ORIGINAL RESEARCH

Metabolic Adaptation of Airway Smooth Muscle Cells to an SPHK2 Substrate Precedes Cytostasis

Pascale Blais-Lecours[1], Sofien Laouafa[1], Christian Arias-Reyes[1], Webster L. Santos[2], Vincent Joseph[1,3], Janette K. Burgess[4,5], Andrew J. Halayko[6,7], Jorge Soliz[1,3], and David Marsolais[1,3]

[1]Centre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec and [3]Faculty of Medicine, Université Laval, Québec City, Québec, Canada; [2]Department of Chemistry and Virginia Tech Center for Drug Discovery, Virginia Tech, Blacksburg, Virginia; [4]Department of Pathology and Medical Biology, Experimental Pulmonology and Inflammation Research and [5]GRIAC (Groningen Research Institute for Asthma and COPD), University Medical Center Groningen, University of Groningen, Groningen, the Netherlands; [6]Department of Physiology and Pathophysiology, University of Manitoba, Winnipeg, Manitoba, Canada; and [7]Biology of Breathing Group, Children's Hospital Research Institute of Manitoba, Winnipeg, Manitoba, Canada

ORCID ID: 0000-0002-8935-6657 (D.M.).

Abstract

Thickening of the airway smooth muscle is central to bronchial hyperreactivity. We have shown that the sphingosine analog (*R*)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol (AAL-R) can reverse preestablished airway hyperreactivity in a chronic asthma model. Because sphingosine analogs can be metabolized by SPHK2 (sphingosine kinase 2), we investigated whether this enzyme was required for AAL-R to perturb mechanisms sustaining airway smooth muscle cell proliferation. We found that AAL-R pretreatment reduced the capacity of live airway smooth muscle cells to use oxygen for oxidative phosphorylation and increased lactate dehydrogenase activity. We also determined that SPHK2 was upregulated in airway smooth muscle cells bearing the proliferation marker Ki67 relative to their Ki67-negative counterpart. Comparing different stromal cell subsets of the lung, we found that high SPHK2 concentrations were associated with the ability of AAL-R to inhibit metabolic activity assessed by conversion of the tetrazolium dye MTT. Knockdown or pharmacological inhibition of SPHK2 reversed the effect of AAL-R on MTT conversion, indicating the essential role for this kinase in the metabolic perturbations induced by sphingosine analogs. Our results support the hypothesis that increased SPHK2 levels in proliferating airway smooth muscle cells could be exploited to counteract airway smooth muscle thickening with synthetic substrates.

Keywords: asthma; sphingolipids; metabolism; AAL-R; FTY720

Asthma is characterized by a thickening of the airway smooth muscle (ASM), which contributes prominently to airway narrowing (1). There is no existing pharmacological treatment specifically designed to impede ASM thickening in asthma, although corticosteroid can accelerate its resorption under specific conditions (2, 3). Both hyperplasia and hypertrophy of ASM cells are suspected to contribute to asthma pathogenesis (4). *In vitro* ASM cells from patients with asthma display a hyperproliferative and a hypersecretory phenotype (5, 6). Moreover, a number of studies have documented, at least in the distal airways, that cellular alterations compatible with heightened oxidative phosphorylation are associated with ASM enlargement, including increased mitochondrial numbers, mass, and oxygen consumption (7, 8).

Of critical importance is the notion that enhanced mitochondrial functions are

(Received in original form December 5, 2018; accepted in final form June 26, 2019)

Supported by Canadian Institutes of Health Research grant 274357, funding from the Respiratory Health Network of Québec, a scholarship from the Sentinel North Strategy of the Canada First Research Excellence Fund (P.B.-L.), NIH grant R01GM121075 (W.L.S.), and an FRQ-S Respiratory Health Network J2 research scholar award (D.M.).

Author Contributions: Participated in research design: P.B.-L., S.L., C.A.-R., W.L.S., V.J., A.J.H., J.S., and D.M. Conducted experiments: P.B.-L., S.L., and C.A.-R. Contributed new reagents: W.L.S., J.K.B., and A.J.H. Performed data analysis: P.B.-L., S.L., C.A.-R., and D.M. Wrote or contributed to manuscript writing: P.B.-L., S.L., C.A.-R., W.L.S., V.J., J.K.B., A.J.H., J.S., and D.M.

Correspondence and requests for reprints should be addressed to David Marsolais, Ph.D., Université Laval, 2725 Chemin Ste-Foy, Quebec, QC G1V 4G5 Canada. E-mail: david.marsolais@criucpq.ulaval.ca.

This article has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Cell Mol Biol Vol 62, Iss 1, pp 35–42, Jan 2020

Originally Published in Press as DOI: 10.1165/rcmb.2018-0397OC on June 27, 2019
Internet address: www.atsjournals.org

required to sustain instrumental aspects of tissue hypertrophy such as protein synthesis (9) and proliferation (10). Interfering with mitochondrial functions was shown to alleviate vascular smooth muscle hyperproliferation in the context of pulmonary hypertension (11), and oxidative phosphorylation is increased during stromal cell proliferation (12). It is thus conceivable that dysregulating energy metabolism in ASM cells could produce salutary effects in the context of asthma.

Increasing evidence obtained in the context of oncology suggests that synthetic sphingosine analogs can act through SPHK2 (sphingosine kinase 2)-dependent and SPHK2-independent pathways to hamper cellular accumulation (13, 14). We recently demonstrated that a sphingosine analog as well as substrate for SPHK2 could reverse ASM thickening in experimental asthma and that subtoxic concentrations of this agent could induce ASM cell cytostasis *in vitro* (15). Nevertheless, the mechanisms underlying the propensity of SPHK2 substrates to impact ASM thickening remain unclear.

In this study, we determined that the SPHK2 substrate (*R*)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol (AAL-R) causes metabolic alterations that precede the reduction of ASM cell accumulation *in vitro*. These include the alteration of overall oxidoreductase activity, the impairment of oxidative phosphorylation, and the enhancement of lactate dehydrogenase (LDH) activity. Importantly, we found that SPHK2 expression was increased in ASM cells bearing the proliferation marker Ki67 and that SPHK2 knockdown reversed the ability of AAL-R to alter ASM cells' metabolic activity.

Methods

Cell Culture

Human primary ASM cells (CC-2576; Lonza) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS (unless otherwise indicated), nonessential amino acids, and 1% penicillin/streptomycin and plated at a density of 1.5×10^4 cells/cm^2. Human primary epithelial cells obtained from a normal patient's biopsy at the Chakir laboratory were characterized and cultured as described previously (16), and they were plated at a density of 2.5×10^4 cells/cm^2. Human primary arterial endothelial cells (302-05a; Cell Applications, Inc.) were cultured in endothelial cell growth medium (CC-3202; Lonza) and plated at a density of 2.5×10^4 cells/cm^2. Primary hTERT (human telomerase reverse transcriptase)-immortalized ASM cells from patients with asthma were cultured as described elsewhere (5). Cells were used at passages 3–8. Crystal violet (17), bromodeoxyuridine, MTT, LDH activity, and JC-10 assays, as well as p21 western blotting, were performed according to the manufacturer's instructions (described in the data supplement).

Reagents

AAL-R and SLM6031434 were graciously provided by Hugh Rosen and Webster Santos, respectively. 2-Amino-*N*-(3-octylphenyl)-3-(phosphonooxy)-propanamaide (VPC 23019) was purchased from Tocris Bioscience, and recombinant human FGF2 (fibroblast growth factor 2) and TGF-β1 (transforming growth factor-β1) were purchased from PeproTech.

siRNA Transfection

ASM cells were seeded at a density of 1.5×10^4 cells/cm^2 and transfected using calcium phosphate (18) for 24 hours with 25 nM of mock siRNA (Thermo Fisher Scientific) or SPHK2 siRNA (s32285; Thermo Fisher Scientific). Cells were incubated with vehicle (VEH) or increasing concentrations of AAL-R for 24 hours for the MTT assay or with VEH or 1 μM AAL-R for 5 hours for lipid quantification using liquid chromatography–tandem mass spectrometry as described previously (15). SPHK2 concentrations were assessed by western blotting (polyclonal; Proteintech) (described in the data supplement).

Flow Cytometry

SPHK2 immunoreactivity on ASM, epithelial, and endothelial cells was assessed as described in the data supplement. ASM surface expression of GLUT1 (glucose transporter 1) (polyclonal; Novus Biologicals) was measured after a 24-hour incubation with 1 μM AAL-R. Apoptosis was assessed after 72-hour incubation with AAL-R 1, 5, and 10 μM using Pacific Blue Annexin V (BioLegend).

Mitochondrial Content and Activity

Mitochondrial DNA quantification was assessed by quantitative PCR as described by Rooney and colleagues (19) and further detailed in the data supplement. Mitochondrial respiration was measured in intact ASM cells incubated in complete DMEM with VEH or 1 μM AAL-R for 24 hours using the Oxygraph-2K instrument (Oroboros Instruments) according to variations of published methods (20, 21). *See* the data supplement for the detailed protocol.

Statistical Analyses

Data were expressed using the average ± SEM. After verifying homogeneity of variances and the assumption of normality, unpaired *t* test or ANOVA was performed. *Post hoc* analyses were performed using the Sidak correction. When necessary, data were logarithmically transformed before parametric tests were performed. Data of oxygen consumption for mitochondrial respiration were evaluated using repeated measures ANOVA followed by Fisher's multiple comparisons test. All tests were performed with Prism version 6.01 software (GraphPad Software, Inc.). The significance level was set at 0.05 for all the tests.

Results

Metabolic Alterations Induced by AAL-R Precede Cytostasis

Increasing evidence supports the hypothesis that alteration of energy metabolism by sphingosine analogs contributes to inhibition of proliferation (13). We observed that AAL-R, at a concentration of 1 μM, diminished the tetrazolium dye MTT reduction to formazan by 38% after 24 hours of incubation compared with cells incubated with VEH (Figure 1A), suggesting a decreased cell number or hampered metabolic activity. We determined that accumulation of ASM cells, measured using crystal violet staining, was not decreased by AAL-R at that time point (Figure 1B), showing that the metabolic

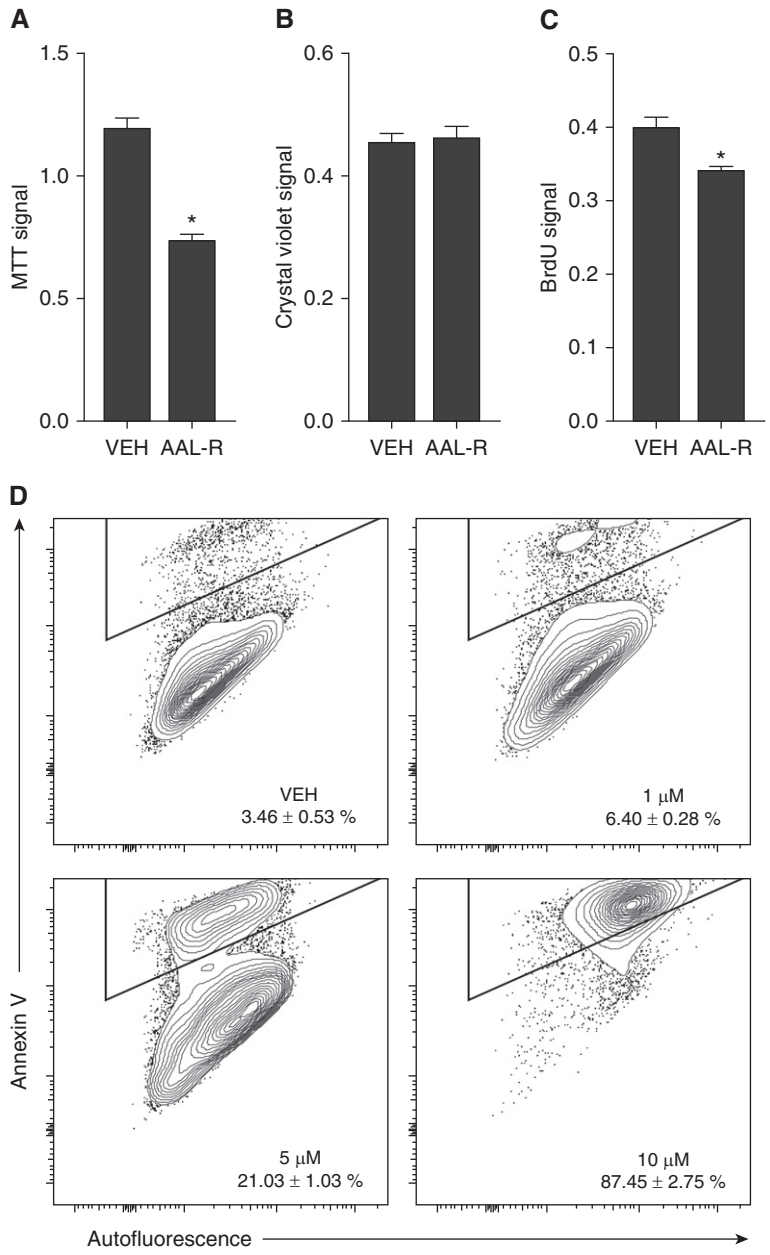

Figure 1. A cytostatic concentration of (*R*)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol (AAL-R) inhibits metabolic activity of airway smooth muscle (ASM) cells. Primary human ASM cells were incubated with vehicle (VEH) or AAL-R in complete media containing 10% FBS. (*A*) A 24-hour incubation with AAL-R (1 μM) decreased MTT conversion, which occurred (*B*) without modulation of ASM cell accumulation. (*C*) After 48 hours of incubation, AAL-R (1 μM) reduced bromodeoxyuridine (BrdU) incorporation compared with VEH. (*D*) These alterations occurred without significant induction of apoptosis over 72 hours. $n = 3–8$. $^*P < 0.05$ compared with VEH.

activity rather than the accumulation of cells was in play at this early time point. In agreement with our previous results (15), AAL-R significantly altered bromodeoxyuridine incorporation relative to VEH at 48 hours (Figure 1C), which occurred in the absence of striking accumulation of annexin V–positive cells over a 72-hour incubation period (Figure 1D). Thus, it appears that a concentration of AAL-R sufficient to cause cytostasis but not apoptosis rapidly impacts determinants of oxidoreductase activity in ASM cells.

AAL-R Interferes with Oxidative Phosphorylation and Promotes LDH Activity

Because MTT reduction depends on oxidoreductase activity, which can involve both mitochondria-dependent and mitochondria-independent events, we determined if AAL-R impacted the ability of proliferating ASM cells to use oxygen (Figure 2). We first compared, as a surrogate for mitochondrial numbers per cell, the relative amount of the $tRNA^{Leu(UUR)}$ mitochondrial gene versus the β_2-*microglobulin* nuclear gene between experimental conditions. We found that AAL-R did not reduce the $tRNA^{Leu(UUR)}/\beta_2$-*microglobulin* ratio, which provides evidence that reduced numbers of mitochondria per cell did not account for the altered MTT conversion (Figure 2A). However, we determined that routine oxygen consumption was reduced by nearly 15% as a result of the altered capacity to use oxygen to generate ATP (oxidative phosphorylation; 27% decrease), rather than as a result of increasing proton leak (not different from VEH) (Figure 2B). This was accompanied by a shift toward a glycolytic metabolism that was evidenced by increased cell surface expression of GLUT1 (Figure 2C) and increased LDH activity (Figure 2D). Consistent with increased markers of glycolysis, we determined that AAL-R increased mitochondrial membrane potential (Figure 2E) measured by incorporation of the JC-10 dye (22). We did not observe modulation of p21 concentrations in response to AAL-R (Figures 2F and E1 in the data supplement), indicating that it did not operate by influencing nuclear targets of SPHK2 products. Together, these results support the hypothesis that AAL-R mitigates energy metabolism in ASM cells.

Proliferative ASM Cells Are Susceptible to AAL-R–induced Inhibition of MTT Conversion

Most sphingosine analogs are preferentially phosphorylated by SPHK2 over SPHK1 (22). Because proliferation likely contributes to ASM thickening in asthma (4, 5, 23), we assessed the immunoreactivity for SPHK2 in ASM cells bearing the proliferation marker Ki67 (Figure 3). Ki67-positive ASM cells

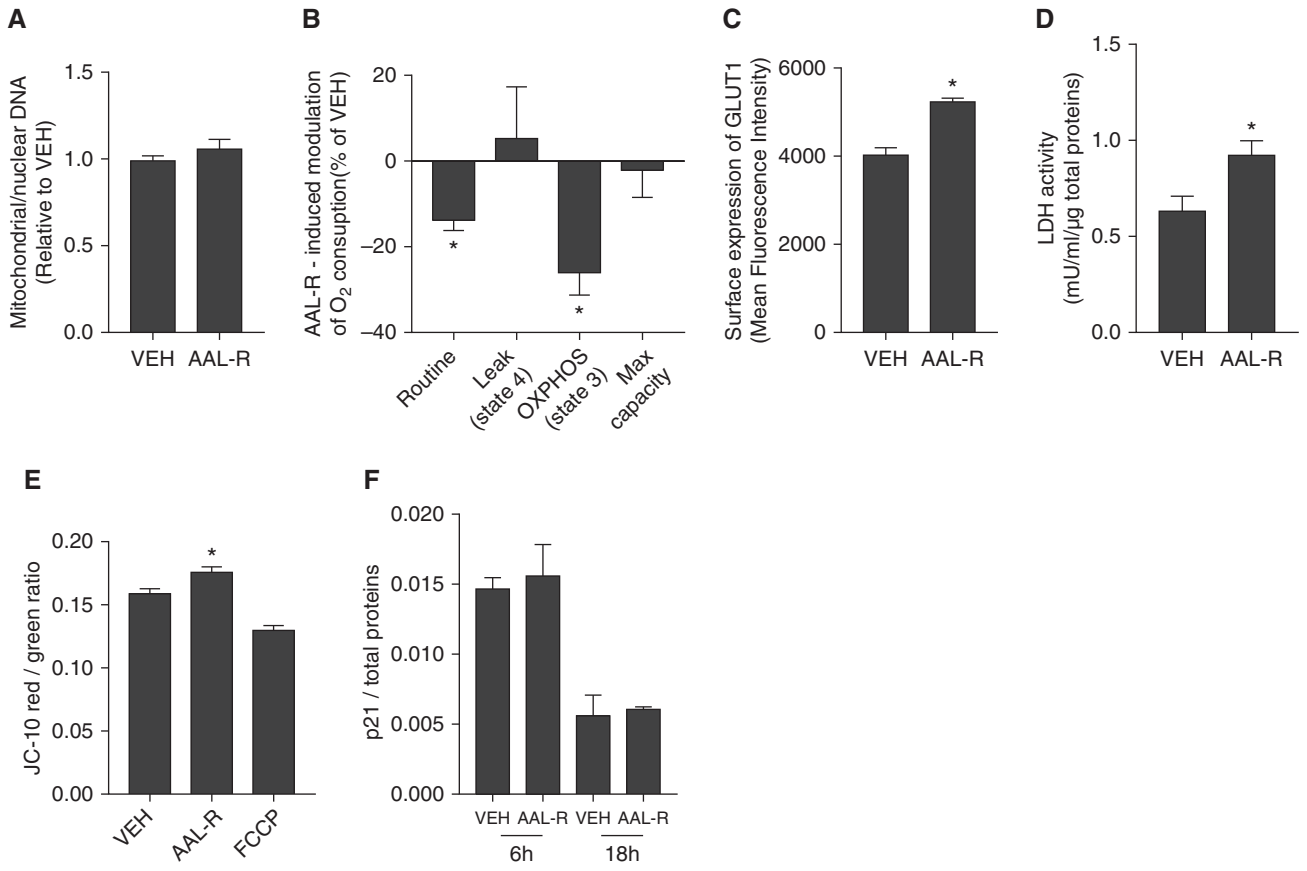

Figure 2. AAL-R causes a shift toward glycolytic metabolism. (*A* and *B*) Primary ASM cells were incubated for 24 hours with VEH or AAL-R (1 μM) in complete medium containing 10% FBS. (*A*) AAL-R did not modify the ratio between genomic mitochondrial and nuclear housekeeping gene levels. (*B*) Microoximetry was performed in VEH- and AAL-R–treated cells to derive O_2 consumption associated with routine respiration, proton leak (state 4), oxidative phosphorylation (OXPHOS) (state 3), and maximal respiration, as described in the METHODS. (*C* and *D*) AAL-R (1 μM) led to an increase of (*C*) GLUT1 and (*D*) lactate dehydrogenase (LDH) activity after 24 hours. AAL-R (*E*) increased JC-10 aggregates (red/green ratio) at 24 hours [carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) 20 μM was used as a positive control] but (*F*) had no impact on p21 expression at 6 and 18 hours. $n = 3$–8. $^*P < 0.05$ compared with VEH. GLUT1 = glucose transporter 1.

showed a more than twofold increase in SPHK2 immunoreactivity compared with their Ki67-negative counterparts (Figures 3A and 3B), suggesting that proliferating ASM cells might feature an enhanced sensitivity to SPHK2 substrates. We thus tested the impact of AAL-R under two culture conditions known to promote ASM cell proliferation *in vitro*, namely 10% FBS (24, 25) and combined FGF2/TGF-β (17), as well as in ASM cells cultured in complete medium containing 1% FBS, into which they do not expand (Figure 3C). We found MTT conversion to be enhanced, regardless of the anabolic stimulus, compared with the cells cultured in 1% FBS. Moreover, AAL-R decreased the conversion of MTT by nearly 40% under both anabolic stimuli compared with VEH. Of note, AAL-R marginally affected MTT conversion in cells incubated with 1% FBS, suggesting that proliferative cells featuring high SPHK2 concentrations are susceptible to AAL-R–induced metabolic alterations.

AAL-R Preferentially Affects Cells with High SPHK2 Levels

Phosphorylatable sphingosine analogs can be delivered in the airways of mice with limited histological impacts (26, 27). Yet, we recently showed that the dysregulated ASM compartment, as seen in the context of asthma, resorbs in the presence of phosphorylatable sphingosine analogs such as AAL-R (15), indicating that the ASM features elements of sensitivity to this type of agent. We thus compared the expression of SPHK2 in ASM cells and other prevalent stromal cell subsets of the lung, namely endothelial and epithelial cells. We found that immunoreactivity for SPHK2 is twice as high in ASM cells as in epithelial or endothelial cells (Figures 4A and 4B). In line with the pattern of SPHK2 expression of ASM cells and epithelial and endothelial cell lines, we observed that AAL-R potently inhibited the conversion of MTT by 45% in ASM cells while having limited effects in endothelial and epithelial cell lines (Figure 4C). These results suggest that stromal cells with high SPHK2 concentrations are susceptible to metabolic perturbations induced by SPHK2 substrates. We also confirmed that AAL-R reduced MTT conversion in ASM cells from patients with asthma (Figure 4D).

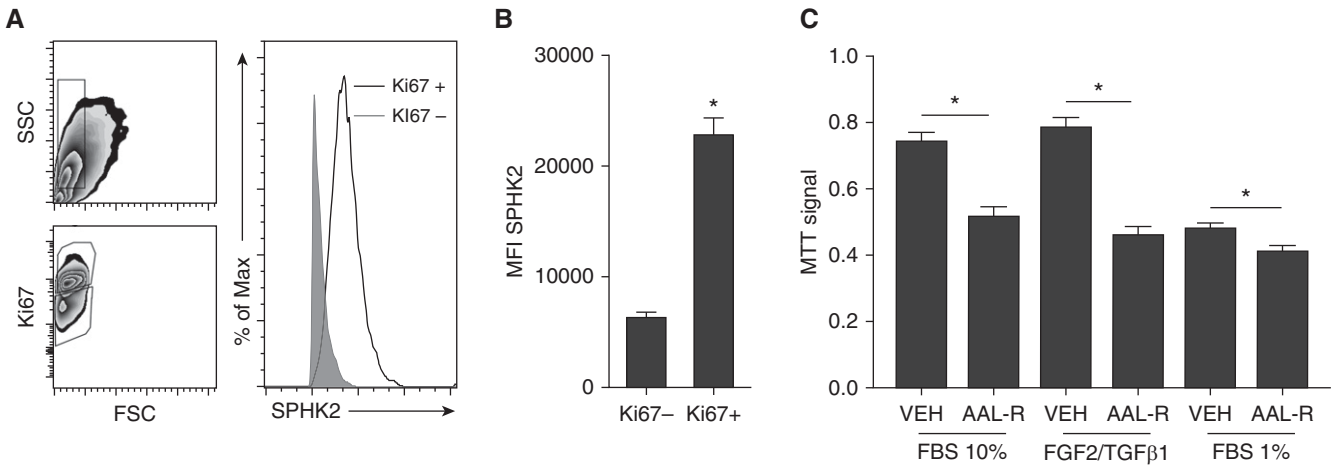

Figure 3. SPHK2 overexpression in proliferative ASM cells sensitizes to inhibition of MTT conversion by AAL-R. (*A* and *B*) Median fluorescence intensity (MFI) for SPHK2 is higher in Ki67-positive ($Ki67^+$) proliferative primary human ASM cells than in Ki67-negative ($Ki67^-$) cells. (*C*) The impact of a 24-hour exposure to VEH or AAL-R (1 μM) on MTT conversion was assessed in primary human ASM cells incubated in complete medium containing 10% FBS in FBS-free medium containing FGF2 (fibroblast growth factor 2) and TGF-β1 (transforming growth factor-β1) or in medium containing 1% FBS. $n = 3–8$. $^*P < 0.05$ compared with VEH. FSC = forward scatter; SPHK = sphingosine kinase 2; SSC = side scatter.

AAL-R–induced Alteration of MTT Conversion Depends on SPHK2

To determine whether the impact of AAL-R on MTT conversion is dependent on SPHK2, we employed complementary strategies. We first demonstrated that low concentrations of SLM6031434, an SPHK2 inhibitor known to interfere with the phosphorylation of sphingosine analogs *in vitro* (28), rescued proliferating ASM cells from AAL-R–induced inhibition of MTT conversion (Figure 5A). Similarly, the lack of effect of AAL-R was recapitulated by knocking down SPHK2 with siRNAs (Figure 5B). In line with the contention that changes in the kinetics of (*R*)-2-amino-4-(4-(heptyloxy)phenyl)-2-methylbutyl dihydrogen phosphate (AFD-R) accumulation (the phosphorylated form of AAL-R) critically affect the impact of AAL-R on ASM cell function, we determined that SPHK2 knockdown reduced the AFD-R/AAL-R ratio by nearly 50% (Figure 5C). Of note, we also confirmed the efficacy of siRNAs to reduce the content of SPHK2 (Figure 5D).

ASM cells express sphingosine-1-phosphate (S1P) receptors 1–3 ($S1P_1$, $S1P_2$, and $S1P_3$ receptors, respectively) (15). Because AFD-R can bind to all S1P receptors except $S1P_2$ (29), we investigated these receptors' involvement in the AAL-R–mediated effects (Figure 5E). As expected, AAL-R diminished MTT conversion in proliferating cells compared with VEH. Against a prominent role for endogenous/medium-containing S1P acting on $S1P_1$ and $S1P_3$ for FBS-driven enhancement of MTT conversion,

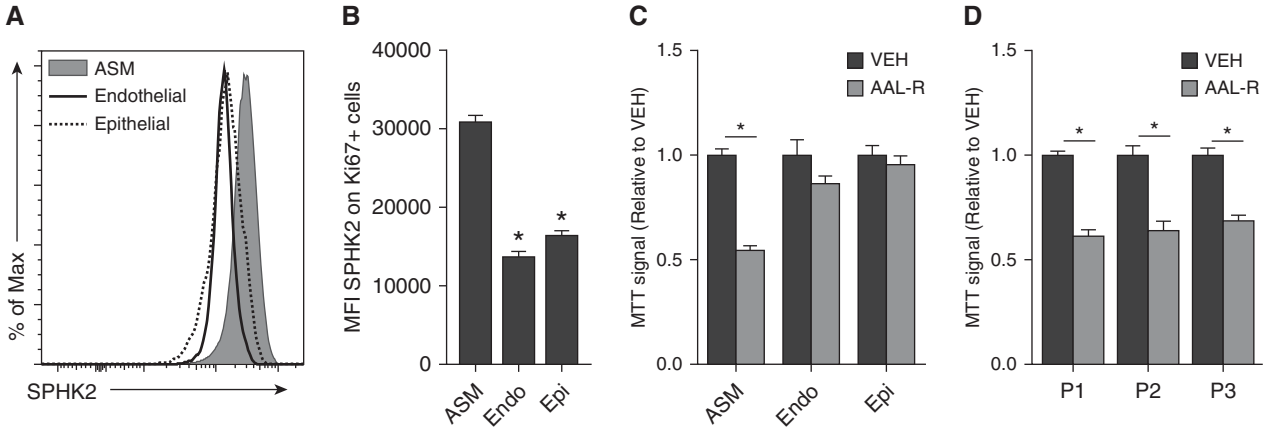

Figure 4. The inhibition of MTT conversion by AAL-R preferentially impacts stromal cells with high SPHK2 expression. Primary human ASM, endothelial (Endo), epithelial (Epi), and hTERT (human telomerase reverse transcriptase)-immortalized ASM cells isolated from patients with asthma were incubated in complete media containing 10% FBS. (*A* and *B*) Flow cytometric analyses of SPHK2 immunoreactivity in proliferative primary human ASM cells compared with primary human endothelial cells and epithelial cells. (*C*) ASM cells, but not endothelial and epithelial cells, show a decreased MTT conversion in response to AAL-R (1 μM) after 24 hours compared with VEH. (*D*) AAL-R (1 μM; 24 h) induces a decreased MTT conversion in hTERT-immortalized ASM cells from three patients with asthma (P1–P3). $n = 3–8$. $^*P < 0.05$.

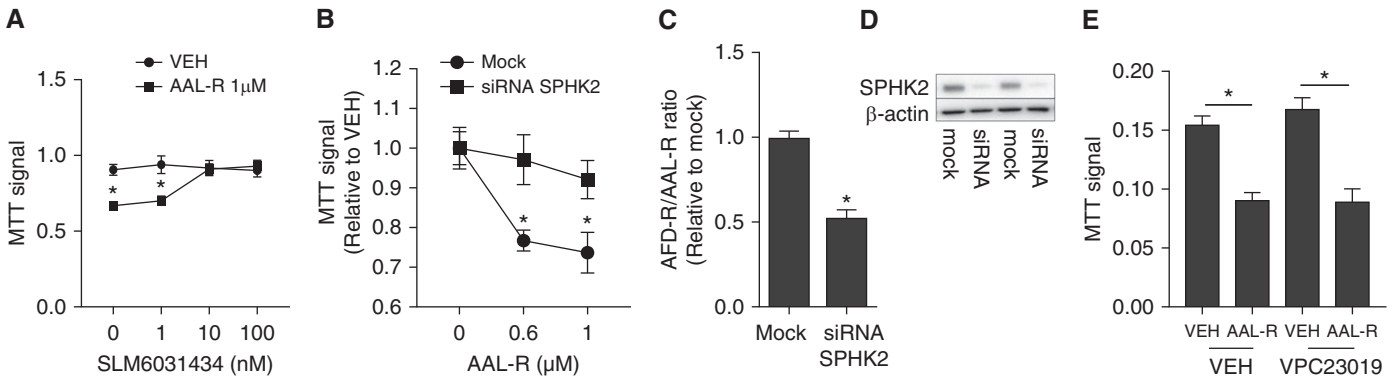

Figure 5. The inhibition of MTT conversion by AAL-R depends on SPHK2 but does not involve S1P (sphingosine-1-phosphate) receptors 1 to 3. (*A*) Primary human ASM cells were incubated for 24 hours with VEH or AAL-R (1 μM) in complete medium containing 10% FBS and the sphingosine kinase inhibitor SLM6031434. (*B*) SPHK2 knockdown by siRNAs interferes with the loss of MTT signal induced by AAL-R compared with mock-transfected cells. (*C*) Compared with mock-transfected cells, SPHK2 siRNA transfection reduced the phosphorylation of AAL-R to (*R*)-2-amino-4-(4-(heptyloxy)phenyl)-2-methylbutyl dihydrogen phosphate (AFD-R). (*D*) The efficiency of siRNAs against SPHK2 was confirmed by Western blots. (*E*) The impact of a 24-hour incubation with AAL-R (1 μM) on MTT conversion is not modified by the dual $S1P_{1–3}$ antagonist VPC23019 (10 μM). $n = 3–8$. $^*P < 0.05$ compared with VEH.

we found that, compared with VEH, the dual $S1P_1$ and $S1P_3$ antagonist VPC 23019 (10 μM) had no effect on MTT conversion in the absence of AAL-R. Moreover, VPC 23019 failed to alleviate the AAL-R–induced alteration of MTT conversion, refuting S1P receptors' involvement.

Discussion

ASM is at the core of asthma symptoms, and its thickening results from perpetual stimulation with numerous anabolic stimuli, including growth factors, spasmogens, and immune modulators (30, 31). The persistent reduction of ASM thickening associated with the long-term benefits of bronchial thermoplasty indicates that punctual interventions directed against ASM bear therapeutic potential (32–34). We showed that the SPHK2 substrate AAL-R potently reversed airway hyperresponsiveness in a corticosteroid-resistant model of asthma and induced human ASM cell cytostasis *in vitro* (15), suggesting that SPHK2 could be a therapeutically amenable target for interfering with ASM enlargement.

The present study furthers this contention by unraveling the preferential efficacy of AAL-R to alter energy metabolism when ASM cells are under anabolic conditions. For that matter, we found that ASM cells undergoing proliferation upregulate SPHK2 and that pulmonary stromal cell subsets with the highest concentrations of SPHK2 are more sensitive to metabolic perturbations induced by AAL-R. Critically, we demonstrate that knocking down or inhibiting SPHK2 makes AAL-R impotent at interfering with the activity of ASM cells, showing that beneficial effects depend on SPHK2.

If SPHK2 is increased in ASM cells under anabolic conditions, then why hasn't it come out in the "omic" studies of asthma? In our view, the most important factor masking the relationship between SPHK2 and asthma is that mild but not severe SPHK2 overexpression sustains hyperproliferation (35). These findings are consistent with our own observation that ASM cells upregulate SPHK2 by merely twofold, when proliferating. SPHK2 could thus be required for altering anabolic responses without affecting quiescent cells. In line with the usual caveats of omic studies performed on whole-tissue samples, SPHK2 is not equally expressed in the different cell types of the lung, which is confirmed by our *in vitro* findings exposing unequal SPHK2 immunoreactivity between ASM, endothelial, and epithelial cells. Although our study was performed with a limited number of cell lines, our results clearly demonstrate that AAL-R can alter MTT signal in ASM cells from patients with asthma and that cell lines with the highest concentrations of SPHK2 are the most susceptible to the metabolic perturbations induced by an SPHK2 substrate. Together with our previous *in vivo* findings showing that AAL-R does not cause aversive effects in the airways while reversing hyperresponsiveness (15), the results of the present study argue that mild overexpression of SPHK2 in ASM cells under anabolic conditions increases their susceptibility to AAL-R–induced metabolic perturbations.

This notion is also corroborated by the fact that SPHK2 substrates with the intrinsic propensity to rapidly accumulate in cells are the most potent at inhibiting MTT conversion and/or cell accumulation (13, 36, 37). In the same line of thought, the sensitivity to rapidly phosphorylated SPHK2 substrates correlates with the amount of SPHK2 expression in cancer cells (13). The correlation between SPHK2 expression and sensitivity to SPHK2 substrates also aligns with our observation that a partial knockdown of SPHK2, which leads to a 50% decrease of the AFD-R/AAL-R ratio, abrogated the impact of low micromolar concentrations of AAL-R on MTT conversion. In addition, concentrations of the SPHK2 inhibitor SLM6031434 lower than its documented half-maximal inhibitory concentration were sufficient to halt AAL-R–induced perturbation of MTT conversion (28). Therefore, our study strengthens the notion that asthma-related increase of SPHK2, or, alternatively, means of promoting SPHK2 expression in the ASM, could serve as a basis to counteract asthma with SPHK2 substrates.

The present study also supports the contention that the ability of sphingosine analogs to interfere with ASM thickening likely do not involve the activation/reactivation of protein phosphatases. Indeed, it was shown that (*S*)-2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol, an isomer of AAL-R that is not an SPHK2 substrate, inhibited the development of experimental asthma by a mechanism involving the promotion of protein phosphatase activity, leading to the inhibition of inflammatory mediator release (38). Importantly, intracellular accumulation of the nonphosphorylated form of sphingosine analogs was also shown to promote protein phosphatase activity in cancer cell lines, which led to their decreased accumulation through the inhibition of nutrient intake (37). For that matter, it is increasingly clear that sphingosine analogs poorly metabolized by SPHK2 preferentially promote protein phosphatase activity compared with rapidly metabolized substrates such as AAL-R (37). Our finding that AAL-R loses its ability to interfere with ASM cell metabolism when SPHK2 is knocked down, combined with no inhibition of cell surface expression of GLUT1, argues that the mechanisms of action likely do not rely on the promotion of protein phosphatase activity.

Although cells deficient in SPHK2 were documented to display an altered mitochondrial function (39), we were not surprised that SPHK2 knockdown or pharmacological inhibition did not impact basal MTT conversion. In fact, it appears that mitochondrial impacts of SPHK2 knockdown are highly dependent on the system, with fully knocked out cardiomyocytes displaying a mere 20% inhibition of complex IV–associated O_2 consumption and partial SPHK2 knockdown in HeLa cells yielding a more than 50% inhibitory effect (39). Our results suggest that the metabolic effects of SPHK2 substrates exceed the impact of SPHK2 modulators in perturbing mitochondrial functions under the current experimental conditions.

Our study indicates that the mechanisms of action of synthetic SPHK2 substrates overlap with some of the functions of the endogenous substrate (sphingosine). Indeed, we determined that the decreased oxygen consumption caused by AAL-R led to mitochondrial membrane hyperpolarity. Because endogenous S1P inhibits the depolarization of the mitochondrial membrane in response to hypoxic episodes (40), it is possible that the phosphorylated form of AAL-R acts as an intracellular S1P mimic to prevent the loss of mitochondrial membrane potential and thus prevents cell death. Although this theory will require further investigation, we determined that AAL-R induced an upregulation of GLUT1 at the cell surface, an event that also confers protection against apoptosis in situations in which oxidative metabolism is altered (41, 42). Altogether, our results support the hypothesis that SPHK2 substrates are modulators of energy metabolism and provide evidence that they trigger compensatory mechanisms usually associated with hypoxia that likely prevent mitochondrion-associated cell death.

Conclusions

Although reversing the thickening of ASM is likely to improve asthma in a majority of patients, there are currently no clinically approved pharmacological strategies that directly target ASM. The task of identifying such strategies is made complex by the subtle phenotypic changes seen in ASM cells from patients *in vitro* and by the fact that mechanisms promoting and sustaining ASM thickening are numerous, interacting, and thus not finite. Our findings have unraveled that SPHK2 concentrations are increased in ASM cells subjected to anabolic stimuli. In line with the central role of sphingolipids in regulating cell fate and metabolism, our study supports the concept that this upregulation could be employed to interfere with the anabolically skewed profile of the ASM in asthma. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Sophie Plante and Jamila Chakir for providing the epithelial cells.

References

1. Lauzon AM, Martin JG. Airway hyperresponsiveness; smooth muscle as the principal actor. *F1000Res* 2016;5(F1000 Faculty Rev):306.
2. Berair R, Brightling CE. Asthma therapy and its effect on airway remodelling. *Drugs* 2014;74:1345–1369.
3. Leclere M, Lavoie-Lamoureux A, Joubert P, Relave F, Setlakwe EL, Beauchamp G, *et al*. Corticosteroids and antigen avoidance decrease airway smooth muscle mass in an equine asthma model. *Am J Respir Cell Mol Biol* 2012;47:589–596.
4. James AL, Elliot JG, Jones RL, Carroll ML, Mauad T, Bai TR, *et al*. Airway smooth muscle hypertrophy and hyperplasia in asthma. *Am J Respir Crit Care Med* 2012;185:1058–1064.
5. Burgess JK, Ketheson A, Faiz A, Limbert Rempel KA, Oliver BG, Ward JPT, *et al*. Phenotype and functional features of human telomerase reverse transcriptase immortalized human airway smooth muscle cells from asthmatic and non-asthmatic donors. *Sci Rep* 2018;8:805.
6. Hassan M, Jo T, Risse PA, Tolloczko B, Lemiere C, Olivenstein R, *et al*. Airway smooth muscle remodeling is a dynamic process in severe long-standing asthma. *J Allergy Clin Immunol* 2010;125:1037–1045, e3.
7. Girodet PO, Allard B, Thumerel M, Begueret H, Dupin I, Ousova O, *et al*. Bronchial smooth muscle remodeling in nonsevere asthma. *Am J Respir Crit Care Med* 2016;193:627–633.
8. Trian T, Benard G, Begueret H, Rossignol R, Girodet PO, Ghosh D, *et al*. Bronchial smooth muscle remodeling involves calcium-dependent enhanced mitochondrial biogenesis in asthma. *J Exp Med* 2007;204:3173–3181.
9. Vishnu N, Jadoon Khan M, Karsten F, Groschner LN, Waldeck-Weiermair M, Rost R, *et al*. ATP increases within the lumen of the endoplasmic reticulum upon intracellular Ca^{2+} release. *Mol Biol Cell* 2014;25:368–379.
10. Yu M, Shi Y, Wei X, Yang Y, Zhou Y, Hao X, *et al*. Depletion of mitochondrial DNA by ethidium bromide treatment inhibits the proliferation and tumorigenesis of T47D human breast cancer cells. *Toxicol Lett* 2007;170:83–93.
11. Boucherat O, Peterlini T, Bourgeois A, Nadeau V, Breuils-Bonnet S, Boilet-Molez S, *et al*. Mitochondrial HSP90 accumulation promotes vascular remodeling in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2018;198:90–103.
12. Yao CH, Wang R, Wang Y, Kung CP, Weber JD, Patti GJ. Mitochondrial fusion supports increased oxidative phosphorylation during cell proliferation. *Elife* 2019;8:e41351.
13. McCracken AN, McMonigle RJ, Tessier J, Fransson R, Perryman MS, Chen B, *et al*. Phosphorylation of a constrained azacyclic FTY720 analog enhances anti-leukemic activity without inducing S1P receptor activation. *Leukemia* 2017;31:669–677.

14. Romero Rosales K, Singh G, Wu K, Chen J, Janes MR, Lilly MB, *et al*. Sphingolipid-based drugs selectively kill cancer cells by down-regulating nutrient transporter proteins. *Biochem J* 2011;439: 299–311.
15. Gendron DR, Lecours PB, Lemay AM, Beaulieu MJ, Huppé CA, Lee-Gosselin A, *et al*. A phosphorylatable sphingosine analog induces airway smooth muscle cytostasis and reverses airway hyperresponsiveness in experimental asthma. *Front Pharmacol* 2017;8:78.
16. Goulet F, Boulet LP, Chakir J, Tremblay N, Dubé J, Laviolette M, *et al*. Morphologic and functional properties of bronchial cells isolated from normal and asthmatic subjects. *Am J Respir Cell Mol Biol* 1996; 15:312–318.
17. Bossé Y, Thompson C, Stankova J, Rola-Pleszczynski M. Fibroblast growth factor 2 and transforming growth factor beta1 synergism in human bronchial smooth muscle cell proliferation. *Am J Respir Cell Mol Biol* 2006;34:746–753.
18. Margolskee RF, McHendry-Rinde B, Horn R. Panning transfected cells for electrophysiological studies. *Biotechniques* 1993;15:906–911.
19. Rooney JP, Ryde IT, Sanders LH, Howlett EH, Colton MD, Germ KE, *et al*. PCR based determination of mitochondrial DNA copy number in multiple species. *Methods Mol Biol* 2015;1241:23–38.
20. Gnaiger E. Mitochondrial pathways and respiratory control: an introduction to OXPHOS analysis. 4th ed. Innsbruck, Austria: OROBOROS MiPNet Publications; 2014.
21. Laouafa S, Roussel D, Marcouiller F, Soliz J, Bairam A, Joseph V. Role of estradiol receptor beta (ERβ) on brain mitochondrial functions and cardio-respiratory control in aged female mice [abstract]. *FASEB J* 2018;32(1 Suppl):879.2
22. Billich A, Bornancin F, Dévay P, Mechtcheriakova D, Urtz N, Baumruker T. Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *J Biol Chem* 2003;278:47408–47415.
23. Johnson PR, Roth M, Tamm M, Hughes M, Ge Q, King G, *et al*. Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med* 2001;164:474–477.
24. Chen G, Khalil N. TGF-β1 increases proliferation of airway smooth muscle cells by phosphorylation of map kinases. *Respir Res* 2006;7:2.
25. Mahn K, Hirst SJ, Ying S, Holt MR, Lavender P, Ojo OO, *et al*. Diminished sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) expression contributes to airway remodelling in bronchial asthma. *Proc Natl Acad Sci USA* 2009;106:10775–10780.
26. Gendron D, Lemay AM, Tremblay C, Lai LJ, Langlois A, Bernatchez É, *et al*. Treatment with a sphingosine analog after the inception of house dust mite-induced airway inflammation alleviates key features of experimental asthma. *Respir Res* 2015;16:7.
27. Idzko M, Hammad H, van Nimwegen M, Kool M, Müller T, Soullié T, *et al*. Local application of FTY720 to the lung abrogates experimental asthma by altering dendritic cell function. *J Clin Invest* 2006;116: 2935–2944.
28. Kharel Y, Morris EA, Congdon MD, Thorpe SB, Tomsig JL, Santos WL, *et al*. Sphingosine kinase 2 inhibition and blood sphingosine 1-phosphate levels. *J Pharmacol Exp Ther* 2015;355:23–31.
29. Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, *et al*. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem* 2002;277:21453–21457.
30. Elliot JG, Noble PB, Mauad T, Bai TR, Abramson MJ, McKay KO, *et al*. Inflammation-dependent and independent airway remodelling in asthma. *Respirology* 2018;23:1138–1145.
31. Bossé Y. Asthmatic airway hyperresponsiveness: the ants in the tree. *Trends Mol Med* 2012;18:627–633.
32. Donovan GM, Elliot JG, Green FHY, James AL, Noble PB. Unraveling a clinical paradox: why does bronchial thermoplasty work in asthma? *Am J Respir Cell Mol Biol* 2018;59:355–362.
33. Pretolani M, Dombret MC, Thabut G, Knap D, Hamidi F, Debray MP, *et al*. Reduction of airway smooth muscle mass by bronchial thermoplasty in patients with severe asthma. *Am J Respir Crit Care Med* 2014;190:1452–1454.
34. Wechsler ME, Laviolette M, Rubin AS, Fiterman J, Lapa e Silva JR, Shah PL, *et al*.; Asthma Intervention Research 2 Trial Study Group. Bronchial thermoplasty: long-term safety and effectiveness in patients with severe persistent asthma. *J Allergy Clin Immunol* 2013; 132:1295–1302.
35. Neubauer HA, Pham DH, Zebol JR, Moretti PA, Peterson AL, Leclercq TM, *et al*. An oncogenic role for sphingosine kinase 2. *Oncotarget* 2016;7:64886–64899.
36. Don AS, Martinez-Lamenca C, Webb WR, Proia RL, Roberts E, Rosen H. Essential requirement for sphingosine kinase 2 in a sphingolipid apoptosis pathway activated by FTY720 analogues. *J Biol Chem* 2007;282:15833–15842.
37. Kim SM, Roy SG, Chen B, Nguyen TM, McMonigle RJ, McCracken AN, *et al*. Targeting cancer metabolism by simultaneously disrupting parallel nutrient access pathways. *J Clin Invest* 2016;126:4088–4102.
38. Collison A, Hatchwell L, Verrills N, Wark PA, de Siqueira AP, Tooze M, *et al*. The E3 ubiquitin ligase midline 1 promotes allergen and rhinovirus-induced asthma by inhibiting protein phosphatase 2A activity. *Nat Med* 2013;19:232–237.
39. Strub GM, Paillard M, Liang J, Gomez L, Allegood JC, Hait NC, *et al*. Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. *FASEB J* 2011;25:600–612.
40. Gomez L, Paillard M, Price M, Chen Q, Teixeira G, Spiegel S, *et al*. A novel role for mitochondrial sphingosine-1-phosphate produced by sphingosine kinase-2 in PTP-mediated cell survival during cardioprotection. *Basic Res Cardiol* 2011;106:1341–1353.
41. Malhotra R, Tyson DG, Sone H, Aoki K, Kumagai AK, Brosius FC III. Glucose uptake and adenoviral mediated GLUT1 infection decrease hypoxia-induced HIF-1α levels in cardiac myocytes. *J Mol Cell Cardiol* 2002;34:1063–1073.
42. Zhu H, Xue H, Jin QH, Guo J, Chen YD. *MiR-138* protects cardiac cells against hypoxia through modulation of glucose metabolism by targetting pyruvate dehydrogenase kinase 1. *Biosci Rep* 2017;37: BSR20170296.