acid content between asthmatic and non-asthmatic BSM did not reach statistical significance ($p=0.06$) (figure 5d). To assess the BSM tissue specificity of this increased consumption of fatty acids, we performed the same analysis for the bronchial epithelium of the same biopsies. There were no alterations in the contents of any of the tested fatty acids between asthmatic and non-asthmatic bronchial epithelial layers (figure 5e–h).

Taken together, these results demonstrated that the fatty acid consumption-mediated increase in mitochondrial metabolism was specifically increased in asthmatic BSM both *in vitro* and *ex vivo*.

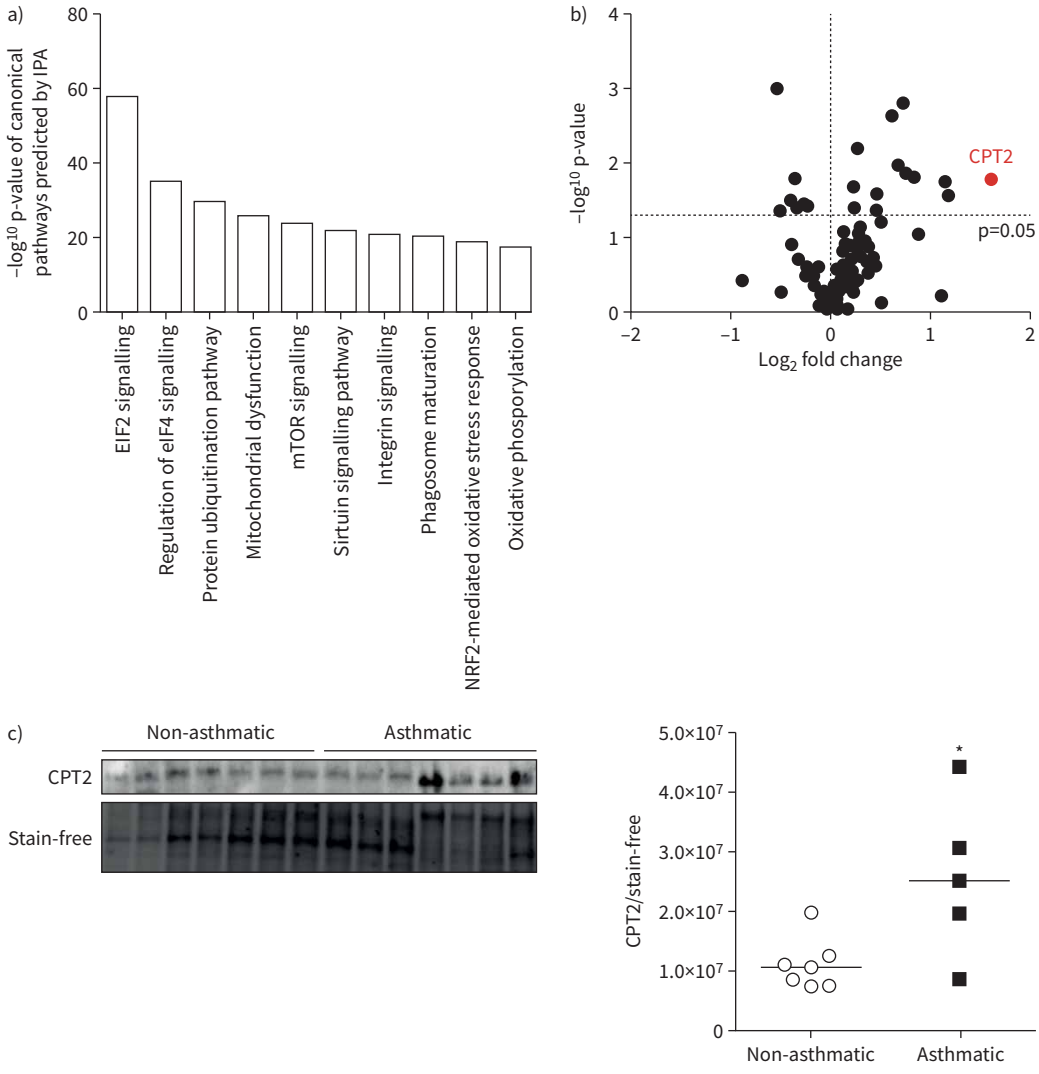

FIGURE 3 Proteome remodelling promotes bioenergetics in asthmatic bronchial smooth muscle (BSM). a) Proteome comparison between non-asthmatic and asthmatic BSM cells *in vitro* (n=5) using ingenuity pathway analysis (IPA) from the raw proteomic data. Proteins with a different expression levels were organised based on pre-defined categories suggested by IPA. Then, the proteins with different expression levels were assigned to these IPA categories based on the IPA database. Categories were ranked according to their frequency of identification in the proteome (−log p-value). b) Volcano plots of differentially expressed genes in the non-asthmatic (n=5) and asthmatic (n=5) BSM cells using mass spectrometry analysis. y=1.3 represents p=0.05. c) Immunoblot of carnitine palmitoyl transferase (CPT)2 protein expression in non-asthmatic (n=7) and asthmatic (n=7) BSM cells. Stain-Free gel technology was used for loading control expression quantification. Data are presented as dot plots with the median. *: p<0.05, using Mann–Whitney test.

Inhibition of fatty acid oxidation specifically impedes asthmatic BSM cell proliferation

Next, we assessed whether the specific increase in fatty acid metabolism within asthmatic BSM was required for BSM cell proliferation. To shut down the fatty acid oxidation pathway, we first inhibited CPT1 and CPT2 activities using etomoxir (figure 6a and b) and perhexilin (figure 6c and d), which both significantly decreased the proliferation of asthmatic BSM cells. However, neither compound affected non-asthmatic BSM cell proliferation. Moreover, the proliferation of bronchial epithelial cells was not affected by either etomoxir or perhexilin (supplementary figure S2a and b), again suggesting a specific role of mitochondrial fatty acid metabolism in asthmatic BSM cell proliferation. Subsequently, we demonstrated that both etomoxir and perhexilin significantly decreased basal respiration in asthmatic, but not non-asthmatic BSM cells (figure 6e). To further confirm the crucial role of CPT2 in asthmatic BSM cell proliferation, we used a silencing strategy with a lentivirus producing short hairpin (sh)RNA against CPT2.

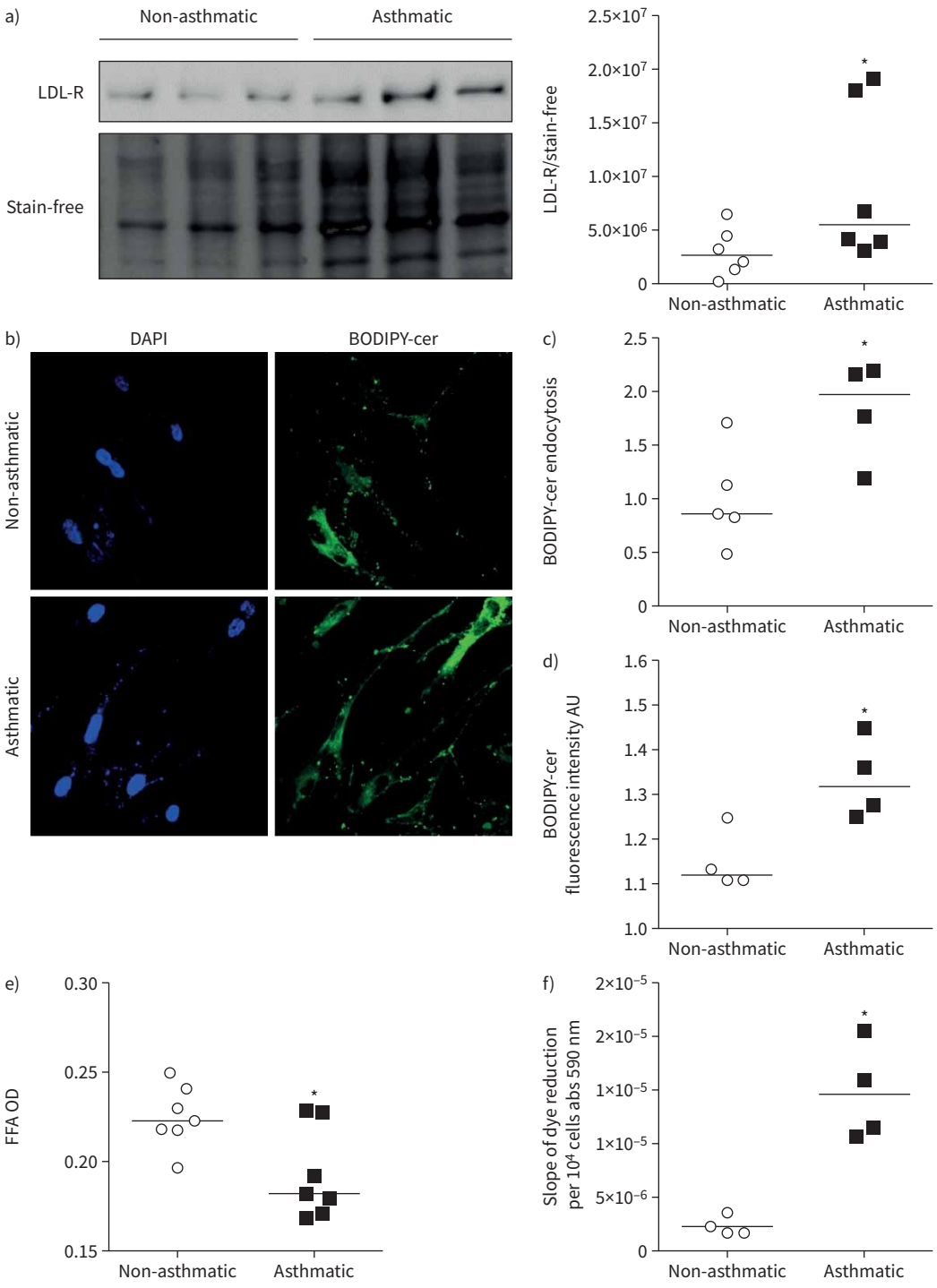

FIGURE 4 Fatty acid uptake is increased in asthmatic bronchial smooth muscle (BSM) cells. a) Immunoblot of low-density lipoprotein receptor (LDL-R) protein expression in non-asthmatic (n=7) and asthmatic (n=7) BSM cells. Stain-Free gel technology was used for loading control expression quantification. b) Representative images of incorporated fluorescent ceramide (cer) in non-asthmatic and asthmatic BSM cells. Nuclei were stained in blue with 4′,6-diamidino-2-phenylindole (DAPI). c) Quantification of endocytosis of fluorescent ceramide 20 min after its addition to the cell culture medium in non-asthmatic (n=5) and asthmatic (n=5) BSM cells. Fluorescence was normalised to cell number. Data are expressed above the mean non-asthmatic value. d) Incorporation of fluorescent ceramide was measured by fluorimetry in non-asthmatic (n=4) and asthmatic (n=4) BSM cells. e) Free fatty acid (FFA) resting concentration in the medium of non-asthmatic (n=7) and asthmatic (n=7) BSM cells was measured by absorbance. f) Palmitoyl-DL-carnitine reduction of cytochrome c using Biolog

assay in non-asthmatic (n=4) and asthmatic (n=4) BSM cells. Data are presented as dot plots with the median. AU: arbitrary unit; OD: optical density. *: p<0.05, using Mann–Whitney test.

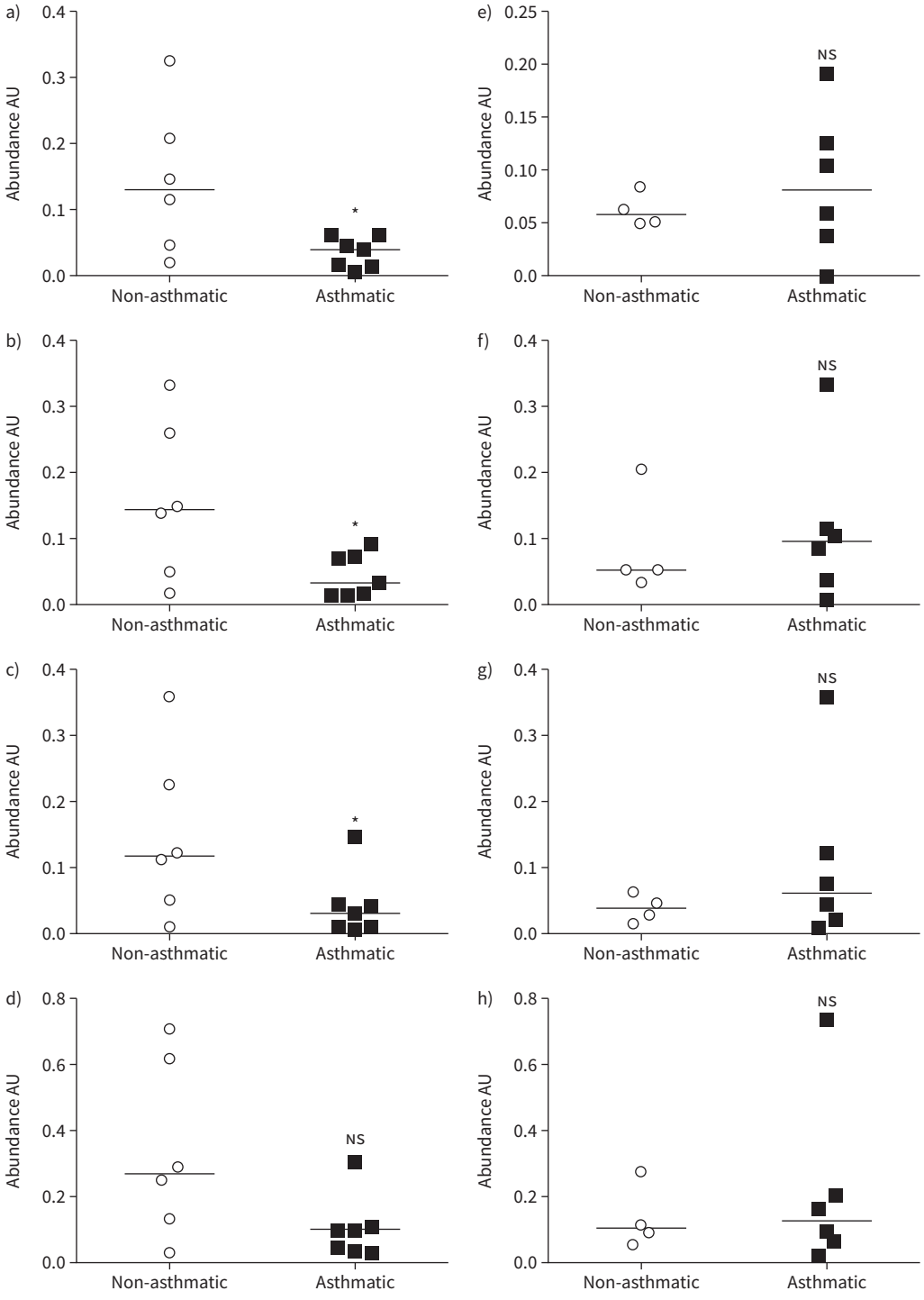

FIGURE 5 *Ex vivo* demonstration of fatty acid consumption by asthmatic bronchial smooth muscle (BSM). Arachidonate C_{20}:4 (a and e), stearate C_{18}:0 (b and f), linoleate C_{18}:2 (c and g) and palmitate C_{16}:0 (d and h) levels were quantified in BSM (a–d) and in bronchial epithelium (BE) (e–h) from non-asthmatic (n=6) and asthmatic (n=7) biopsies using matrix-assisted laser desorption/ionisation (MALDI)–mass spectrometry imaging analysis. Data are presented as dot plots with the median. AU: arbitrary units; NS: nonsignificant. *: p<0.05, using Mann–Whitney test.

>90% of asthmatic BSM cells were effectively transduced by the lentivirus, as assessed by the percentage of green fluorescent protein-positive cells using flow cytometry (supplementary figure S3a). This lentivirus significantly reduced CPT2 protein expression by 59.4±3.6% in asthmatic BSM cells, as assessed by Western blot analysis (supplementary figure S3b), but it did not significantly alter CPT1 expression (supplementary figure S3c). The specific silencing of CPT2 was shown to reduce both asthmatic (figure 6f and g) and non-asthmatic BSM cell proliferation (supplementary figure S4a and b), as determined by the percentage of Ki67-positive cells and by cell number. We further demonstrated that blocking LDL-R drastically decreased proliferation in asthmatic BSM cells but not in the non-asthmatic cells (figure 6h). In addition, we demonstrated that etomoxir, perhexilin and the anti-LDL-R antibody did not modify asthmatic BSM cell apoptosis compared to that observed for the non-asthmatic cells (supplementary figure S5). Moreover, blocking fatty acid entry using an LDL-R-blocking antibody or inhibiting fatty acid entry into mitochondria using etomoxir, perhexilin or dedicated lentivirus-producing shRNA against CPT2 did not normalise mitochondrial mass in asthmatic BSM cells (supplementary figure S6). Finally, we assessed the effect of optimal concentrations of steroids (*i.e.* fluticasone, budesonide) alone or in combination with long-acting β_2-agonists (*i.e.* salmeterol, formoterol) on asthmatic BSM cells. None of these compounds, either alone or in combination, was able to significantly alter either CPT2 or LDL-R expression (supplementary figure S7) or ATP production (supplementary figure S8) in asthmatic BSM cells.

Discussion

Taken together, the results of the present study demonstrate, for the first time, that hyperproliferative asthmatic BSM is characterised by a switch towards mitochondrial fatty acid metabolism both *in vitro* and *ex vivo*. Moreover, this switch is responsible for the hyperplasia observed in asthmatic BSM. Finally, two transport proteins implicated in this increased fatty acid metabolism were identified namely CPT2 and LDL-R, suggesting that they may represent novel therapeutic targets for asthmatic BSM remodelling.

In the present study, which initially confirmed previous findings related to the increase in mitochondrial mass and BSM cell proliferation in asthma [8], we focused on mitochondrial bioenergetics to identify specific pathways in these phenomena. In this regard, a key role of the fatty acid metabolism pathway in asthmatic BSM, both *in vitro* and *ex vivo*, was demonstrated using several complementary approaches. The first one was a metabolomic approach, using ^{13}C-labelled glucose. Metabolite production in asthmatic and non-asthmatic BSM cells was analysed, revealing a significant increase in pyruvate, succinate and malate production by asthmatic BSM cells. Consequently, ATP production was increased in asthmatic BSM cells, a finding in agreement with our previous result regarding the increase in cellular respiration within asthmatic BSM cells [8]. With respect to reactive oxygen species (ROS) implication in ATP production, unlike in bronchial epithelial cells, where mitochondrial dysfunction is related to increased ROS exposure causing electron transport chain subunit inhibition leading to ATP depletion [22–24], in asthmatic BSM cells, metabolic rewiring appeared independent of ROS production. Indeed, we showed that protein carbonylation was not different between non-asthmatic and asthmatic BSM cells. The second approach was based on an unbiased proteomic analyses of BSM cells. Proteome remodelling is an important aspect of cell phenotypic change [25], and we showed that the major proteome alterations occurred in clusters representative of mitochondrial and bioenergetic pathways. These findings have not been reported previously in asthmatic BSM, even though similar proteome remodelling has already been observed in cancer cells with energetic metabolism changes [26]. However, it could be argued that such mitochondrial proteome rearrangements may be related to specific mutations of mitochondrial genes. Thus we examined genome-wide association study databases obtained from asthmatic patients (GCST010043 and GCST009798) and identified a single mutation within the genes implicated in the mitochondrial dysfunction box (*i.e.* aconitase 2 (ACO2)) [27, 28]. Indeed, ACO2 catalyses the isomerisation of citrate to isocitrate within mitochondria during the TCA cycle. Specifically, the proteomic analysis revealed that CPT2, a mitochondrial fatty acid transporter allowing fatty acids to follow the β-oxidation pathway for ATP production was the most significantly upregulated protein in asthmatic BSM cells compared to that observed in the non-asthmatic BSM cells, as discussed later. The third approach analysed the expression of the transport system of the precursor of fatty acids, namely LDL-R, which was also specifically increased in asthmatic BSM.

Fatty acid content was directly measured in BSM *in vitro* and through high-resolution MALDI–mass spectrometry imaging *ex vivo*. Fatty acid consumption was increased in asthmatic BSM compared to non-asthmatic BSM in both experimental conditions. Similarly, mass spectrometry has been recently used to assess lipidome rearrangements, in colorectal cancer, where Mika *et al*. [14] observed decreased levels of monounsaturated fatty acids (*i.e.* oleic acid). Fatty acid oxidation occurs within mitochondria and enables cells to produce large amounts of ATP [29, 30]. Our findings, obtained using human patient

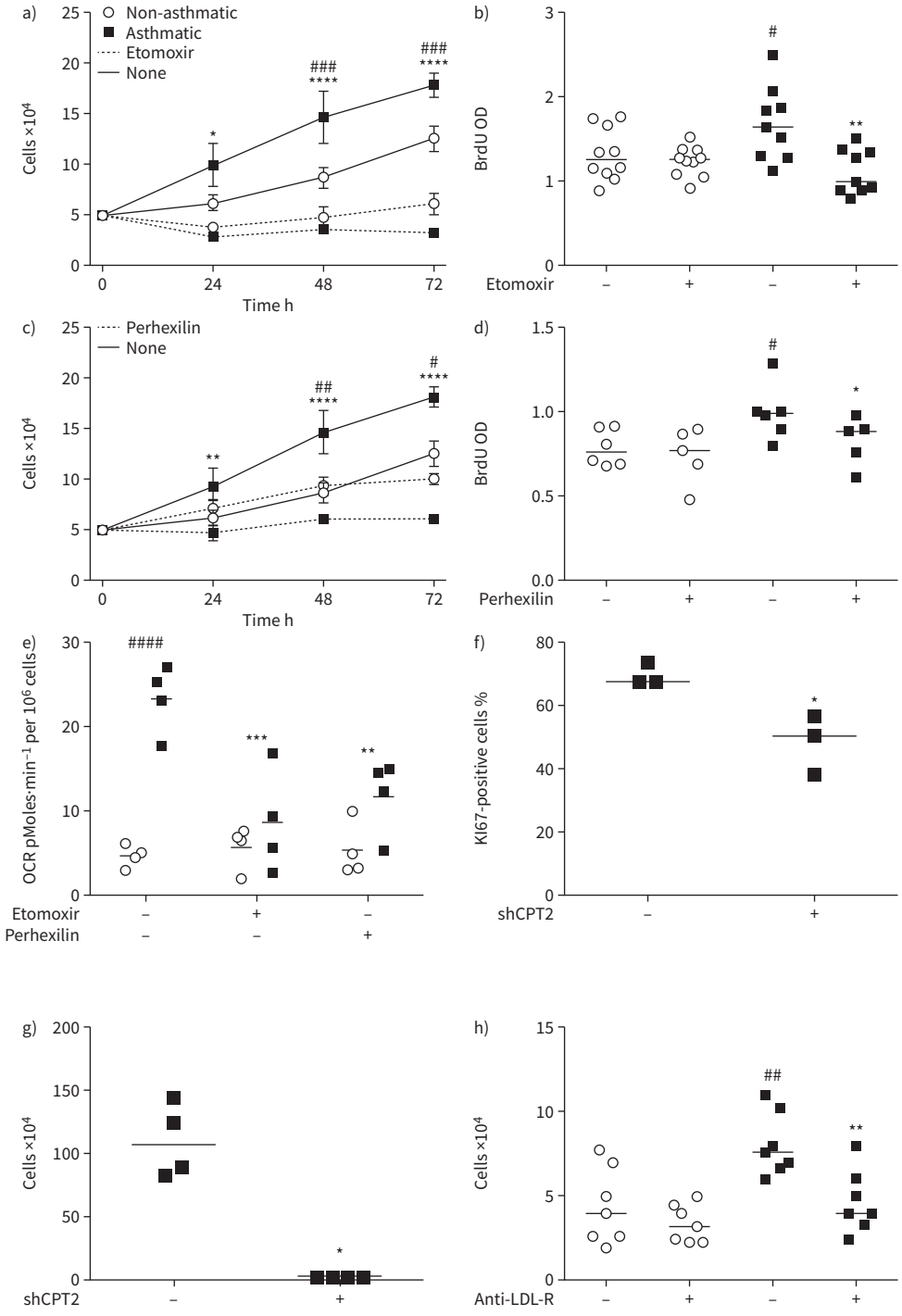

FIGURE 6 Blocking fatty acid oxidation dramatically decreases asthmatic bronchial smooth muscle (BSM) cell proliferation. a) Growth curves of non-asthmatic (n=6) and asthmatic (n=6) BSM cells were obtained by performing cell counting with or without 10 nM etomoxir for 72 h (two-way ANOVA). b) Bromodeoxyuridine (BrdU) incorporation assay in non-asthmatic (n=10) and asthmatic (n=10) BSM cells with (+) or without (–) 10 nM etomoxir for 24 h (one-way ANOVA). c) Growth curves of non-asthmatic (n=6) and asthmatic (n=6) BSM cells were obtained by performing cell counting with or without 10 nM perhexilin for 72 h (two-way ANOVA). d) BrdU incorporation assay of non-asthmatic (n=10) and asthmatic (n=10) BSM cells with (+) or without (–) 10 nM perhexilin for 24 h (one-way ANOVA). e) Oxygen consumption rates (OCR) were measured under basal conditions (DMEM 5 mM glucose) with (+) or without (–) 10 nM etomoxir or 10 nM perhexilin. BSM cells were obtained from non-asthmatic (n=4) and asthmatic patients (n=4) (two-way ANOVA). Cell proliferation was assessed in asthmatic BSM cells (n=3) transduced with either a scramble short hairpin (sh)RNA lentivirus (–) or

a CPT2 shRNA lentivirus (+), by f) Ki67-positive staining using flow cytometry and g) cell counting (Wilcoxon test). h) Cell counting of non-asthmatic (n=7) and asthmatic (n=7) BSM cells cultured in the presence (+) or in the absence (–) of blocking antibody against low-density lipoprotein receptor (LDL-R) (one-way ANOVA). Data are presented as dot plots with the median. #: p<0.05, ##: p<0.01, ###: p<0.001 between non-asthmatic and asthmatic patients; *: p<0.05, **: p<0.01, ***: p<0.001; ****: p<0.0001 between groups with and without treatment.

samples, appeared to be restricted to BSM both *in vitro* and *in vivo*, since fatty acid consumption was similar in asthmatic and non-asthmatic bronchial epithelium.

We next demonstrated a causal link between fatty acid metabolism and the proliferation of asthmatic BSM cells. Using both pharmacological and lentiviral strategies, blocking fatty acid transport decreased asthmatic BSM cell proliferation. In contrast, the use of steroids alone or in combination with LABA, was unable to decrease CPT2 or LDL-R expression or ATP production in asthmatic BSM cells. These results are in agreement with those previously published on the absence of a significant effect of steroids on asthmatic BSM cell proliferation [31]. Indeed, as the protein expression of both CPT2 and LDL-R was increased in asthmatic BSM, blockade of either CPT2 or LDL-R significantly decreased asthmatic BSM cell proliferation. CPT2, in association with its isoform CPT1, is responsible for fatty acid transport from the cytoplasm to the mitochondria, whereas LDL-R transports lipoprotein-enriched triglycerides, a major source of fatty acids, into the cytoplasm [15–17]. A similar link between fatty acid metabolism and cell proliferation has been previously demonstrated in hepatocytes [13] and cancer cells [14]. However, in our study, the effect of blocking fatty acid transport was restricted to asthmatic BSM cells, since we did not observe any effect of etomoxir or perhexilin in non-asthmatic BSM or bronchial epithelial cells. Indeed, the cancer-induced inhibition of CPT2 has been shown to be associated with an accumulation of fatty acids in the cytoplasm of hepatocellular carcinoma cells [21]. The role of LDL-R is to transport lipoprotein-enriched triglycerides, a major source of fatty acids, into the cytoplasm [22, 24, 25]. Moreover, apolipoprotein B and LDL levels have been shown to be negatively correlated with FEV_1 in asthma, indicating a strong link between lipoprotein-enriched triglycerides and worse respiratory function in asthma [18]. LDL-R expression is observed not only in the liver, but also in various other tissues, including the lung [32], and we demonstrated its expression in BSM cells.

To compare our proteomic results with previous publications, we examined various public proteomic and RNA-sequencing databases. Surprisingly, we did not identify any proteomic dataset and only a single RNA-sequencing study performed on asthmatic BSM (online dataset GSE119579) [33]. However, Fong *et al.* [33] did not detect any difference in CPT2 and LDL-R mRNA expression between asthmatic and non-asthmatic BSM. The discrepancy between these mRNA data and our observations of increased protein expression of both CPT2 and LDL-R in asthmatic BSM has to be related to the fact that previous studies have highlighted that the metabolic cell switch can be characterised by protein expression alterations without mRNA expression modifications [34]. Other fatty acid receptors, FFAR1 and FFAR4, have been described in BSM cells [35] and associated with increased BSM cell proliferation induced by oleic and linoleic acids [36]. However, these additional fatty acid receptors and transporters were not increased in the asthmatic BSM as observed using our unbiased proteomic analyses.

In summary, BSM remodelling is a key feature of asthma pathophysiology, but no pharmacological compound has been identified that inhibits this process [7]. We previously demonstrated that blocking abnormal calcium entry within asthmatic BSM cells using gallopamil was also able to normalise BSM cell proliferation *in vitro* [8], although its effect was less impressive *in vivo* [11]. In the present study, we demonstrated that asthmatic BSM is characterised by an increased mitochondrial metabolism that specifically involves fatty acid oxidation both *in vitro* and *ex vivo* and identified two new targets (*i.e.* CPT2 or LDL-R). Blocking CPT2 or LDL-R dramatically decreased asthmatic BSM cell proliferation *in vitro*. Thus, mitochondrial metabolism may represent a new target for the treatment of asthmatic BSM remodelling, although further studies are needed to elucidate the driving force responsible for metabolic remodelling in asthmatic BSM cells.

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