RAPID COMMUNICATION

Tuberous Sclerosis Complex 2 Loss Increases Lysophosphatidylcholine Synthesis in Lymphangioleiomyomatosis

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Abstract

Lymphangioleiomyomatosis (LAM) is a destructive lung disease affecting women. LAM is caused by mutations in the tuberous sclerosis complex (TSC) genes. The TSC protein complex inhibits the mechanistic/mammalian target of rapamycin complex 1 (mTORC1), which is a master regulator of cellular metabolism. Using mass spectrometry–based lipid profiling, we analyzed plasma from patients with LAM and discovered elevated levels of four lysophosphatidylcholine (LPC) species (C16:0, C18:0, C18:1, and C20:4) compared with those in healthy control women. To investigate whether these lipids are generated in a TSC2-dependent manner, we profiled *in vitro* preclinical models of TSC/LAM and found significant LPC accumulation in TSC2-deficient cells relative to TSC2-expressing control cells. These lysoglycerophospholipid changes occurred alongside changes in other phospholipid and neutral lipid species. Treatment with rapamycin or torin1 or down-regulation of sterol regulatory element-binding protein (SREBP), a lipogenic transcription factor, did not suppress LPC in TSC2-deficient cells. Inhibition of distinct isoforms of phospholipase A2 decreased the proliferation of TSC2-deficient cells. Collectively, these results demonstrate that TSC2-deficient cells have enhanced choline phospholipid metabolism and reveal a novel function of the TSC proteins in choline lysoglycerophospholipid metabolism, with implications for disease pathogenesis and targeted therapeutic strategies.

Keywords: lipidomics; mTORC1; mass spectrometry; phospholipase

Clinical Relevance

Collectively, these results reveal a novel function of the tuberous sclerosis complex proteins in choline lysoglycerophospholipid metabolism, with implications for disease pathogenesis and targeted therapeutic strategies in tuberous sclerosis complex–related disorders.

Lymphangioleiomyomatosis (LAM) is a multisystem disease affecting women. LAM is characterized by the proliferation of TSC2-deficient smooth muscle–like "LAM cells." In the lung, LAM can cause extensive, progressive cystic destruction, leading to lung collapse, supplemental oxygen dependence, and respiratory failure. LAM occurs in a sporadic form or in women with tuberous sclerosis complex (TSC), an autosomal dominant disease caused by germline mutations in the *TSC1* or *TSC2* tumor suppressor genes (1, 2).

Aberrant activation of the mechanistic/mammalian target of rapamycin complex 1

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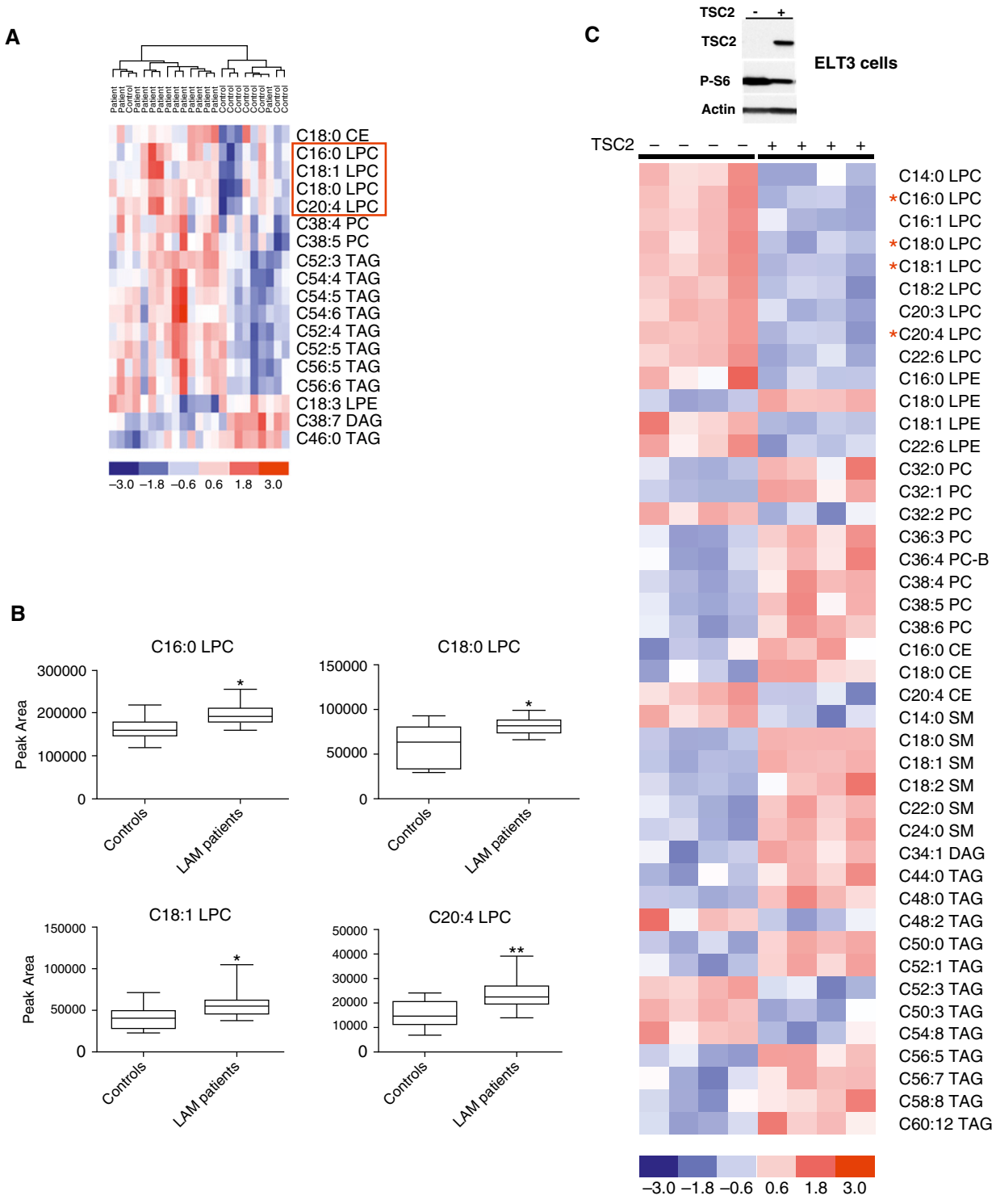

Figure 1. Lipidomic signature of tuberous sclerosis complex (TSC) 2 deficiency. (*A*) Liquid chromatography–mass spectrometry lipidomic profiling was performed on plasma from patients with lymphangioleiomyomatosis (LAM) ($n = 14$) and healthy women ($n = 9$). The heat map represents all differential lipid species (*rows*; $P < 0.05$) and is ordered upon unsupervised clustering of patients and control subjects (*columns*). The *red box* highlights the lysophosphatidylcholine (LPC) species significantly elevated in patients. (*B*) Box plots of LPC measurements. Student's *t* test was applied (*$P < 0.05$; **$P < 0.01$). (*C*) Lipidomic profiling of ELT3 cells (V3 = TSC2-deficient; T3 = TSC2 re-expressing) revealed 43 differential lipid species ($q < 0.05$), which are represented in the heat map. The *red asterisks* indicate the LPC species that were altered in the patient plasma profile (*A*). Four independent samples per group were analyzed. Heat maps of log2 values were generated using dCHIP 2008_05. Immunoblotting of TSC2 and phospho-S6 shows activation of mechanistic/mammalian target of rapamycin complex 1 (mTORC1) signaling in TSC2-deficient cells and control cells.

(mTORC1) occurs in LAM cells because of inactivating mutations in the *TSC1/2* genes (3, 4). mTORC1 is a master regulator of cell growth, proliferation, and metabolism (5–12). A downstream effector of mTORC1, the sterol regulatory element-binding protein (SREBP), regulates the transcription of glucose metabolism and *de novo* fatty acid synthesis enzymes (8, 12, 13). However, the impact of loss of the TSC proteins on the lipidome and how complex lipid species are affected by rapamycin and its analogs are unknown.

Lysophosphatidylcholines (LPC) are a class of bioactive lipids generated by phospholipase A (PLA) activity. PLA2 hydrolyzes phosphatidylcholine (PC), releasing LPC and arachidonic acid, which can be further metabolized to synthesize eicosanoids. PLA2 isoforms have been shown to play roles in lung injury, cancer, inflammation, and angiogenesis through the production of bioactive lipids (14–19). The PLA2 superfamily of lipolytic enzymes comprises 16 groups and at least 22 isoforms, which are classified based on their cellular localization (cytosolic and secretory) and chemical properties (calcium dependent and calcium independent). LPC can also be reacylated into PC by LPC acyltransferase enzymes (PC remodeling). Remodeling is critical for lipid composition of pulmonary surfactant (20) and to maintain lipid homeostasis to prevent toxic levels of cellular ER stress (21).

Here, we report the first systematic study of the LAM lipidome. Analyzing the plasma metabolite profile of patients with LAM, we discovered that plasma of women with LAM had elevated levels of four LPC species. TSC2-deficient cells also had increased LPC. LPC were not suppressed by rapamycin, Torin1, or down-regulation of SREBP1/2. Finally, down-regulation of the isoform PLA2G16 led to a decrease in proliferation selectively in $Tsc2^{-/-}$ mouse embryonic fibroblasts (MEFs) compared with $Tsc2^{+/+}$ MEFs. These data suggest that distinct isoforms of PLA2 play a role in TSC2-dependent glycerophospholipid metabolism and potentially in the pathogenesis and treatment of LAM and TSC.

Materials and Methods

Human Samples

Plasma samples from 14 patients with LAM who did not receive rapamycin and nine healthy control subjects were obtained through the Center for LAM Research and Clinical Care at Brigham and Women's Hospital under Institutional Review Board approval.

Cell Lines, Drugs, and Short Hairpin RNA

$Tsc2^{+/+}p53^{-/-}$, $Tsc2^{-/-}p53^{-/-}$ MEFs, Eker rat–derived ELT3 cells, and patient-derived 621-101 cells were cultured in Dulbecco's modified Eagle medium with 4.5 g/l glucose supplemented with 10% FBS. MEFs and ELT3 cells were provided by David J. Kwiatkowski and Cheryl L. Walker, respectively. AACOCF3 and LY311727 were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX). Short hairpin RNAs (shRNAs) against Pla2g16 or nontargeting green fluorescent protein (shGFP) control were obtained from the RNAi Consortium (Cambridge, MA).

Small Interfering RNA Transfection and shRNA

Small interfering RNA (siRNA) Smartpools targeting SREBP1 (L-040814–01–0005), SREBP2 (L-050073–01–0005), and nontargeting controls (D-001810–10–20) obtained from Dharmacon (Lafayette, CO) were used at 25 nM final concentration. Cells were transfected with Lipofectamine-RNAiMAX Reagent (Invitrogen, Carlsbad, CA) using the reverse transfection protocol.

Crystal Violet Staining

Cells were plated into 12- or 96-well plates. After treatment or 48 hours of culture, cells were fixed with 10% formalin for 5 minutes, stained with 0.05% crystal violet in distilled water for 30 minutes, washed two times with tap water, and drained. Crystal violet was solubilized with 100 μl of methanol, and the plate was read with a plate reader (OD 540; BioTek, Winooski, VT).

Targeted Metabolite Profiling and Isotopic Labeling Experiments

Lipid profiles of plasma or cell extracts were obtained using liquid chromatography–mass spectrometry methods as described previously. Details are provided in the online supplement.

mRNA Expression Analysis

Two micrograms of total RNA (RNeasy MicroKit; Qiagen Inc., Valencia, CA) were retrotranscribed with the High-Capacity cDNA Reverse-transcription kit (Applied Biosystems, Grand Island, NY) or the Superscript III kit (Life Technologies, Grand Island, NY). Forty nanograms of cDNA per reaction were run using a TaqMan probe (Applied Biosystems) for the PLA2 isoforms or primers for SREBP and stearoyl-CoA desaturase-1 (SCD1) (*see* Table E1 in the online supplement) in an Applied Biosystems Instrument. Results were normalized to actin or m36B4, which had stable expression in our experimental conditions, and analyzed using the ΔΔCt method. Final values were expressed as n-fold the calibrator.

Immunoblotting

Total proteins were extracted through 15-minute incubation with Nonidet P-40 lysis buffer and resolved on NuPage SDS polyacrylamide gels (Life Technologies). Phospho-S6 (Ser235/236), phospho-S6-kinase, total S6-kinase, SCD (2438S), Tuberin, and β-actin antibodies were obtained from Cell Signaling Technology (Danvers, MA). β-actin (A5316) was also obtained from Sigma (St. Louis, MO).

Results

LPC Species Are Elevated in Patients with LAM

To determine whether the metabolic effects of mTORC1 activation in LAM cells are reflected in the plasma of patients with LAM, we used liquid chromatography–mass spectrometry to perform lipidomic profiling of 14 patients with LAM and nine healthy female control subjects. Eighteen out of 131 complex lipid species measured were significantly altered in plasma from patients with LAM ($P < 0.05$) (Table E2; Figure 1A). Fifteen lipid species were elevated in patients with LAM compared with control subjects, including nine triacylglycerol (TAG), two PC, and four LPC species (C16:0, C18:0, C18:1, and C20:4) (Figures 1A and 1B). LPC are derived from PC via hydrolysis of a fatty-acyl at the sn1 or sn2 position. Most commonly, sn1 is a saturated fatty-acyl, and sn2 is an unsaturated fatty-acyl.

LPC and Other Lipid Species Are Elevated in TSC2-Deficient Cells

To determine whether the lipidomic signature found in plasma from patient with LAM could arise from TSC2-deficient cells, we analyzed complex lipids in cells

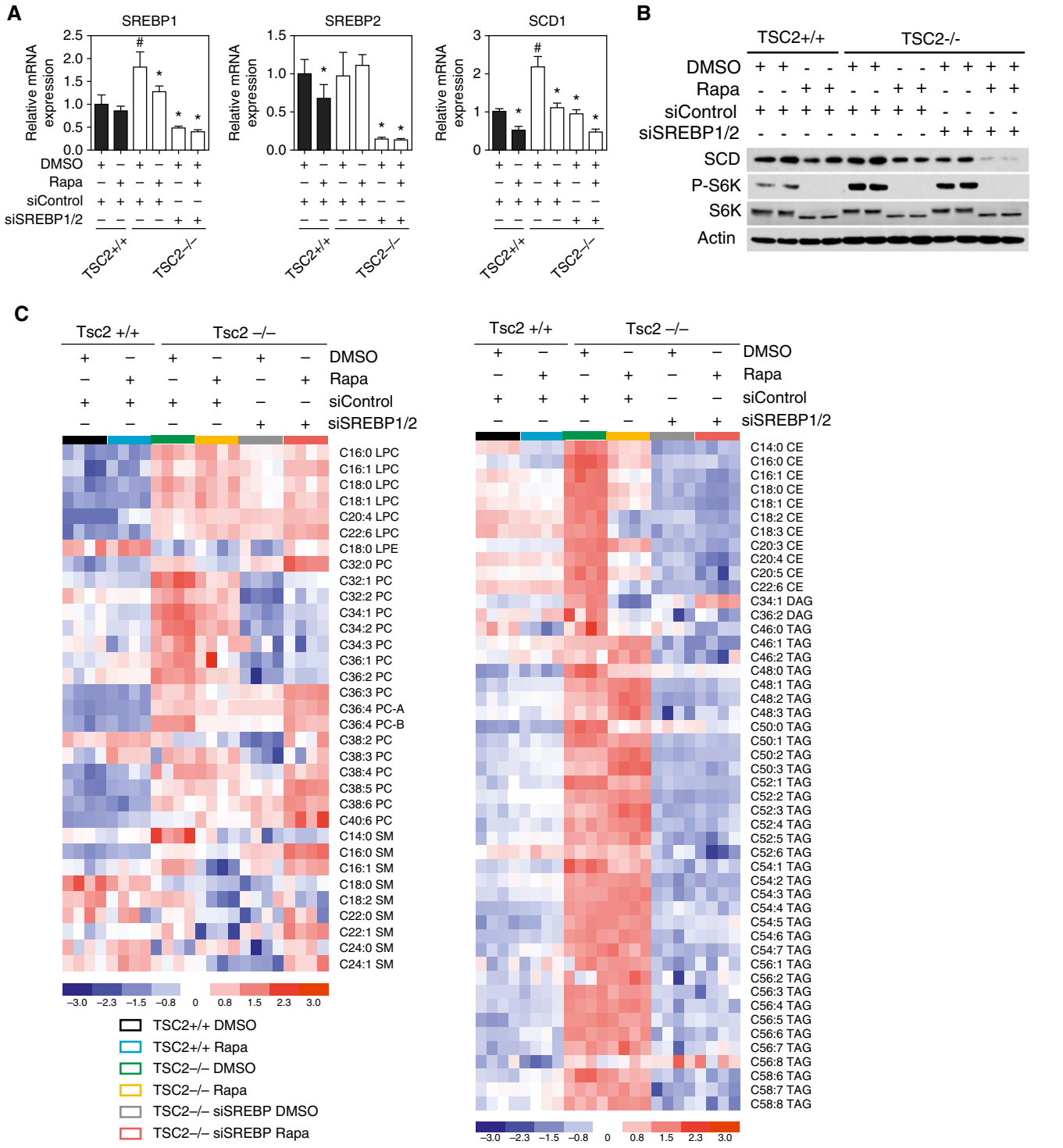

Figure 2. Sterol regulatory element–binding protein (SREBP) 1/2 down-regulation affects levels of complex lipid species but not LPC in TSC2-deficient cells. (*A*) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were transfected with small interfering RNA targeting SREBP1/2 (siSREBP1/2) or nontargeting control (siControl) for 72 hours and treated for the last 20 hours with rapamycin (20 nM) or vehicle (DMSO). Down-regulation of SREBP was confirmed by mRNA levels of SREBP1 and SREBP2 and the SREBP1 target stearoyl-CoA desaturase-1 (SCD1). The mRNA was normalized to m36B4. #Significant difference ($P < 0.05$) between DMSO-treated *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs. *Significant difference ($P < 0.05$) between the sample and the comparable siRNA or DMSO control. (*B*) Immunoblotting shows down-regulation of SCD1 in *Tsc2*$^{-/-}$ MEFs with SREBP knockdown or rapamycin (Rapa) treatment, increased S6 Kinase (S6K) phosphorylation in *Tsc2*$^{-/-}$ compared with *Tsc2*$^{+/+}$ MEFs, and decreased S6K phosphorylation in response to rapamycin. Two samples per condition are included. (*C*) Lipidomic profiling of *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs treated as in (*A*) and (*B*). All of the measured lipid species ($n = 81$) are depicted in the heat maps, ordered by class and saturation status.

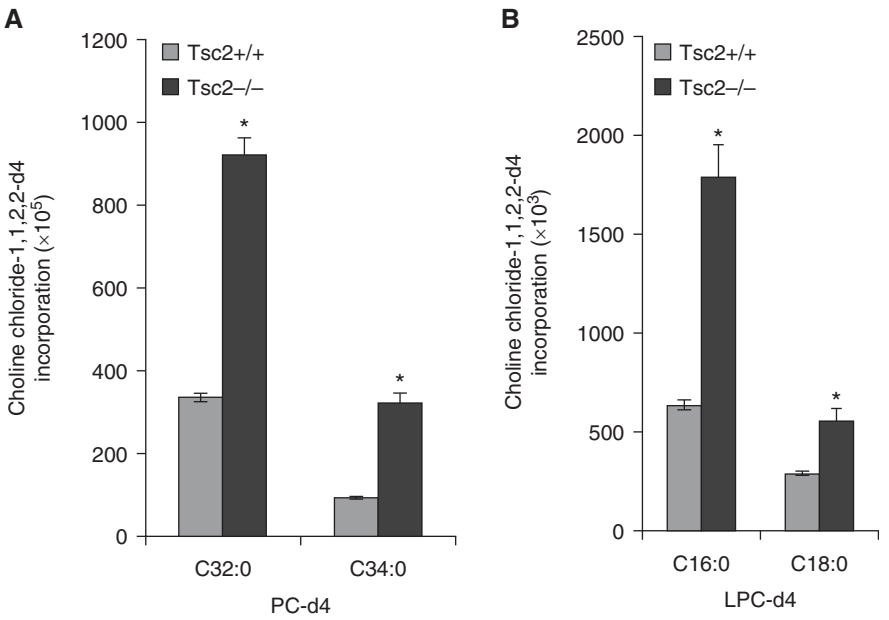

Figure 3. TSC2-deficient cells incorporate higher levels of choline into phosphatidylcholine (PC) and LPC. (*A* and *B*) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were labeled with choline chloride 1,1,2,2-d4 for 6 hours, and lipids were extracted for isotopomer measurements. All PC (*A*) and LPC (*B*) analyzed showed at least 2-fold increase in choline-d4 incorporation in *Tsc2*$^{-/-}$ MEFs. Mann-Whitney test was applied. *$P < 0.05$

isolated from the Eker rat model of TSC (ELT3 cells) (22), which were engineered to express an empty vector (ELT3-V3) or wild-type TSC2 (ELT3-T3). We identified 43 lipid species that were significantly changed ($q < 0.05$) (Table E3) upon TSC2 loss (Figure 1C). Interestingly, all measurable LPC species were significantly elevated in the TSC2-deficient cells, including the LPC species that were elevated in patients with LAM. Multiple other phospholipid (PC and sphingomyelin [SM]) and neutral lipid (TAG and cholesterol ester [CE]) species were differentially regulated in the TSC2-deficient ELT3-V3 cells compared with the TSC2-proficient ELT3-T3 cells ($q < 0.05$). These results suggest that both phospholipid synthesis and metabolism are altered in TSC2-deficient cells.

SREBP Down-Regulation Does Not Affect Levels of LPC Species in TSC2-Deficient Cells

mTORC1 activates the SREBP family of transcription factors (8, 12, 23), which, in turn, induces expression of genes encoding the major enzymes of *de novo* fatty acid synthesis, the oxidative branch of the pentose phosphate pathway (SREBP1) (8) and cholesterol synthesis/metabolism (SREBP2) (24).

To determine how the SREBPs contribute to complex lipid metabolism in TSC2-deficient cells, particularly regarding LPC, *Tsc2*$^{-/-}$ MEFs were transfected with nontargeting control siRNAs (siControl) or those targeting SREBP1 and SREBP2 for 72 hours (Figures 2A and 2B). In *Tsc2*$^{-/-}$ MEFs, SREBP1 mRNA levels were reduced to 26.4 ± 2.1%, and SREBP2 levels were reduced to 14.4 ± 2.4% of the siControl. Depletion of SREBP1 was also validated by a decrease in the expression of its canonical target SCD1 to 43.2 ± 4.8% of the siControl (Figure 2A). Lipid profiles were analyzed after 20 hours of treatment of *Tsc2*$^{-/-}$ and *Tsc2*$^{+/+}$ MEFs with rapamycin (20 nM) or vehicle (DMSO). Consistent with our findings in samples from patients with LAM and the Eker rat–derived cells, *Tsc2*$^{-/-}$ MEFs also had significantly increased levels of the specific LPC versus *Tsc2*$^{+/+}$ MEFs ($P < 0.05$) (Table E4). This change was accompanied by a global increase in phospholipids (PC and SM) and neutral lipids (TAG and CE) ($P < 0.05$). The majority of lipids were unaffected by rapamycin in TSC2-expressing cells (Figure 2C). In *Tsc2*$^{-/-}$ MEFs, rapamycin significantly ($P < 0.05$) decreased specific forms of SM (6/9 species), CE (5/11 species), and PC (2/17 species) but had little to no effect on TAG (2/35 species).

In contrast, knockdown of SREBP1/2 had a significant effect on neutral lipids, decreasing all TAG and CE species as well as some phospholipids (2/9 SM and 11/17 PC species). The stronger effect of the SREBP knockdown compared with rapamycin may reflect the complex regulation of SREBP activity, which is not fully sensitive to rapamycin (12). Interestingly, neither rapamycin nor SREBP1/2 siRNA suppressed the levels of six LPC species that were measured, despite the fact that levels of PC species, which can interconvert with LPC species, were decreased by SREBP inhibition. These results suggest that distinct LPC species could be generated via an SREBP-independent and rapamycin-insensitive mechanism upon TSC2 loss. Next, we analyzed LPC species in *Tsc2*$^{-/-}$ MEFs after treatment with the mTOR catalytic inhibitor Torin1 (25). None of the six LPC species was suppressed, consistent with rapamycin and SREBP down-regulation (Figure E1). Interestingly, the two saturated LPC (C16:0 and C18:0) and the unsaturated LPC C18:1 were further increased by Torin1 treatment (Figure E1).

LPC species are produced from PC via phospholipase activity. PLA2 hydrolyzes the acyl moiety at the sn-2 position, most commonly an unsaturated fatty acid, releasing saturated LPC species. To determine whether TSC2-deficient cells generate saturated LPC using *de novo* synthesized PC, we labeled *Tsc2*$^{-/-}$ and *Tsc2*$^{+/+}$ MEFs with deuterated choline (choline chloride 1,1,2,2-d4) for 6 hours and isolated lipids to measure choline incorporation into two LPC species (C16:0 and C18:0) and two PC species (C32:0 and C34:0). TSC2-deficient cells incorporated higher levels of deuterated choline into all of these species compared with TSC2-expressing cells ($P < 0.05$) (Figures 3A and 3B).

PLA2 Regulates the Proliferation of TSC2-Deficient Cells

These results suggest that specific rapamycin-insensitive phospholipids represent a fundamental metabolic trait of TSC2-deficient cells. We next investigated the impact of rapamycin in human TSC2-deficient cells derived from the renal

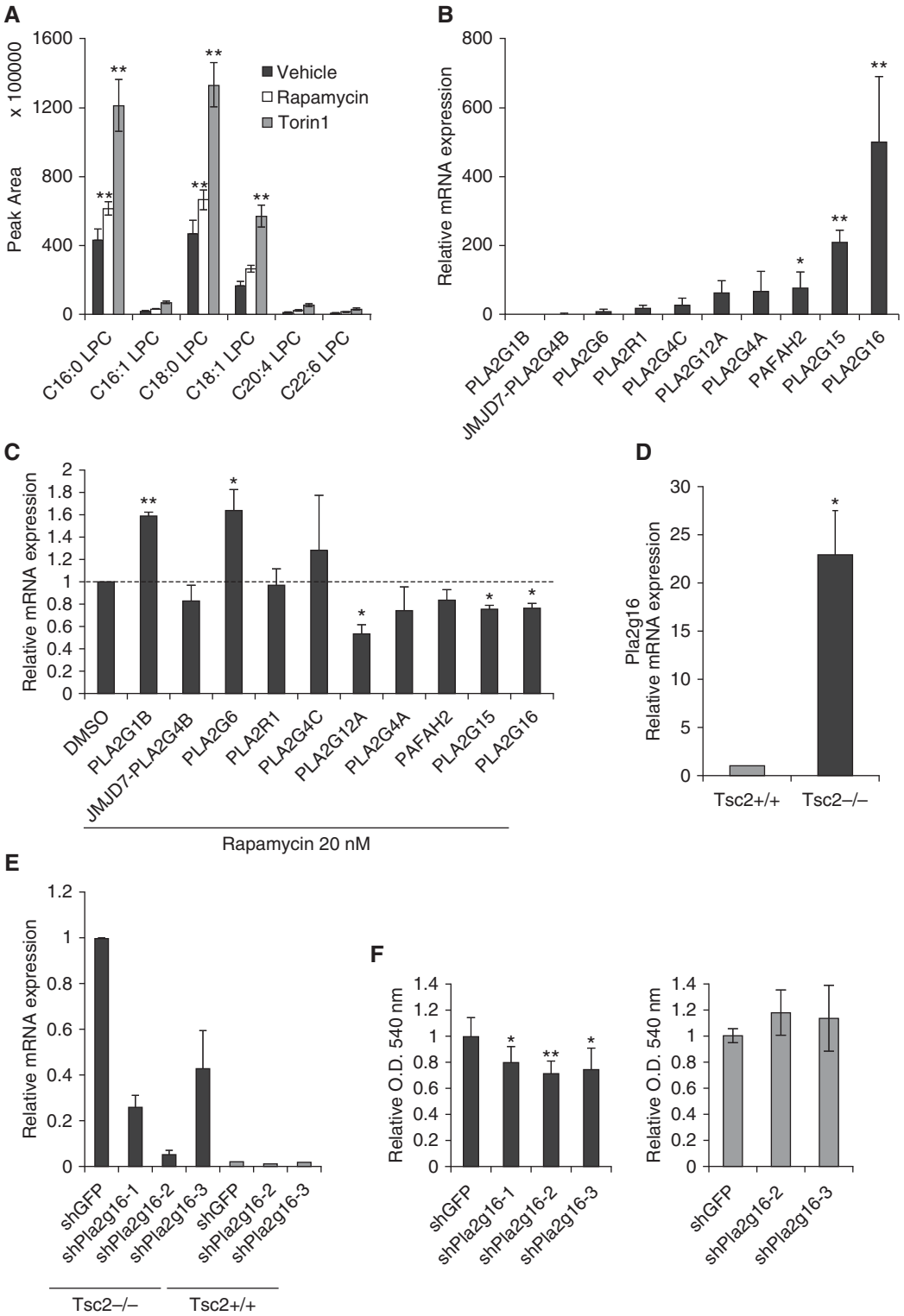

Figure 4. Distinct phospholipase A2 (PLA2) isoforms are regulated upon TSC2 loss. (*A*) LPC species were analyzed in LAM patient angiomyolipoma-derived 621-101 cells (three independent samples) following 20-hour culture in the presence of rapamycin (20 nM), Torin1 (250 nM), or vehicle control (DMSO). *Bar graph* represents mean ± SD of liquid chromatography–mass spectrometry peak area values (two-way ANOVA; $^{**}P < 0.01$). (*B*) Quantitative PCR expression levels of PLA2 isoforms relative to the least expressed isoform, PLA2G1B, in 621-101 cells. *Bar graph* represents means ± SD of relative values. (*C*) Expression of PLA2 isoforms was analyzed upon rapamycin treatment (20 nM for 20 h). *Bar graph* represents means ± SD of values of rapamycin-treated samples relative to their vehicle (DMSO) control. (*D*) Pla2g16 expression in *Tsc2*$^{-/-}$ and *Tsc2*$^{+/+}$ MEFs. *Bar graph* represents

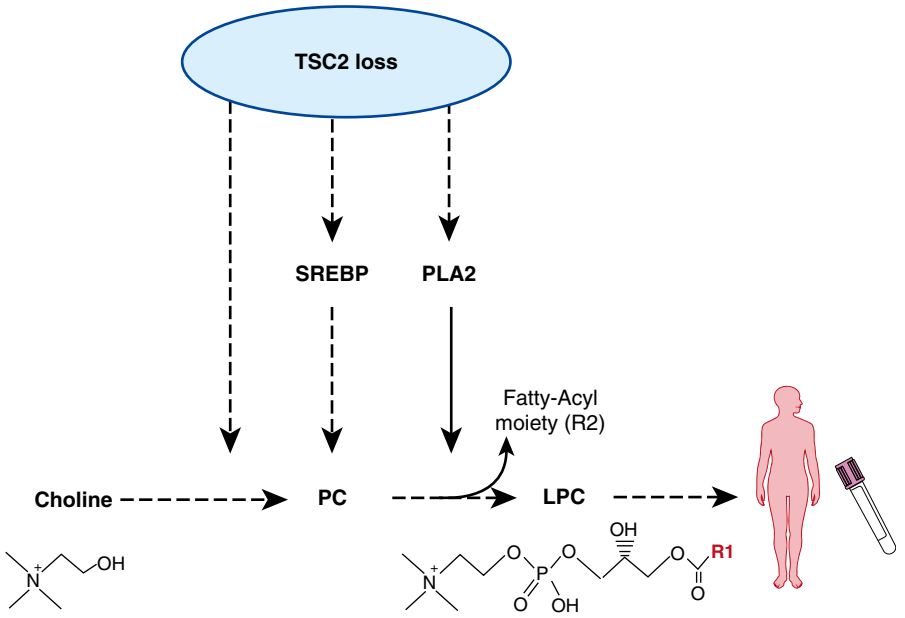

Figure 5. Simplified working model. TSC2-deficient cells show an increase in (*1*) LPC species, which derive from the hydrolysis of PC catalyzed by PLA2; (*2*) choline incorporation into PC; and (*3*) SREBP-mediated PC synthesis. Distinct LPC species were elevated in the plasma of patients with LAM compared with healthy women.

angiomyolipoma of a patient with LAM (621-101 cells). These cells carry biallelic somatic TSC2 mutations that are identical to the patient's pulmonary LAM cell mutations (26, 27). Similarly to MEFs, 20-hour treatment with rapamycin or Torin1 failed to suppress levels of LPC species and led to increased levels of LPC C16:0, C18:0, and C18:1 ($P < 0.05$) (Figures 4A and E2). In contrast, no increase in these LPC species was found in human TSC2-expressing cells treated with rapamycin (Figure E3).

Several isoforms of PLA2 have been described (14). These can be classified based on their cellular localization (cytosolic or secretory) and sensitivity to calcium (Ca^{2+} dependent or Ca^{2+} independent). To determine whether PLA2 inhibition affects the proliferation of TSC2-deficient cells, we treated $Tsc2^{-/-}$ MEFs or 621-101 cells with inhibitors of cytosolic (AACOCF3) or secretory (LY311727) PLA2 isoforms for 48 hours. Intriguingly, LY311727 did not affect proliferation (Figures E4A and E4C), whereas AACOCF3 inhibited the proliferation of 621-101 cells by 52 ± 9% (10 μM; $P < 0.01$) (Figure E4A) and of $Tsc2^{-/-}$ MEFs by 54 ± 12% (15 μM; $P < 0.01$) (Figure E4B), leading to a 30 to 50% decrease in the levels of LPC species C16:0 and C18:0 in $Tsc2^{-/-}$ MEFs (Figure E5). AACOCF3 also inhibited the proliferation of $Tsc2^{+/+}$ MEFs to a similar extent (61 ± 13% at a dosage of 15 μM; $P < 0.01$) (Figure E4B). However, because AACOCF3 may inhibit several isoforms of cytosolic PLA2 and also PLA1, these results do not rule out a model in which selective PLA2 isoforms play a role in the proliferation of TSC2-deficient cells.

To identify PLA2 isoforms that may participate in choline lysophospholipid metabolism in the patient-derived 621-101 cells, we used quantitative PCR to measure the expression of 22 PLA2 isoforms and related genes in the presence or absence of rapamycin. Ten isoforms and the PLA2 receptor1 (PLA2R1) were detected. Three isoforms were significantly expressed relative to the least expressed PLA2: PLA2G16 (Ca^{2+} independent with PLA1/2 activity in adipose tissue, also called HREV107), PLA2G15 (lysophospholipase), and PAFAH2 (platelet-activating factor acetyl-hydrolase) (Figure 4B). Rapamycin decreased the expression ($P < 0.05$) of PLA2G16 by 23 ± 4%, PLA2G15 by 25 ± 3%, and PLA2G12A by 46 ± 7%. Interestingly, expression of PLA2G1B and PLA2G6 was increased ($P < 0.05$) by rapamycin by 64 ± 18% and 59 ± 3%, respectively (Figure 4C). We next examined the expression of PLA2 genes in $Tsc2^{-/-}$ and $Tsc2^{+/+}$ MEFs. Interestingly, Pla2g16 expression was greater than 20-fold higher in $Tsc2^{-/-}$ MEFs compared with $Tsc2^{+/+}$ MEFs ($P < 0.05$) (Figure 4D).

Finally, to determine the effect of specific PLA2 isoforms in TSC2-deficient cells, we measured the proliferation of $Tsc2^{-/-}$ and $Tsc2^{+/+}$ cells infected with a control shRNA and three independent shRNA targeting Pla2g16 (Figure 4E). Inhibition of Pla2g16 suppressed proliferation by 20 to 30% in $Tsc2^{-/-}$ cells, but not in $Tsc2^{+/+}$ MEFs, in serum-deprivation conditions (Figure 4F).

Discussion

LAM is a rare disease affecting women. In LAM, smooth muscle–like cells that express melanocytic markers invade the lungs, causing extensive cystic lung destruction. Cystic lung disease is observed in up to 80% of women with TSC, and 10 to 20% of these women develop respiratory symptoms, including spontaneous pneumothorax, dyspnea, supplemental oxygen dependence, and respiratory failure. In both patients with TSC and in the sporadic form of LAM, inactivating mutations of the TSC genes are present in LAM cells (2, 26, 28), leading to aberrant activation of mTORC1, a master regulator of cellular metabolism. We sought to determine whether the plasma of patients with LAM had elevated levels of metabolites reflecting activation of mTORC1 in LAM cells. We identified a lysophospholipid signature in LAM plasma, with significantly elevated levels of four LPC species. Importantly, levels of plasma LPC have been shown to be

Figure 4. (Continued). means ± SD of values relative to $Tsc2^{+/+}$ MEFs. In *B*, *C*, and D, one-sample *t* test was applied (*$P < 0.05$; **$P < 0.01$). (*E*) $Tsc2^{-/-}$ and $Tsc2^{+/+}$ MEFs were infected with independent shRNA targeting Pla2g16 or nontargeting green fluorescent protein (shGFP) control. *Bar graph* represents means ± SD of values relative to $Tsc2^{-/-}$ MEFs shGFP. The mRNA (*B–E*) was normalized to actin. (*F*) Proliferation of $Tsc2^{-/-}$ (*black bars*) *and* $Tsc2^{+/+}$ (*light gray bars*) MEFs infected with shRNA targeting Pla2g16 or nontargeting shGFP control was measured with a colorimetric assay (crystal violet). *Bar graph* represents means ± SD of values relative to the shGFP control. Mann-Whitney test was applied (*$P < 0.05$; **$P < 0.01$). O.D., optical density.

independent of age, body mass index, and relative fat mass (29) and to be stable in healthy subjects over 1- to 2-year follow-up (30). In addition, LPC have been found to be decreased in the blood of patients with colorectal cancer (31). Therefore, distinct plasma LPC species could represent potential candidates for future biomarker studies in LAM. The lipidomic signature of LAM could ultimately have an impact as a diagnostic, prognostic, or therapeutic response indicator and could be combined with other known or emerging biomarkers, including VEGF-D (32–34) and clinical phenotypes.

We also found the same signature in two independent TSC2-deficient cellular models, suggesting that the plasma LPC may be specifically related to the LAM cells. LPC are lipolytic products of PC (Figure 5). *De novo* glycerophospholipid synthesis generates PC species via the "Kennedy pathway," and PLA2 generates LPC acting on PC. This process is critical for two reasons: (*1*) the remodeling of membrane phospholipids, which requires LPC to be reacylated to PC by lysophospholipid acyltransferases, and (*2*) the release of bioactive lipids, including LPC and arachidonic acid, a precursor of eicosanoids. Although phospholipases and their downstream bioactive lipids have been previously studied primarily in the context of inflammation and atherosclerosis, a role for this large class of enzymes in proliferative disorders is emerging (14, 35–37). For example, a direct role for LPC in tumor angiogenesis has been shown in preclinical models of glioblastoma and lung cancer (35). In LAM, the remodeling and bioactive lipid release functions of PLA could be relevant to disease pathogenesis in several ways. First, membrane phospholipid remodeling is critical for the lipid composition of pulmonary surfactant (20) and to maintain lipid homeostasis to prevent toxic levels of ER stress (21). TSC2-deficient cells are known to have an increased susceptibility to ER stress (38). Second, pulmonary and extrapulmonary LAM is characterized by abnormal blood and lymphatic vasculature, which could be influenced by bioactive lipids, including LPC. Third, up-regulation of COX2, an enzyme responsible for the production of prostaglandins downstream of PLA2, and PLA2G16 has been recently found in LAM nodules (39, 40). Therefore, in addition to the PLA2-dependent proliferation demonstrated here, we hypothesize that other LPC-dependent pathways may participate in LAM pathogenesis.

Interestingly, our analysis of the TSC lipidome revealed how distinct lipid classes, including the LPC precursors PC, are differentially regulated in a rapamycin- or SREBP-dependent manner upon TSC2 loss. Although an increase in several phospholipid and neutral lipid species was dependent upon TSC2 loss, rapamycin decreased only a minority of these lipid species. This could be due to the fact that rapamycin does not entirely suppress mTORC1 activity (41), which is further substantiated by the result that SREBP down-regulation had a major effect on most lipid species. In fact, SREBP activation by mTORC1 is only partially affected by rapamycin (12). Surprisingly, despite a decrease in several PC species, higher levels of LPC were maintained in $Tsc2^{-/-}$ MEFs upon SREBP down-regulation. This could suggest an unchecked activity of PLA downstream of TSC2, targeting selective PC species, for which even low endogenous PC levels or exogenous lipids could provide a substrate. In conclusion, the TSC complex regulates both phospholipids and neutral lipids via multiple pathways, involving SREBPs and a potential TSC2-linked rapamycin-insensitive mechanism. Indeed, evidence of noncanonical functions of the TSC complex has been reported by multiple groups (42). Finally, inhibition of specific PLA2 isoforms can affect the proliferation of TSC2-deficient cells, suggesting that elucidating the pathways through which phospholipid metabolism is deregulated in TSC-related disorders could improve our understanding of disease pathogenesis and targeted therapy. ■

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

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