Activation of Sterol-response Element-binding Proteins (SREBP) in Alveolar Type II Cells Enhances Lipogenesis Causing Pulmonary Lipotoxicity

Laurent Plantier††, Valérie Besnard‡, Yan Xu§, Machiko Ikegami†, Susan E. Wert‡, Alan N. Hunt§, Anthony D. Postle†, and Jeffrey A. Whitsett‡‡

From the ††Section of Neonatology, Perinatal and Pulmonary Biology, Perinatal Institute, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio 45229 and the §Division of Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton SO16 6YD, United Kingdom

Background: Lung function is dependent upon the regulation of tissue and alveolar lipids.
Results: Activation of SREBP in alveolar cells caused neutral lipid accumulation, inflammation, and tissue remodeling.
Conclusion: The accumulation of neutral lipids in the lung caused inflammation consistent with findings in lipid storage disorders.
Significance: Pulmonary lipotoxicity may contribute to lung dysfunction associated with diabetes, obesity, and metabolic disorders.

Pulmonary inflammation is associated with altered lipid synthesis and clearance related to diabetes, obesity, and various inherited metabolic disorders. In many tissues, lipogenesis is regulated at the transcriptional level by the activity of sterol-response element-binding proteins (SREBP). The role of SREBP activation in the regulation of lipid metabolism in the lung was assessed in mice in which both Insig1 and Insig2 genes, encoding proteins that bind and inhibit SREBPs in the endoplasmic reticulum, were deleted in alveolar type 2 cells. Although deletion of either Insig1 or Insig2 did not alter SREBP activity or lipid homeostasis, deletion of both genes (Insig1/2−/− mice) activated SREBP1, causing marked accumulation of lipids that consisted primarily of cholesterol esters and triglycerides in type 2 epithelial cells and alveolar macrophages. Neutral lipids accumulated in type 2 cells in association with the increase in mRNAs regulating fatty acid, cholesterol synthesis, and inflammation. Although bronchoalveolar lavage fluid phosphatidylcholine was modestly decreased, lung phospholipid content and lung function were maintained. Insig1/2−/− mice developed lung inflammation and airspace abnormalities associated with the accumulation of lipids in alveolar type 2 cells, alveolar macrophages, and within alveolar spaces. Deletion of Insig1/2 activated SREBP-enhancing lipogenesis in respiratory epithelial cells resulting in lipotoxicity-related lung inflammation and tissue remodeling.

Precise regulation of alveolar lipid homeostasis is required for lung function. Lack of surfactant phospholipids and proteins causes respiratory distress syndrome in neonates and participates in the pathogenesis of acute lung injury in adults. The fibrosing sequellae that often accompany these syndromes can cause chronic respiratory insufficiency. Lack of pulmonary surfactant, related to mutations in genes critical for surfactant synthesis and activity, causes both acute respiratory failure and life-threatening chronic lung disorders, including pulmonary fibrosis (1–4). Conversely, excess accumulation of surfactant lipids occurs in the lungs of patients with pulmonary alveolar proteinosis, a disorder caused by defects in GM-CSF signaling that impair clearance of surfactant by alveolar macrophages (5). Lipid storage diseases are often accompanied by chronic inflammation and fibrosing alveolitis (6, 7).

The abundance and composition of lung lipids are precisely regulated. Pulmonary surfactant is enriched in phosphatidylcholine (PC) and phosphatidylglycerol that play important roles in reduction of surface tension. Surfactant PC is relatively enriched in saturated species. Although cholesterol is present in varying quantities in mammalian surfactants, excess cholesterol impairs the surface tension reducing properties of surfactants (8). Triacylglycerols, an important storage form of lung lipids, are normally present in small amounts in alveolar tissues and are thought to serve as substrates for phospholipid synthesis by alveolar type 2 cells during development (9). The role of neutral lipid pools in surfactant lipid homeostasis has not been clearly defined in the adult lung. Surfactant lipid composition and abundance are influenced by lung injury, likely mediated by intracellular and extracellular processes that control synthesis and catabolism of lipids present in pulmonary surfactant (10). Thus, a complex regulatory system has evolved to precisely control lung lipid content and composition in the alveolus.

The abbreviations used are: PC, phosphatidylcholine; SREBP, sterol-regulatory element-binding protein; SCAP, SREBP cleavage-activating protein; BALF, bronchoalveolar lavage fluid; SP-C, surfactant protein C; RXR, retinoid X receptor.
Pulmonary Lipotoxicity

Lung lipid homeostasis is regulated by multitiered processes influencing 1) the uptake of lipids from the systemic circulation, 2) synthesis and storage of lipids by epithelial and stromal compartments in the alveolus, and 3) secretion, reuptake, reutilization, and catabolism of surfactant lipids, which together precisely control intracellular and extracellular surfactant pools before and after birth. As in other tissues, lipid homeostasis in the lung is influenced, in part, by sterol-regulatory element-binding proteins (SREBPs). SREBP1c expression increases before birth in association with increased lipogenesis in type 2 epithelial cells (11). Deletion of the Scap gene (encoding the SREBP cleavage-activating protein) inhibited SREBP activity in type 2 cells and enhanced neutral lipid accumulation in lung fibroblasts in the fetal and postnatal mouse lung (12), demonstrating important roles for the SREBP pathway in lung lipid homeostasis.

SREBP is activated at the post-transcriptional level by SCAP. SCAP serves as a lipid sensor that in the absence of sterols/phospholipids transports SREBPs from the endoplasmic reticulum to the Golgi where S1P and S2P proteases release a tran-

slation, and catabolism of surfactant lipids, which together precisely control intracellular and extracellular surfactant pools before and after birth. As in other tissues, lipid homeostasis in the lung is influenced, in part, by sterol-regulatory element-binding proteins (SREBPs). SREBP1c expression increases before birth in association with increased lipogenesis in type 2 epithelial cells (11). Deletion of the Scap gene (encoding the SREBP cleavage-activating protein) inhibited SREBP activity in type 2 cells and enhanced neutral lipid accumulation in lung fibroblasts in the fetal and postnatal mouse lung (12), demonstrating important roles for the SREBP pathway in lung lipid homeostasis.

SREBP is activated at the post-transcriptional level by SCAP. SCAP serves as a lipid sensor that in the absence of sterols/phospholipids transports SREBPs from the endoplasmic reticulum to the Golgi where S1P and S2P proteases release a transcriptionally active N-terminal fragment of SREBP. Conversely, SREBP activity is inhibited by insulin-induced gene proteins 1 and 2 that anchor the SCAP-SREBP complex to the endoplasmic reticulum membrane in a lipid-dependent manner. Insig1 and Insig2 share structural similarities and have partially redundant functions (13). Although germ line deletion of Insig1 in the mouse caused death at birth, germ line deletion of Insig2 did not influence survival. Deletion of both Insig genes in hepatic tissues increased transcription of SREBP target genes causing accumulation of cholesterol and triglycerides (13). There is increasing evidence that the accumulation of lipid droplets in various tissues during substrate excess causes tissue inflammation and is inhibited in part by the recruitment and activation of tissue macrophages (14).

This study was designed to further define the roles of SREBP and insulin-induced genes in the regulation of lung lipid homeostasis and to test whether enhanced lipogenesis influenced surfactant homeostasis, lung lipid content, or lung inflammation. Deletion of Insig1 and -2 induced SREBP1 in alveolar type 2 cells, causing neutral lipid accumulation in type II cells and in the alveoli. The accumulation of lung lipids caused inflammation and airspace remodeling with pathological findings similar to those associated with lipid storage disorders, diabetes mellitus, and obesity.

EXPERIMENTAL PROCEDURES

Transgenic Animals—Insig1<sup>−/−</sup> and Insig2<sup>−/−</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME). In Insig1<sup>−/−</sup> mice, loxp sites flank exon 1 of the Insig1 gene. The Insig2 gene is disrupted by replacement of exons II and III with a neo cassette eliminating the first 123 of 225 amino acids (13). Mice were mated with SFTPC-rtTA<sup>WT/Tg</sup>/(tetO)<sub>2</sub>CMV-Cre<sup>WT/Tg</sup> to generate the following: 1) single transgenic Insig2<sup>−/−</sup> mice; 2) triple transgenic SFTPC-rtTA<sup>WT/Tg</sup>/(tetO)<sub>2</sub>CMV-Cre<sup>WT/Tg</sup>/Insig<sup>−/−</sup> mice; and 3) quadruple transgenic SFTPC-rtTA<sup>WT/Tg</sup>/(tetO)<sub>2</sub>CMV-Cre<sup>WT/Tg</sup>/Insig1<sup>−/−</sup>/Insig2<sup>−/−</sup> mice. Doxycycline-exposed, SFTPC-rtTA<sup>WT/Tg</sup>/(tetO)<sub>2</sub>CMV-Cre<sup>WT/Tg</sup>/Insig<sup>−/−</sup>/Insig2<sup>−/−</sup> mice were exposed to doxycycline in utero, Insig1 was conditionally deleted in the Insig2<sup>−/−</sup> background; Insig1<sup>−/−</sup>/Insig2<sup>−/−</sup> mice were compared with Insig1<sup>−/−</sup>/Insig2<sup>−/−</sup> littermates lacking either SFTPC-rtTA or (tetO)<sub>2</sub>CMV-Cre; and 3) quadruple transgenic SFTPC-rtTA<sup>WT/Tg</sup>/(tetO)<sub>2</sub>CMV-Cre<sup>WT/Tg</sup>/Insig1<sup>−/−</sup>/Insig2<sup>−/−</sup> mice. Doxycycline-exposed, SFTPC-rtTA<sup>WT/Tg</sup>/(tetO)<sub>2</sub>CMV-Cre<sup>WT/Tg</sup>/Insig<sup>−/−</sup>/Insig2<sup>−/−</sup> mice were exposed to doxycycline in utero, Insig1 was conditionally deleted in the Insig2<sup>−/−</sup> background; Insig1<sup>−/−</sup>/Insig2<sup>−/−</sup> mice were compared with Insig1<sup>−/−</sup>/Insig2<sup>−/−</sup> littermates lacking either SFTPC-rtTA or (tetO)<sub>2</sub>CMV-Cre. With this system, recombination of the loxp flanked allele is typically obtained in most alveolar type 2 cells (15). Genotypes were identified by PCR from genomic tail DNA as described previously (13, 16).

Animal Husbandry and Doxycycline Administration—Mice were maintained in a pathogen-free environment in accordance with protocols approved by the Institutional Animal Care and Use Committee of Cincinnati Children’s Hospital Research Foundation. Gestation was dated by detection of the vaginal plug, and dams bearing pups carrying the Insig1<sup>lox</sup> allele were maintained on doxycycline in food (625 mg/kg dry weight; Harlan Teklad, Madison, WI) from embryonic days 6.5 to 12.5 to delete Insig1 in progenitor cells that form the peripheral lung and to minimize effects of Cre-recombinase (Cre) or reverse tetracycline transactivator that can occur later in gestation. Mice were killed at 2, 6, and 8 months of age for study.

Preparation of Tissue for Morphologic Analysis—Mice were killed by overdose of anesthetic and exsanguinated by sectioning the abdominal aorta. The anterior thoracic wall was removed. The trachea was cannulated, and the lungs were inflated at a pressure of 25 cm of H<sub>2</sub>O with 4% paraformaldehyde in phosphate-buffered saline for 1 min. The trachea was ligated, and the heart and lungs were removed and immersed in the same fixative at 4 °C for 18 h. After three rinses in cold PBS, the right lung and the upper half of the left lung were dehydrated in graded ethanol and embedded in paraffin; the lower half of the left lung was immersed in 30% sucrose in PBS for 24 h, immersed in a 2:1 mixture of 30% sucrose and OCT sectioning medium (Sakura, Torrance, CA) for another 24 h, and then embedded in OCT and stored at −80 °C. Five-μm-thick sections of paraffin-embedded tissue were prepared for Movat’s pentachrome or hematoxylin and eosin stains (Poly Scientific, Bay Shore, NY) and immunohistochemistry studies. Eight-μm-thick frozen sections were prepared for Oil Red O staining, Nile Red staining, and immunofluorescence studies.

Whole Lung Protein Extraction, Immunoblotting—The left atrium was cut open, and the lungs were rinsed with 10 ml of PBS injected through the right ventricle and snap-frozen in liquid nitrogen. The lungs were homogenized in 50 mM Tris, pH 7.4, with 150 mM NaCl, 2 mM EDTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.1 mM sodium vanadate, 0.2% Triton X-100, 0.3% Nonidet P-40, and 0.1% protease inhibitor mixture (P8340, Sigma) and centrifuged at 13,000 rpm for 15 min. The supernatant was stored at −20 °C. Proteins (10 μg) were separated by SDS-PAGE on polyacrylamide gels and transferred onto 0.2-μm nitrocellulose membranes (Bio-Rad). Membranes were probed with antibodies targeting SREBP1 (sc-8984, Santa Cruz Biotechnology, Santa Cruz, CA), fatty-acid synthase (FASN) (BD Transduction Laboratories), and GAPDH (A300-641A, Bethyl Laboratories, Montgomery, TX) as a loading con-
control. Band density was analyzed using ImageJ (National Institutes of Health, Bethesda).

**Oil Red O Staining**—Frozen lung sections or BALF cells were fixed in 4% paraformaldehyde for 15 min and rinsed three times in distilled water. Slides were immersed for 2 min in absolute propylene glycol and incubated for 1 h (BALF cells) or overnight (lung sections) at room temperature in Oil Red O solution (Poly Scientific). Slides were then immersed in 85% propylene glycol, rinsed twice in distilled water, and counterstained with Harris’ hematoxylin.

**pro-SP-C Immunofluorescence and Nile Red Staining**—To identify lipid-laden cells, frozen lung sections were rehydrated in PBS, blocked with 4% normal goat serum, and stained with an anti-pro-SP-C antibody (WRAB-SPC, Seven Hills Bioreagents, Cincinnati, OH) followed by an Alexa Fluor 488-conjugated goat anti-rabbit antibody (Invitrogen). Sections were then counterstained for 5 min with 2.5 mg/ml Nile Red in 75% aqueous glycerol and coverslipped. Nile Red fluorescence was analyzed using a 535–580 nm excitation filter and a 591–667 nm emission filter. With this filter set, fluorescence emitted by Nile Red predominantly reflects neutral lipids over phospholipids. The number of Nile Red-positive cells relative to the number of pro-SP-C-positive cells in five microscopic fields at ×10 magnification was determined.

**Electron Microscopy**—Lungs from 8-week-old mice were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer and 0.1% calcium chloride, pH 7.3. Tissue was postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin (EMbed 812, Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections were viewed in a Hitachi H7600 transmission electron microscope, and digitized images were collected with an AMT Advantage Plus 2k × 2k digital camera (Advanced Microscopy Techniques, Danvers, MA).

**BALF**—Mice were anesthetized; the trachea was cannulated, and the lungs were lavaged twice with 1 ml of PBS, pH 7.4. The total number of cells was determined using trypan blue exclusion, and 10,000 cells were plated onto glass slides using a CytoSpin centrifuge (Thermo, Waltham, MA) and stained with hematoxylin and eosin for differential counting or kept at −20°C for Oil Red O staining and immunostaining. In other experiments, BALF cells were lysed, and total RNA was extracted.

**Isolation and Sorting of Alveolar Type 2 Cells**—Mouse alveolar type 2 cells were isolated as described previously (17). Briefly, the lungs were perfused to remove blood, inflated with Dispase (Stem Cell Technologies, British Columbia, Canada) for 45 min, and trimmed manually. The resulting suspension was filtered on 100-, 40-, and 20-μm filters and incubated for 2 h on 100-μm cultures dishes coated with anti-CD45 and anti-CD16/32 antibodies to eliminate leukocytes. Alveolar type 2 cells were recovered from the supernatant by centrifugation. Total alveolar type 2 cells from 2-month-old animals were used for mRNA microarray and RT-PCR.

Type 2 cells were purified from 6-month-old Insig1/2ΔΔ mice and subjected to flow cytometry and sorting using the lipid probe Nile Red with an excitation wavelength of 488 nm. Phospholipid-rich cells were sorted using a 660/20 nm emission filter. Neutral lipid-rich cells were sorted using a 545/35-nm emission filter (18). Neutral lipid-positive cells were gated based on side scatter and intensity of Nile Red fluorescence. Cells were analyzed and sorted by a FACSAria II cell sorter (BD Biosciences).

**Cytokine and Chemokine Assays**—Lung homogenates were centrifuged at 2,000 × g for 10 min, and supernatants were used for cytokine protein analyses. IL-6, IL-1β, TNF-α, keratinocyte chemotactant, and MIP2 were analyzed using immunoassays (MILLIPLEX™ Map, Millipore, Billerica, MA).

**RNA Extraction, RT-PCR Analysis**—RNA was extracted using RNAeasy reagents (Qiagen, Valencia, CA). Potentially contaminating genomic DNA was removed by exposure to recombinant DNase I (Roche Applied Science). RNAs were retrotranscribed to complementary DNAs using Moloney murine leukemia virus reverse transcriptase (Invitrogen), according to the manufacturer’s instructions, and analyzed by RT-PCR using TaqMan reagents (Applied Biosystems, Foster City, CA) or iQ SYBR Green Supermix (Bio-Rad). Gene expression was reported relative to that of 18S RNA as an internal control. Primer sequences and thermal cycling conditions are listed in supplemental Table 1. IL-1B, IL-12B, and TNF-α mRNA was assessed by RT-PCR using StepOne Plus Time PCR (Applied Biosystems) and normalized to 18 S RNA using RNA isolated from alveolar macrophages isolated from control and Insig1/2-deleted mice, n = 8 per group.

**RNA Microarray Analysis in Alveolar Type 2 Cells**—For microarray analysis, 700 ng of alveolar type 2 cell RNA from Insig1/2ΔΔ (n = 3) and Insig1flox/flox/Insig2−/− (n = 3) mice were hybridized to the murine genome 430 2.0 array (Affymetrix, Santa Clara, CA). The RNA quality and quantity assessment, probe preparation, labeling, hybridization, and image scan were carried out in the Cincinnati Children’s Hospital Medical Center Affymetrix Core using standard procedures. Data were analyzed using BRB Array Tools software package (linus.nci.nih.gov). Differentially expressed mRNAs were identified using a random variance t test, which permits sharing information among genes about within-class variation without assuming that all genes have the same variance (19). mRNAs were considered differentially expressed with a p value of ≤0.05 and a fold change of ≥1.5. Affymetrix “Present Call” in at least two of three replicates was set as a prerequisite for gene selection.

Differentially expressed genes were subjected to gene ontology, promoter analysis, and pathway analysis. Gene Ontology analysis was performed using the publicly available web-based tool David (data base for annotation, visualization, and integrated discovery) (21). Over-represented transcription factor-binding site analyses were performed using 2 kb of promoter sequences upstream of transcription start sites of differentially expressed genes (Genomatix). Gene ontology categories were considered to be significant when a Fisher’s exact test p value was ≤0.01 and gene hits were ≥10. Over-represented functions, diseases, known pathways, as well as potential protein/protein or protein/DNA interactions were identified using ingenuity pathway analysis (Ingenuity). Customized gene networks were constructed by integrating microarray results, literature mining, and experimental observations. Changes in
mRNA expression were compared with those obtained in the lungs of Scap-deleted animals (12). Differentially expressed genes in the Insig deletion array were compared with published gene expression data in the L2L data base.

**Lipid Mass Spectrometry Analysis**—To assess PC synthesis, turnover, and secretion, animals were injected intraperitoneally with 6.7 μmol of [methyl-9-2H]choline chloride (CDN Isotopes, Pointe-Claire, Canada). Mice were anesthetized; the trachea was cannulated, and the lungs were lavaged five times with 1 ml of PBS. Lavaged lungs were homogenized in 2 ml of 1.2 M sucrose, and the lamellar body fraction was isolated by sucrose gradient density centrifugation as described previously (20). Lipids were extracted with chloroform and methanol, as described previously (21), from lung homogenate, lamellar bodies, and BALF and from isolated alveolar type 2 cells in an independent experiment. Dimyristoylphosphatidylcholine (PC 14:0/14:0, 15 nmol) and a deuterated triglyceride (glyceryl tri(octadecanoate-18,18,18-d5), CDN Isotopes, 10 nmol) were added as internal standards. The dried lipid extract was divided in two portions for separate electrospray ionization mass spectrometry (ESI-MS) analysis of phospholipids and neutral lipids. PC was analyzed by ESI-MS as described previously (22), employing precursor ion scans of the m/z 184 phosphocholine headgroup for endogenous PC species and of the corresponding m/z 193 for newly synthesized PC species. A neutral lipid fraction was isolated as the flow-through from 100 mg of aminopropyl (NH2) BondElut columns (Agilent Technologies UK Ltd.) after application of the second portion of the lipid extract dissolved in chloroform. The neutral lipid fraction was dissolved in 250 μl of 50:50 (v/v) chloroform/methanol solution containing 1% NH4OH (0.880) and introduced into the mass spectrometer by direct infusion at 5 μl/min. Neutral lipids were quantified from the ESI+ scan with a series of fatty acid neutral loss scans for 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:4, 22:5, and 22:6 used to identify the dominant fatty acid profiles of triacylglycerol, diacylglycerol, monoa-cylglycerol, and cholesterol ester species. Specific precursor scans at m/z 369+ were used to confirm the identity of cholesterol ester species, whereas neutral loss of m/z 35 fragments identified diacylglycerol species.

**Measurement of Lung Mechanics**—Lung mechanics were studied in anesthetized and tracheostomized mice using the FlexiVent System (SCIREQ Scientific Respiratory Equipment, Montreal, Quebec, Canada), as described previously (23). Dynamic resistance, elastance, and compliance of the airways were measured as well as tissue damping, tissue compliance, and tissue hysteresivity.

**Statistical Analysis**—Data were expressed as means ± S.D. Student’s t test was used for comparisons of continuous variables. Association between categorical variables was tested using the χ2 test. A p value <0.05 was considered statistically significant.

**RESULTS**

**Germ Line Deletion of Insig2 or Conditional Deletion of Insig1 Alone Did Not Activate SREBP Signaling in the Lung—Insig1+/+ and Insig2−/− mice were bred to generate littermate Insig2+/+ and Insig2−/− mice. As reported previously (13), Insig2−/− mice were born at the expected Mendelian ratio from heterozygous parents. Deletion of Insig2 had no effect on the levels of the 72-kDa activated SREBP1 (aSREBP1) in the lung and did not change mRNAs of known SREBP target genes, including Fasn, Scdl, Ldrl, and Hmgcs1; compensatory increases in Insig1 mRNA were not observed in the lungs of Insig2−/− mice (data not shown). At 2 and 6 months of age, lungs of Insig2−/− mice were morphologically indistinguishable from the lungs of Insig2+/+ mice by light microscopy and Oil Red O staining. BALF cell counts were similar in both groups of mice, and there were no changes in Oil Red O-stained alveolar macrophages in Insig2−/− and Insig2+/+ mice (13.5 ± 3.5 vs. 11.0 ± 0.6%, p = 0.59) at 2 months of age; at 6 months of age, percent of Oil Red O-stained macrophages in BALF was 22.3 ± 3.8 and 19.7 ± 4.7%, respectively (p = 0.51). These data indicate that SREBP signaling was not activated in lungs of Insig2−/− mice, suggesting either that Insig2 has no detectable function in the lung or that Insig1 fully compensates for the lack of Insig2.

Mice in which Insig1 was conditionally deleted with the hsFSTPC-rtTA/(otet)Cre system in alveolar type 2 cells (Fig. 1A) were compared with Insig1+lox/lox mice. Again, deletion of the single Insig1 gene in the respiratory epithelium did not cause detectable changes in lung by histology and Oil Red O staining (data not shown). Altogether, these data indicate that deletion of Insig1 or Insig2 alone did not activate SREBP, suggesting that Insig1 or Insig2 can fully compensate for each other.

**Deletion of Both Insig1- and Insig2-induced SREBP Activity and Caused Lipid Accumulation in Alveolar Type 2 Cells—**Insig1/2Δ/Δ mice were produced in which Insig1 was deleted in the alveolar epithelium in addition to germ line deletion of Insig2. Insig1 mRNA was reduced by 72% in purified alveolar type 2 cells from Insig1/2Δ/Δ mice compared with Insig1+lox/lox/Insig2−/− mice. Western blot analysis demonstrated that activated SREBP1 levels were increased 2.2-fold, p = 0.021 (Fig. 1B). At 2 months of age, lung morphogenesis was generally preserved in Insig1/2Δ/Δ mice; however, a subset of alveolar type 2 cells were hypertrophic and contained large cytoplasmic inclusions containing neutral lipids as demonstrated by Oil Red O staining (Fig. 2A). The cells were stained with Nile Red, at a fluorescence concentration with neutral lipids, and by pro-S-P-C antibody, an alveolar type 2 cell-specific marker (Fig. 2B). At 2 months of age, 30.9 ± 2.6% of alveolar type 2 cells contained cytoplasmic neutral lipid inclusions in the lungs of Insig1/2Δ/Δ mice, compared with 1.75 ± 1.75% in Insig1+lox/lox/Insig2−/− mice (p = 0.049). At 6 months of age, 30.4 ± 4% of alveolar type 2 cells contained neutral lipid inclusions in Insig1/2Δ/Δ mice versus 3.04 ± 3.04% in Insig1+lox/lox/Insig2−/− mice (p = 0.034) (Fig. 2C).

**Ultrastructural Alterations of Alveolar Type 2 Cells in Insig1/2Δ/Δ Mice—**Although ultrastructural abnormalities were not seen in Insig1Δ/Δ or Insig1+lox/lox/Insig2−/− mice as compared with wild type mice, extensive ultrastructural changes were observed in alveolar type 2 cells in lungs from Insig1/2Δ/Δ mice (Fig. 3A) wherein large electron lucent lipid droplets were observed (Fig. 3, B and C). Normal, but smaller, lamellar bodies were present in the cytoplasm of lipid-laden cells. Type 2 epi-
thelial cells with normal morphology were also observed, perhaps indicating incomplete deletion of Insig1 in some cells. The morphology of pulmonary lipofibroblasts located in alveolar septa of Insig1/2Δ/Δ mice was unaltered (data not shown), and the ultrastructure of tubular myelin and other forms of secreted surfactant was not changed (data not shown) in Insig1/2Δ/Δ mice, consistent with the preservation of lung function.

Accumulation of Triacylglycerols and Cholesterol Esters in Lungs of Insig1/2Δ/Δ Mice—Although there was no difference in total PC concentration in either lung homogenate or isolated type 2 alveolar cells (Fig. 4, A and B), marked increases in cholesterol esters and triacylglycerols were observed in both fractions for Insig1/2Δ/Δ mice, consistent with the intracellular accumulation of Oil Red O-stained material seen in alveolar type 2 cells of these mice. By contrast, neither neutral lipid was increased in purified lamellar bodies, confirming minimal contamination of this subcellular preparation with lipid vesicles (Fig. 4C). Although triacylglycerol concentration was not elevated in BALF from Insig1/2Δ/Δ mice, cholesterol esters were significantly increased (Fig. 4D). Molecular species analysis demonstrated that BALF cholesterol ester composition was very different from that in serum, confirming a local lung synthetic origin in both Insig1/2Δ/Δ and Insig1flox/flox/Insig2 mice (results not shown). The presence of elevated cholesterol ester in BALF combined with its absence in lamellar bodies suggests that, at least in Insig1/2Δ/Δ mice, it may either be secreted independently from lamellar bodies or be subject to considerably decreased intra-alveolar catabolism and turnover.

Synthesis de Novo and Secretion of Phosphatidylcholine in Insig1/2Δ/Δ Mice—Lipid mass spectrometry analysis revealed significant quantitative and qualitative changes in lung phospholipid contents in Insig1/2Δ/Δ mice. Most strikingly, a 35% (p = 0.002) decrease in total PC was observed in BALF (Fig. 4D), in contrast to the unchanged total PC content of lung homogenate (Fig. 4A) and alveolar type 2 cells (Fig. 4B).
The fractional incorporation of \([\text{methyl-9-}^{2}\text{H}]\)choline (expressed as % total labeled + unlabeled PC) was unaltered in lung tissue, lamellar body, and alveolar type 2 cells but increased in BALF PC from \(\text{Insig1/2}^{-/}\) mice (Fig. 5A). As lamellar body and BALF PC have a precursor/product relationship, we used fractional incorporation of \([\text{methyl-9-}^{2}\text{H}]\)choline in lamellar body PC to calculate the apparent amount of PC secreted into the lung airspace over the 3-h incubation with label. Although the absolute values of this calculation need to be regarded with caution, as an accurate assessment of secretion would have required incorporation data from multiple time points, the apparent rate of secretion of lamellar body PC was unaltered in \(\text{Insig1/2}^{-/}\) compared with \(\text{Insig1}^{\text{flox/flox}}/\text{Insig2}^{-/}\) mice (Fig. 5B).

In the lungs, synthesis of PC is achieved by a combination of biosynthesis de novo following the Kennedy pathway, with the enzyme choline phosphotransferase (Chpt1) catalyzing the incorporation of CDP-choline with diacylglycerol or by acyl remodeling of unsaturated PC species by two concerted reactions as follows: decylation of unsaturated PC by a phospholipase \(A_{2}\), followed by reacylation of the resultant 1-palmitoyl-2-lysophosphatidylcholine (lyso-PC) with palmitoyl-CoA by lysophosphatidylcholine (lyso-PC) acyltransferase 1 (Lpcat1) (24). In addition, the final composition of secreted surfactant PC is also modulated by the ABCA3-dependent selection of PC species into lamellar bodies (25), and also by the specificity of the ABCA1-dependent basolateral secretion of unsaturated PC species from type II alveolar epithelial cells (26). Consequently, the pattern of incorporation of \([\text{methyl-9-}^{2}\text{H}]\)choline into lung PC molecular species will reflect the net sum of all these activities. This pattern was essentially identical to the endogenous PC compositions of whole lung, alveolar type II cells, and lamellar bodies, suggesting that newly synthesized PC in all three compartments was at equilibrium composition with endogenous PC by 3 h (27). By contrast, the composition of newly synthesized PC secreted into BALF was, for both \(\text{Insig1}^{\text{flox/flox}}/\text{Insig2}^{-/}\) and \(\text{Insig1/2}^{-/}\) mice, enriched in unsaturated PC species containing 18 carbon atoms or greater, compared with the total BALF PC composition (Fig. 5C). A proportion of newly synthesized surfactant PC is secreted before the processes of acyl remodeling and completion of species selection, suggesting a similar initial elevated fractional synthesis of long chain unsaturated PC species in both groups of mice. The major difference caused by deletion of Insig1/2 was the increased fractional content and label incorporation of PC16:0/16:1 in BALF PC from \(\text{Insig1/2}^{-/}\) mice at the expense of decreased content and incorporation into PC16:0/16:0. As lamellar bodies can select and package PC16:0/16:1 equally well as PC16:0/16:0 (24), this contrasting specificity suggests that the altered PC composition in \(\text{Insig1/2}^{-/}\) mice was possibly due to altered specificity of acyl remodeling related to increased substrate CoA16:1 as a consequence of increased expression of Scd1 (Fig. 7B). Altered activity of the acyl remodeling pathway would be expected to alter the kinetics of lyso-PC labeling, but neither the concentration nor the \([\text{methyl-9-}^{2}\text{H}]\)choline labeling of lyso-PC in lung tissue were altered by Insig deletion (data not shown). Although BALF PC16:0/16:0 was decreased, lung mechanics were similar in 6-month-old \(\text{Insig1/2}^{-/}\) mice and...
Insig1\textsuperscript{flx/flx}/Insig2\textsuperscript{−−/−} mice (data not shown), indicating maintenance of surfactant activity.

RNA Microarray Analysis Revealed Activation of Fatty Acid and Cholesterol Biosynthetic Pathways in Insig1/2\textsuperscript{Δ/Δ} Alveolar Type 2 Cells—RNA microarray analysis of purified alveolar type 2 cells from 8-week-old Insig1/2\textsuperscript{Δ/Δ} mice has been submitted to Gene Expression Omnibus, accession number GSE 31797. We identified 650 mRNAs that were significantly altered ($p < 0.05$ and a fold change of $>1.5$) in comparison with Insig1\textsuperscript{flx/flx}/Insig2\textsuperscript{−−/−} mice. Of these, 181 mRNAs were significantly decreased and 469 induced (Fig. 6A). Of the 469 induced mRNAs, 99 were related to lipid metabolism. mRNAs encoding proteins regulating fatty acid synthesis, acyl chain elongation, and cholesterol biosynthesis were increased in alveolar type 2 cells from the Insig1/2\textsuperscript{Δ/Δ} mice. Approximately 100 mRNAs related to inflammatory processes were increased in the Insig1/2\textsuperscript{Δ/Δ} mice (Fig. 10 and supplemental Table 2). Decreased expression of Insig1 and increased expression of Hmgs1, Fasn, and Stard4 were verified by RT-PCR (Fig. 6B). Consistent with RNA microarray data, Western blot analyses (Fig. 6C) indicated that FASN was significantly increased in Insig1/2\textsuperscript{Δ/Δ} lungs (9.5-fold increase, $p = 0.0004$). Although LPCAT1 activity was not measured, LPCAT1 mRNA was not altered ($p = 0.38$), consistent with preservation of acyl chain remodeling seen in the analysis of lung lipids.

According to Gene Ontology classification, the biological processes most induced by deletion of Insig1/2 in type 2 cells were related to “immunity and defense,” “lipid, fatty acid, and steroid metabolism,” “macrophage-mediated immunity,” “cytokine- and chemokine-mediated signaling pathway,” “T-cell mediated immunity,” and “cholesterol metabolism.”

mRNA expression profiles from Insig1/2\textsuperscript{Δ/Δ} mice were compared with those in which SREBP was inhibited by deletion of Scap in type 2 cells (12). We identified 30 mRNAs that were both induced by Insig deletion and decreased by Scap deletion (supplemental Table 2). These genes were highly enriched in pathways regulating lung “lipid metabolism,” e.g. fatty acid biosynthesis, metabolism, steroid synthesis, and liver X receptor/RXR activation, many of which are regulated by SREBP in liver or lung, consistent with the important role of the SREBP pathways in lipid metabolism. A number of genes in this list, including Abca1, Fasn, Fdft1, Hmgs1, Lpin1, Rora, Srebf1, and Steap2, are known to be associated with dyslipidemia, a disorder of high cholesterol and high blood triglycerides and hypercholesterolemia.

Gene Expression Profile of Alveolar Type 2 Cell Subpopulations—Because ultrastructural studies identified subsets of cells that either were lipid-laden or normal in appearance, subpopulations of alveolar type 2 cells were sorted according to their neutral lipid content (Fig. 7A). Insig1/2\textsuperscript{Δ/Δ} neutral-lipid
positive cells (NL) lacked Insig1 mRNA, whereas neutral lipid-negative (NL) cells expressed Insig1 at levels similar to Insig1^{flox/flox}/Insig2^{-/-} alveolar type 2 cells (Fig. 7B), indicating that Insig1 was not deleted in the NL cells. Consistent with SREBP activation in NL cells, levels of mRNAs implicated in neutral lipid, phospholipid, and surfactant apoprotein synthesis were considerably increased in NL cells compared with either NL cells or normal alveolar type 2 cells from Insig1^{flox/flox}/Insig2^{flox/flox} mice (Fig. 7C). A number of mRNAs were induced in NL alveolar type 2 cells in the Insig1^{flox/flox}/Insig2^{flox/flox} mice, perhaps indicating an adaptive response to the induction of lipid synthesis caused by deletion of Insigs. These mRNAs included Fabbp4 (a lipid transporter), Lipa (a lipid catabolism enzyme), and pro-inflammatory molecules Cxcl3, Ccl6, and Il1b (Fig. 7D).

Analysis of the promoter regions of the group of genes induced in the NL versus NL cells identified SREBP as the most enriched common regulatory cis-element in the NL (Insig1/2^{flox/flox}) cells (p = 3.17E-13), whereas the promoters of genes induced in NL (nondeleted) cells were enriched in common cis-elements for peroxisome proliferator-activated receptor/RXR (p = 5.51E-04) and TR4 (NR2C2, p = 2.73E-05), a member of nuclear receptor subfamily 2 known to inhibit peroxisome proliferator-activated receptor a/RXRα (27). The genes altered in NL cells are likely to represent direct effects of insulin-induced gene/SREBP. In contrast, mRNAs induced in NL type II cells may represent compensatory responses regulated in part via peroxisome proliferator-activated receptor/RXR signaling.

Differentially expressed genes identified after Insig1/2 deletion were similar to those in lungs of lysosomal acid lipase (Lipa) knock-out mice (28). LIPA hydrolyzes triglycerides and cholesterol esters in lysosomes. Phenotypic changes in Insig1/2^{flox/flox} mice share similarities with the Lipa^{-/-} mice, including accumulation of triglycerides and cholesterol esters in type II

**Figure 4. Concentrations of PC, triacylglycerol (TAG), and cholesterol ester (CE).** Lipid concentrations are shown for lavaged lungs (A), isolated alveolar type 2 cells (B), isolated lamellar body preparations (C), and BALF from Insig1^{flox/flox}/Insig2^{-/-} (open bars) and Insig1/2^{flox/flox} mice (closed bars) (D) *p < 0.05. n = 6. E, precursor scan of m/z 369 shows that BALF and serum BALF cholesterol ester compositions were distinct, indicating that BALF cholesterol ester was not blood-derived. F demonstrates that decreased PC16:0/16:0 was the major contributor to the lower total BALF PC concentration in Insig1/2^{flox/flox} mice, n = 6, p < 0.05.
cells, progressive inflammation, foamy macrophages, lung remodeling, and epithelial hyperplasia (29). In humans, mutation of LIPA causes Wolman disease, a cholesterol ester storage disease in which cholesterol esters and triglycerides accumulate in most tissues (30). In this study, Lipa mRNA was significantly reduced in NL+ and induced in NL− cells, correlating with Insig1 mRNA levels in the two subpopulations of type II cells. We speculate that reduction of Lipa mRNA contributes to the accumulation of cholesterol esters and triglycerides in Insig1/2-deficient type II cells. The expression of mRNAs encoding a number of lipid transport proteins was increased in Insig1Δ/Δ/Insig2−/− alveolar type 2 cells, including Abca3, Abcg1, Abca1, Stard4, and Stard2 (supplemental Table 2A) (31–33).

Inflammation in Lungs of Insig1/2Δ/Δ Mice—Although inflammatory infiltrates were not observed in lung sections of 2-month-old Insig-deficient mice, mRNA levels of many inflammatory response genes were modestly induced in type II epithelial cells isolated from the mice (Fig. 6 and supplemental Table 2). RNAs associated with the IL-12 signaling pathway were modestly but significantly induced, including Il12b, Irf8, Stat4, and Stats5a mRNAs. Increased expression of mRNAs encoding a number of cytokines, chemokines, and associated receptors and transcriptional regulators was observed, consistent with a proinflammatory environment caused by deletion of Insig1/2. At 6 months of age, focal inflammatory lesions were evident in the lungs of Insig1/2Δ/Δ but not in Insig1Δ/Δ/Insig2−/− mice. Accumulation of foamy and multinucleated macrophages was observed in the alveoli and interstitium in 67% of Insig1/2Δ/Δ mice (p = 0.016, χ2 test, see Fig. 8A). Lymphocyte infiltration was shown by CD3 immunostaining in 40% of Insig1/2Δ/Δ mice (p = 0.078, χ2 test, see Fig. 8B). Moreover, inflammatory changes were accompanied by alveolar type 2 cell hyperplasia in Insig1/2Δ/Δ mice, as shown by pro-SP-C immunostaining (Fig. 8C). Extensive pulmonary pathology was observed in older Insig1/2-deficient mice (8–9 months), and alveoli were filled with lipids, foamy macrophages, and cholesterol crystals (Fig. 8D).

Numbers of BALF cells were markedly increased in Insig1/2Δ/Δ compared with Insig1Δ/Δ/Insig2−/− mice at 2 and 6 months of age (Fig. 9A and data not shown). BALF cells were predominantly (99%) monocytes/macrophages in both genotypes. At 2 and 6 months of age, lipid-laden macrophages as shown by Oil Red O staining were increased (Fig. 9B). The atypical alveolar macrophages stained for anti-arginase1, a marker of alternative macrophage activation, in Insig1/2Δ/Δ mice (14.8 ± 4%) compared with controls (3 ± 1.9%, p = 0.028, Fig. 9C), and expressed high levels of Arg1 mRNA but low levels of Nos2 mRNA (Fig. 9D). BALF PGE2, IL4, IL10, IL13, and IFN-γ levels were similar in both groups of mice (data not shown).

FIGURE 5. Lipid concentrations and incorporation of [methyl-9-2H]choline and secretion of BALF PC. A, fractional incorporation of [methyl-9-2H]choline into lavaged lungs, purified lamellar bodies, and isolated alveolar type 2 cells was unaltered in Insig1/2Δ/Δ mice (closed bars) compared with Insig1Δ/Δ/Insig2−/− (open bars) but was significantly increased in BALF PC. B, correcting the incorporation of [methyl-9-2H]choline in BALF PC for the enrichment of stable isotope label in the substrate lamellar body pool suggested the rate of PC secretion into BALF was unaltered in Insig1/2Δ/Δ mice, n = 7, *p < 0.05. C, comparison of the molecular species compositions of [methyl-9-2H]choline incorporation into BALF PC (closed bars) with endogenous PC compositions (open bars) demonstrated that there was an initial synthesis of unsaturated species with fatty acyl groups containing 18 or more carbon atoms (Unsat PC) and increased acyl remodeling to PC16:0/16:1 compared with PC16:0/16:0 in the Insig1/2Δ/Δ mice.
Concentrations of cytokines and chemokines associated with inflammation in diabetes and obesity were assessed in lung homogenates at 8 weeks of age (Fig. 9F). IL-6 and keratinocyte chemoattractant concentrations were significantly increased in the Insig1/- deleted mice, consistent with the inflammation seen histologically. Likewise, IL-1B and IL-12B mRNAs in alveolar macrophages isolated from BALF were significantly increased at this age (Fig. 9G).
DISCUSSION

Conditional deletion of Insig1 in type 2 alveolar epithelial cells in combination with germ line deletion of Insig2 1) caused constitutive activated SREBP activity in type II alveolar epithelial cells, 2) resulted in the accumulation of neutral lipids in type II epithelial cells, alveolar macrophages, and alveoli, and 3) caused chronic pulmonary inflammation and alveolar remodeling. Despite dramatic changes in lipid homeostasis, surfactant function was preserved in Insig1/2 Δ/Δ mice, supporting the presence of compensatory mechanisms that preserve pulmonary surfactant despite activation of SREBP and marked accumulation of neutral lipids. The present findings demonstrate an important role for Insig1/2 and SREBP activation in the regulation of lipid synthesis in alveolar type II cells. The disruption of
alveolar lipid homeostasis caused by deletion of Insig1/2 and activation of SREBP1 resulted in progressive lung inflammation and alveolar remodeling related to lipotoxicity.

The finding that conditional deletion of either Insig1 in alveolar cells in the lung or germ line deletion of Insig2 alone was not sufficient to significantly alter lipid homeostasis in the lung supports concepts regarding the substantially redundant functions of Insig1 and Insig2 genes previously described in liver metabolism (13). In alveolar type 2 cells bearing deletions of both Insig genes, increased nuclear SREBP1 was associated with increased expression of known SREBP target mRNAs such as Hmgs1, Hmger, Ldlr, Acaca, and Fasn (34). Despite SREBP activation and abnormal lipid accumulation, surfactant function was maintained. The expression of mRNAs encoding a number of lipid transport proteins was increased in Insig1<sup>Δ/Δ</sup>/Insig2<sup>−/−</sup> alveolar type 2 cells, including Abca3, Abcg1, Abca1, Stard4, and Stard2 (supplemental Table 2) (31–33, 35), which may represent a direct response of SREBP activation to handle the increased lipid accumulation. The mRNAs regulating lipid biosynthetic pathways activated by Insig deletion in the lung were similar to those induced by Insig deletion in the liver; nevertheless, quantitative differences were evident between the two organs. In the liver, Insig deletion led to moderate increases in mRNAs coding enzymes involved in cholesterol synthesis (Hmgs1, 1.2-fold; Hmger, 2-fold; Ldlr, 1.2-fold), whereas mRNAs coding enzymes involved in fatty acid synthesis were markedly increased (Abca2, 2.7-fold; Fasn, 4.5-fold). A distinct expression profile was observed after Insig1/2 deletion in the lung, as mRNAs encoding protein in both cholesterol and phospholipid biosynthetic pathways were similarly but modestly induced. These results show that transcriptional responses to SREBPs follow organ-specific patterns. Fig. 10 provides a schematic of a proposed gene network representing changes in mRNAs regulating lipid homeostasis and immune response that were influenced by Insig1/2 deletion in alveolar type II cells.

Although Insig1 mRNA was reduced by 70% in type 2 cells in Insig1/2-deficient mice, heterogeneity in type 2 cell morphology and the cell sorting studies indicated that Insig1 was incompletely targeted in a subset of type 2 cells. Neutral lipids did not accumulate in the nontargeted cells as indicated by cell sorting with Nile Red. In Insig1/2-deleted cells, genes regulating the lipid synthetic pathways (e.g. Abca2, Fasn, Scd1, Elolv1, Hmger, and Ldlr) were substantially increased; likewise, in Insig1/2-deficient cells, mRNAs associated with phospholipid synthesis and surfactant proteins were modestly increased. Conversely, the mRNAs in nontargeted type 2 cells were generally unchanged, although Fabp4, Lipa, Cxcl3, Il1b, and Ccl6 were induced in the nontargeted cells, indicating a potential compensatory response by type 2 cells with remaining Insig1 activity. The induction of surfactant proteins in the Insig1/2-deficient cells is consistent with their regulation by SREBP (12) and may indicate a cell autonomous response to SREBP activation. Likewise, induction of Foxa2 and Cebpα (Fig. 7), genes known to regulate surfactant lipid and protein synthesis in the developing lung, indicates a potential compensatory response by Insig-deleted alveolar type 2 cells. Thus, findings from the mRNA microarray of the total lungs from the Insig1/2-targeted mice are likely to represent the integration of direct and indirect compensatory responses in targeted and nontargeted cells, as well as by other cells within the lung following activation of SREBP. The interactions of targeted and nontargeted type 2 cells may serve to maintain surfactant function despite significant neutral lipid accumulation in the model.

The activation of lipogenic pathways in the lungs of Insig1<sup>Δ/Δ</sup>/Insig2<sup>−/−</sup> mice resulted in the accumulation of neutral lipid species shown morphologically by Oil Red O and Nile Red staining and
by mass spectrometry. Both triglycerides and cholesterol ester were increased in lung tissue but not in lamellar bodies isolated by differential centrifugation from the Insig-deleted mice. Combined with the unaltered triacylglycerol profile in BALF, these results support the presence of distinct pathways organizing neutral lipid storage and lamellar body formation in alveolar type 2 cells. Furthermore, the increased cholesterol ester concentration in BALF but not in lamellar bodies (Fig. 4D) also suggests that neutral lipids in BALF have a different intracellular origin in type 2 alveolar cells other than from surfactant lamellar bodies. The accumulation of triglycerides in the lungs of Insig-deficient mice was not associated with increases in the expression levels of Dgat1 or Dgat2 in alveolar type 2 cells, the enzymes mediating triglyceride synthesis.

In sharp contrast to mice in which other members of the transcriptional network controlling alveolar type 2 cell surfactant lipid homeostasis, e.g. Foxa2, Nfatc3, Cebpa, and Nkx2–1, were deleted or mutated in the respiratory epithelium (36–39), deletion of Insig genes and activation of SREBP did not impair lung formation and maturation. Surfactant function was maintained despite increased SREBP activity and its activation of nonsurfactant lipogenic pathways. The distinct gene expression profiles seen in the subpopulation of deleted and nondeleted alveolar type 2 cells in Insig1/2Δ/Δ mice supports metabolic compensation within and between type 2 cells. Increased numbers and associated lipid catabolic activity of alveolar macrophages in the Insig-deficient mice likely contribute to the increased lung lipid clearance in Insig1/2Δ/Δ mice, consistent
Pulmonary Lipotoxicity

with the important role of alveolar macrophages in lung lipid homeostasis. The unaltered surfactant PC content in lungs of \textit{Insig1/2}\textsuperscript{\textDelta} mice was consistent with preserved expression of proteins known to be essential for lamellar body formation and secretion, for example \textit{Sftpb} or \textit{Abca3}, the latter being induced by deletion of \textit{Insigs}.

The focal accumulation of giant foamy multuncleated macrophages in the alveolar spaces and airspace remodeling was a striking feature of older \textit{Insig1/2}\textsuperscript{\textDelta} mice. This phenomenon is also observed in other models of lung lipid overload, as seen in mice bearing deletion of the \textit{Sftpd} (40) or \textit{Abcg1} (41) genes and in mice with mutations of the \textit{Hps1} or \textit{Ap3} genes that model the Hermanski-Pudlak syndrome in humans (42).

Lipid accumulation and pulmonary infiltration with lipid-laden macrophages increased with age in the \textit{Insig}-deleted mice. In \textit{Insig1/2}\textsuperscript{\textDelta} mice, alveolar macrophages had morphological and functional characteristics consistent with macrophage activation. IL-6 and keratinocyte chemoattractant levels were increased in lung tissue, consistent with the mRNA microarray data from alveolar type II cells isolated from the \textit{Insig1/2}\textsuperscript{\textDelta} mice. Pathway enrichment analysis supports the concept that macrophages were activated via an IL-12/STAT4-dependent signaling pathway.

Changes in expression of pro-inflammatory cytokines and chemokine-associated pathways in lungs from \textit{Abcg1}\textsuperscript{-/-} mice are similar to those induced by deletion of \textit{Insig1/2}, including increased mRNAs encoding Ccl 5–9,17,22, Cxcl 3,5, and chemokine receptors Ccr 5–7, Infg, and Tgfb1. \textit{In vitro} loading of human monocyte-derived macrophages with cholesterol directly induces the expression of IL-1\beta (43). Thus, in the present model, excessive synthesis of cholesterol and other neutral lipids in the type II epithelial cells caused lipid accumulation in both epithelial cells and alveolar macrophages. Although lipid synthetic pathways were induced in \textit{Insig1/2}-deleted alveolar type II cells, Cxcl3, Ccl6, and IL-1\beta mRNAs were induced in the nondepleted cells and in alveolar macrophages (Fig. 7), indicating that both alveolar macrophages and epithelial cells likely contribute to the inflammation and remodeling seen in the \textit{Insig1/2}-deleted mice. Fig. 10 provides a proposed gene network linking changes in lipogenesis and lung inflammation observed in the \textit{Insig1/2}-deleted mice.

Pulmonary inflammation and lung dysfunction is associated with diabetes mellitus, obesity, and in lipid storage disorders (14). Likewise, lung dysfunction, inflammation, and remodeling are associated with lipid droplet accumulation in lungs of diabetic Zucker rats (44). Taken together, the accumulation of neutral lipids related to enhanced lung lipid synthesis or content may represent a form of pulmonary lipotoxicity similar to that associated with obesity and diabetes (45–46).

REFERENCES

1. Whitsett, J. A., Wert, S. E., and Weaver, T. E. (2010) Alveolar surfactant homeostasis and the pathogenesis of pulmonary disease. \textit{Annu. Rev. Med.} \textbf{61}, 105–119

2. van Moorsel, C. H., van Oosterhout, M. F., Barlo, N. P., de Jong, P. A., van der Vis, J. I., Ruven, H. J., van Es, H. W., van den Bosch, J. M., and Grutters, J. C. (2010) Surfactant protein C mutations are the basis of a significant portion of adult familial pulmonary fibrosis in a dutch cohort. \textit{Am. J. Respir. Crit. Care. Med.} \textbf{182}, 1419–1425

3. Bullard, J. E., Wert, S. E., Whitsett, J. A., Dean, M., and Nogee, L. M. (2005) ABCA3 mutations associated with pediatric interstitial lung disease. \textit{Am. J. Respir. Crit. Care. Med.} \textbf{172}, 1026–1031

\textbf{FIGURE 10.} mRNAs induced in type 2 cells from \textit{Insig1/2}\textsuperscript{\textDelta} mice were functionally enriched in the lipid metabolism and immune/inflammatory response. The regulatory relationships linking the two categories of genes were identified through literature mining using Ingenuity knowledge base. Red and green nodes represent the genes induced or repressed in \textit{Insig1/2}\textsuperscript{\textDelta} versus control, respectively. Triangular nodes are genes induced in \textit{Insig1/2}\textsuperscript{\textDelta} and repressed in \textit{Scap}\textsuperscript{\textDelta} mice (22). Color range indicates the relative fold change (the darker the color, the larger the fold change).
surfactant phospholipid homeostasis in normal lung and during endotoxin-
mediated lung injury. J. Appl. Physiol. 104, 1753–1760
24. Bridges, J. P., Ikegami, M., Brilli, L. L., Chen, X., Mason, R. J., and Shannon,
J. M. (2010) LP-CAT1 regulates surfactant phospholipid synthesis and is
required for transitioning to air breathing in mice. J. Clin. Invest. 120,
1736–1748
25. Whitsett, J. A. (2010) Review. The intersection of surfactant homeostasis
and innate host defense of the lung: lessons from newborn infants. Innate
Immunity, 16, 138–142
26. Agassandian, M., Mathur, S. N., Zhou, J., Field, F. J., and Mallampalli, R. K.
(2004) Oxysterols trigger ABCA1-mediated basolateral surfactant efflux.
Am. J. Respir. Cell. Mol. Biol. 31, 227–233
27. Yan, Z. H., Karam, W. G., Staudinger, J. L., Medvedev, A., Ghanayem, B. L.,
and Jetten, A. M. (1998) Regulation of peroxisome proliferator-activated
receptor α-induced transactivation by the nuclear orphan receptor
TAK1/TR4. J. Biol. Chem. 273, 10948–10957
28. Lian, X., Yan, C., Qin, Y., Knox, L., Li, T., and Du, H. (2005) Neutral lipids
and peroxisome proliferator-activated receptor-γ control pulmonary
gene expression and inflammation-triggered pathogenesis in lysosomal
acid lipase knockout mice. Am. J. Pathol. 167, 813–821
29. Lian, X., Yan, C., Yang, L., Xu, Y., and Du, H. (2004) Lysosomal acid lipase
deficiency causes respiratory inflammation and destruction in the lung.
Am. J. Physiol. Lung Cell. Mol. Physiol. 286, L801–L807
30. Du, H., Sheriff, S., Bezerra, J., Leonova, T., and Grabowski, G. A. (1998)
Molecular and enzymatic analyses of lysosomal acid lipase in cholesteryl
ester storage disease. Mol. Genet. Metab. 64, 126–134
31. Wong, J., Quinn, C. M., and Brown, A. J. (2006) SREBP-2 positively regu-
lates transcription of the cholesterol efflux gene, ABCA1, by generating
oxysterol ligands for LXR. Biochem. J. 400, 485–491
32. Soccio, R. E., Adams, R. M., Romanowski, M. J., Sehayek, E., Burley, S. K.,
and Breslow, J. L. (2002) The cholesterol-regulated StarD4 gene encodes a
STAR-related lipid transfer protein with two closely related homologues,
StarD5 and StarD6. Proc. Natl. Acad. Sci. U.S.A. 99, 6943–6948
33. Ecker, J., Langmann, T., Moehle, C., and Schmitz, G. (2007) Isomer-spe-
cific effects of conjugated linoleic acid on macrophage ABCG1 transcrip-
tion by a SREBP-1c-dependent mechanism. Biochem. Biophys. Res. Com-
mun. 352, 805–811
34. Horton, J. D., Shah, N. A., Warrington, J. A., Anderson, N. N., Park, S. W.,
Brown, M. S., and Goldstein, J. L. (2003) Combined analysis of oligonu-
cleotide microarray data from transgenic and knockout mice identifies
direct SREBP target genes. Proc. Natl. Acad. Sci. U.S.A. 100, 12027–12032
35. Besnard, V., Xu, Y., and Whitsett, J. A. (2007) Sterol-response element-
direct SREBP target genes. Proc. Natl. Acad. Sci. U.S.A. 100, 12027–12032
36. Martis, P. C., Whitsett, J. A., Xu, Y., Perl, A. K., Wan, H., and Ikegami, M.
(2006) C/EBPa is required for lung maturation at birth. Proc. Natl. Acad. Sci.
U.S.A. 101, 14449–14454
37. DeFelice, M., Silberschmidt, D., DiLauro, R., Xu, Y., Wert, S. E., Weaver,
T. E., Bachurski, C. J., Clark, J. C., and Whitsett, J. A. (2003) TTF1-Phos-
phorylation is required for peripheral lung morphogenesis, perinatal sur-
vival, and tissue-specific gene expression. J. Biol. Chem. 278,
35574–35583
38. Davy, V., Chilad, T., Xu, Y., Ikegami, M., Besnard, V., Maeda, Y., Wert,
S. E., Neilson, J. R., Crabtree, G. R., and Whitsett, J. A. (2006) Calcineurin/
Nfat signaling is required for perinatal lung maturation and function.
J. Clin. Invest. 116, 2597–2609
39. Wert, S. E., Yoshida, M., LeVine, A. M., Ikegami, M., Jones, T., Ross, G. F.,
Fisher, J. H., Korfhang, T. R., and Whitsett, J. A. (2000) Increased metal-
lipoproteinase activity, oxidant production, and emphysema in surfactant
protein D gene-inactivated mice. Proc. Natl. Acad. Sci. U.S.A. 97,
5972–5977
40. Balád, Á., Gomes, A. V., Ping, P., and Edwards, P. A. (2008) Loss of
ABCG1 results in chronic pulmonary inflammation. J. Immunol. 180,
3560–3568
42. Lyerla, T. A., Rusiniak, M. E., Borchers, M., Jahreis, G., Tan, J., Ohtake, P., Novak, E. K., and Swank, R. T. (2003) Aberrant lung structure, composition, and function in a murine model of Hermansky-Pudlak syndrome. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **285**, L643–L653

43. Feng, Y., Schreiner, G. F., Chakravarty, S., Liu, D. Y., and Joly, A. H. (2001) Inhibition of the mitogen-activated protein kinase, p38alpha, prevents proinflammatory cytokine induction by human adherent mononuclear leukocytes in response to lipid loading. *Atherosclerosis* **158**, 331–338

44. Foster, D. J., Ravikumar, P., Bellotto, D. J., Unger, R. H., and Hsia, C. C. (2010) Fatty diabetic lung. Altered alveolar structure and surfactant protein expression. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **298**, L392–L403

45. Kodolova, I. M., Lysenko, L. V., and Saltykov, B. B. (1982) Changes in the lungs in diabetes mellitus. *Arkh. Patol.* **44**, 35–40

46. Reinila, A., Koivisto, V. A., and Akerblom, H. K. (1977) Lipids in the pulmonary artery and the lungs of severely diabetic rats. A histochemical and chemical study. *Diabetologia* **13**, 305–310