Perinatal expression of genes that may participate in lipid metabolism by lipid-laden lung fibroblasts

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Abstract Although a morphologically distinct population of lipid-laden interstitial cells (lipofibroblasts, LF), has been identified, the origins and functions of this population during lung development and disease remain undefined. Illumination of the developmental and functional characteristics of two other populations of lipid-laden mesenchymal cells, namely adipocytes and hepatic lipocytes, has fashioned tools that can be used to explore similar properties in pulmonary LFs. As the LF is transiently a very abundant cell in the perinatal lung, we elected to study the perinatal ontogeny of the expression of several genes that are involved in the acquisition of lipids by adipocytes, and may be involved in promoting the triglyceride accumulation that is the morphologic hallmark of the pulmonary LF. We found that the maximal expression of peroxisome proliferator-activated receptor-gamma (PPAR-γ), at gestational day 21 in the LF, precedes the rise at birth, in the expression of genes that are involved in the hydrolysis of triglycerides at the plasma membrane (lipoprotein lipase, LPL), transport of fatty acids across the plasma membrane (fatty acid transporter, FAT) and in the cytoplasm (adipocyte lipid binding protein, ALBP). The steady-state levels of LPL, FAT, and ALBP mRNAs that were isolated from whole lung tissue showed a similar temporal pattern. The levels of the protein products of the LPL and ALBP genes changed in tandem with those of their precursor mRNAs in the LF, suggesting that these gene products are under pre-translational control. These findings indicate that characteristic adipocyte genes are also expressed in lipid-laden pulmonary fibroblasts and may participate in triglyceride accumulation and metabolism by these cells.—Chen, H., S. Jackson, M. Doro, and S. McGowan. **Perinatal expression of genes that may participate in lipid metabolism by lipid-laden lung fibroblasts.** *J. Lipid Res.* **1998.** 39: **2483–2492.**

Supplementary key words alveolar type 2 pneumocytes • surfactant • peroxisome proliferators • tropoelastin • elastin • lung development

As early as 1970, a morphologically distinct, lipid-laden interstitial cell was observed in the lungs of fetal and neonatal rats (1). The distinguishing structural and biochemical characteristics of this cell were detailed in the late 1970s and early 1980s, when it was named the lipid interstitial cell (which we will refer to as the lipofibroblast, LF) and its characteristics have recently been reviewed (2). Based on their lipid contents, pulmonary interstitial cells have been divided into two populations, the lipofibroblast and the non-lipid interstitial cell that lacks the characteristic lipid droplets and is located more peripherally in the alveolar septum. The LF is a mesenchymal cell with fibroblastic characteristics and can first be identified by the presence of its characteristic lipid droplets that appear on gestational day 16 in the rat and regress during the third postnatal week. Pregnancy terminates at gestational day 22 in the rat, which will be termed postnatal day 0 (P−0). Therefore, gestational day 16 is postnatal day −6 or P−6 . While some of the lipids may be acquired from plasma triglycerides, the LF appears to independently regulate the accumulation of lipids, as the volume density of droplets does not directly correlate with the perinatal levels of plasma triglycerides in the rat (3). The droplets are a major source of lung triglycerides which increase 7.5-fold between gestational days 16 (P−6) and postnatal day 1, and continue to increase at a slower rate during the first postnatal week. The droplets contain neutral lipids, primarily triglycerides and cholesterol, and only 14% of the lipids are phospholipids. The decrease in the number of LFs prior to weaning results from a decrease in cell proliferation, which declines approximately 9-fold from postnatal day 4 to day 11 (4). Little is known about how LF acquire lipids in vivo, but information is available from studies conducted when the cells are cultured in medium containing triglyceride-rich neonatal rat serum. LF acquire triglycerides when they are presented as triolein, which is preferentially incorporated into intracellular lipid stores and can then be transferred to fetal type 2 alveolar epithelial cells (5). Other types of pulmonary cells such as the alveolar type II cell and the macrophage also directly acquire lipids during the perinatal period.

Abbreviations: LF, lipofibroblasts; LPL, lipoprotein lipase; FAT, fatty acid transporter; ALBP, adipocyte lipid binding protein; PPAR, peroxisome-proliferator-activated receptor; HSL, hormone sensitive lipase; RT-PCR, reverse transcription-polymerase chain reaction; FITC, fluorescein isothiocyanate.

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The LF shares several characteristics with the adipocyte. Both are mesenchymal cells and accumulate triglyceride-rich neutral lipid droplets. Both are absent until late gestation and their number increases markedly perinatally. Messenger RNAs and their proteins that are usually regarded as characteristic of adipocytes, such as lipoprotein lipase (LPL) and adipocyte lipid-binding protein (ALBP, the Ap2 gene product) could also be present in LF (6). The genes encoding both LPL and ALBP contain responsive elements for the nuclear receptor, peroxisome proliferator-activated receptor-gamma (PPAR-γ) which binds as a heterodimer with the retinoid-X receptor (7, 8). 15-Deoxy-Δ-12,14-prostaglandin J_2 has been identified as a natural ligand for PPAR-γ, although it may not be the exclusive natural ligand. Signaling through the PPAR-γ nuclear receptor is apparently able to execute the adipocyte differentiation program in vitro (9). The PPARs are members of the retinoid X-receptor (RXR) heterodimer family of the retinoid/steroid/thyroid hormone superfamily of ligand-activated nuclear receptors (10). In mammals, the PPAR subfamily contains two members in addition to PPAR-γ, namely PPARs-α, and -δ. PPAR-α is primarily expressed in the liver, intestine, and kidney and is activated by multiple ligands including eicosanoids and fatty acids (11). PPAR-α regulates the expression of several peroxisomal enzymes in the liver, particularly those involved in β-oxidation of fatty acids. The function of PPAR-δ, which is widely expressed and is sometimes referred to as the fatty acid activated receptor (FAAR), remains unestablished (10).

The specific functions of the LF during alveolar development remain incompletely understood. However, it is thought that it may supply lipids for surfactant production, the synthesis of membrane phospholipids, and/or energy metabolism, and that it participates in elastin synthesis. These processes all occur during the postnatal period of rapidly expanding alveolar surface area. The primary objective of this study was to define the ontogeny in LF of the expression of several genes that are regarded as adipocyte-specific, in order to lay a groundwork for future studies that may identify the functions of the LF. Because our longer term objective is to learn how fatty acids that are accumulated by LF may be transferred to other cells and be used for the synthesis of surfactant lipids and membrane phospholipids, we chose to study genes that are involved in the uptake, intracellular transport, and release of fatty acids from triglycerides (6, 12). Lipoprotein lipase, an extracellular lipase, is produced by adipocytes and LF and may be involved in the acquisition of fatty acids from the circulation. Fatty acid transporter (FAT), an intrinsic membrane protein, is involved in the transport of medium chain fatty acids into mesenchymal cells including cardiomyocytes and adipocytes and has a high level of similarity at the nucleotide level to CD36, an intrinsic plasma membrane protein that binds thrombospondin, transports fatty acids, and is involved in signal transduction (13, 14). ALBP is a cytoplasmic fatty acid binding protein that is abundantly expressed in adipocytes when they are accumulating triglycerides (12). Hormone sensitive lipase (HSL) is a neutral lipase, also contained in adipocytes, that promotes the hydrolysis of triglycerides during periods of fatty acid export (15). We have examined the steady-state levels of the mRNAs and their protein products for these genes, as well as the PPARs during late gestation and the first 3 postnatal weeks in rat lungs.

MATERIALS AND METHODS

Procurement of fetal and postnatal rat lungs and LF

Timed pregnant, specific pathogen-free, female Sprague-Dawley rats were obtained from Harlan-Sprague Dawley (Madison, WI) or bred within the Animal Care Unit at the VA Medical Center, Iowa City, IA and offered water and food ad libitum. The first day of gestation was assumed to be the day that sperm were identified by a vaginal swab. A hysterotomy was performed on gestational day 17 (P−5), 19 (P−3), or 21 (P−1) and the fetuses were removed and placed at 4°C in 0.145 m NaCl, 0.0015 m KH_2PO_4, 0.0027 m KCl, 0.0086 m Na_2HPO_4 (PBS). The fetal lungs were removed while visualized with a dissecting microscope and placed in Dulbecco's MEM containing 10 mm HEPES. A portion of the lungs was immediately frozen in liquid nitrogen and stored at −75°C and the remainder of the lung tissue was pooled, minced into 1- to 1.5-mm^3 pieces with a razor blade or scissors, and used to isolate LF using a procedure similar to that described previously for neonates (16). This procedure for neonates can be summarized as follows. After mincing, the lung tissue is digested with a mixture of collagenase, trypsin, and DNase for 2 h and the cell suspension is passed through a 10-ml pipet 10 times. The proteolytic enzymes are inactivated with bovine calf serum and the suspension is filtered through 125-mm mesh. The cells are collected after centrifugation at 300 *g* for 10 min. They are then overlaid onto a discontinuous gradient of Percoll with densities of 1.04, 1.06, and 1.07 g/ml and centrifuged at 400 *g* for 20 min. The LF comprise the least dense layer which sediments at 1.04 g/ml. The cell layer is collected, washed, counted, and viability is assessed by trypan blue exclusion. The procedure was altered for fetal lungs in that the enzyme concentrations were 3.25 mg/ml for trypsin, 1.0 mg/ml for collagenase, and 0.15 mg/ml for DNase, and the duration of the digestion was reduced to 70 min. The isolated LF were frozen as cell pellets in liquid N_2 and saved at −75°C until RNA or cytoplasmic proteins were isolated (16).

Characterization of homogeneity of the isolated LF cell population

Immunohistochemistry and freshly isolated LF were used to establish the purity of the LF–cell isolate. Cells isolated from rats at postnatal days 4, 8, 12, and 21 that sedimented in the Percoll gradient at a density of 1.04 g/ml were applied to a glass slide using a Cytospin centrifuge (Shandon Southern, Sewickley, PA) (16). The cells were fixed in 2% paraformaldehyde for 20 min at 4°C and subjected to immunostaining. To determine the percentage of cells that were mesenchymal or epithelial in origin, cells that contained vimentin (mesenchymal), von Willebrand's factor (VWF, endothelial), and cytokeratin (epithelial) were identified immunohistochemically. Anti-OX-41, a mouse monoclonal antibody, was used to recognize a 115 kDa antigen that is specific for rat alveolar macrophages (17). The other antibodies included mouse monoclonal anti-vimentin (clone V9, Sigma Chemical), mouse monoclonal anti-pan cytokeratin (clone C-11, Sigma Chemical), and rabbit-anti human Factor VIII related antigen (VWF, Sigma Chemical). The staining procedure involved blocking for 20 min with 1% goat serum in PBS containing 0.2% bovine serum albumin (BSA). A fluorescent second antibody was

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used to identify the OX-41 and anti-VWF reactive antigens and, in some cases, anti-vimentin. The OX-41 antibody or anti-vimentin (diluted 1:50 in blocking solution) or the anti-VWF (diluted 1:500) were applied for 2 h at room temperature. The slides were again washed and then incubated for 2 h with affinity-purified sheep anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugate $F(ab')_2$ (diluted 1:200, from Sigma Chemical Co.). The slides were viewed using an Olympus BX-40 microscope and a wide interference green filter for FITC. The fluorescent cells were counted and expressed as a percentage of the entire cell population that was visible using phase-contrast. A peroxidase reaction was used to identify cytokeratin and, in some instances, vimentin. The staining procedure was similar to that used for OX-41, except that the endogenous peroxidase was quenched before the blocking serum was applied. Anti-vimentin and anti-cytokeratin, diluted 1:50 and 1:400, respectively, were incubated with the LF isolate overnight at 4°C. The antigen–antibody complexes were detected using an biotin-linked anti-mouse IgG and the ABC anti-peroxidase method (Vectastain Elite kit, Vector Laboratories, Burlingame, CA) and diaminobenzadine as a substrate (18). The peroxidase products were identified using brightfield microscopy, after counterstaining with Gill's hematoxylin. In all cases, 300 cells were counted, the percentage of cells bearing the antigen of interest were identified, and the data were expressed as a percentage of total cells as defined under phase contrast or by hematoxylin staining. Cells containing neutral lipid droplets were identified as described previously using oil red-O (19).

Northern analyses of ALBP, LPL, FAT, HSL, and ribosomal phosphoprotein P-0

Total RNA was isolated from whole lung tissue or LF at various perinatal ages, using guanidine isocyanate, and was subjected to denaturing electrophoresis on 1.5% agarose following previously described procedures (16). Fifteen micrograms of total RNA was loaded in each lane and the filters were successively probed with the following cDNAs. Rat LPL and HSL cDNAs were obtained from the American Type Culture Collection (ATCC, Rockville, MD) repository. The plasmid containing the cDNA for ALBP (aP2) was obtained from Dr. Bruce Spiegelman (9). Plasmids containing cDNA from rat fatty acid transporter and ribosomal phosphoprotein P-0 (RP-0) were prepared using the reverse-transcriptase polymerase chain reaction (RT-PCR) (20). The primers for the fatty acid transporter were forward, TCCGCTCTAGATAACTGTACGTCGT, and reverse complement, CAGCCATCGATTCTTAGATCTGCAAG. The cloned cDNA constructs were sequenced using the dideoxy method to ensure that they represented the desired clones (16). The inserts were excised from the plasmids using *Xba I* and *Cla I*, resolved by electrophoresis on agarose gels, and purified using the Qiaex kit (Qiagen, Chatsworth, CA). The inserts were labeled using random priming and ^{32}P-dCTP and hybridized with the filters at 42°C (16). The filters were washed twice with 1 × SSC at 50°C for 45 min and then with 0.1 × SSC at 60°C for 10 min for HSL and three times with 1 × SSC at 50°C for LPL, FAT, and ALBP, and as described for RP-0 (20). Autoradiography and densitometry were performed as described previously (21). The densities of the bands representing mRNAs for LPL, FAT, and ALBP were normalized to the density of the band for RP-0 in the same sample of RNA. This allowed accommodation for inadvertent differences in the amounts of RNA loaded in the various lanes.

Ribonuclease protection assays (RPA) of PPAR mRNAs

RPA were used to quantify the steady-state levels of the mRNAs for PPAR-α, PPAR-γ, and PPAR-δ in lung tissue and LF that were isolated from rats at various gestational and postnatal ages. Specific segments of cDNA from each of the rat genes were cloned using RT-PCR and primers from the ligand binding domains, in regions of low degrees of similarity among the cDNA sequences for the three PPARs. The primers, which contain either *Xba I* or *Cla I* recognitions sites, were as follows: PPAR-α, forward GCAAACTGCAGGATTGTGCACGTGCT and reverse complement, ATCGGGGTACCAGGCCATCTGTGCAA; PPAR-γ, forward, TGAAACTGCAGTGGCCCACCAACTTCG and reverse complement, ATCGGGGTACCCAATCGGATGGTTCT; and PPAR-δ (FAAR) forward CTAGCTCTAGAGTGGCAGAGCTATGA and reverse complement TAGCCATCGATGAGCTTCATGCGGAT. Our RPA probe does not distinguish between the γ1 and γ2 isoforms, as it encompasses a portion of the ligand binding domain and not the 5′-terminus of the cDNA, where the alternative splicing occurs. The amplified cDNAs were subjected to restriction enzyme digestion with *XbaI* and *ClaI* and ligated into pBluescript SK-. The cloned cDNA constructs were sequenced using the dideoxy method to ensure that they represented the desired clones (16). After linearizing the plasmids with *Xba* I, the antisense cRNAs were synthesized using T7 viral RNA polymerase and an in vitro transcription kit from Boehringer Mannheim (Indianapolis, IN). The transcripts were subjected to denaturing polyacrylamide gel electrophoresis and the full-length transcripts were excised from the gel and eluted (16). The RPA was performed using the method described previously and the protected ^{32}P-cRNA species were resolved on 5%, 0.4-mm thickness, denaturing polyacrylamide gels. Yeast tRNA was used as a control for hybridization to RNA in a sequence-independent manner. Under the hybridization and RNase treatment conditions that were used, tRNA did not protect the probes from digestion. The gels were dried, autoradiograms were prepared and subjected to densitometry (16). The sizes of the protected mRNA species were 340, 362, and 403 bp, respectively, for PPAR-α, δ, and γ. The quantities of the various protected PPAR cRNAs were normalized to the quantity of β-actin mRNA for the corresponding RNA sample, to account for differences in the quantities of RNA that were assayed (16).

Enzymatic assays and immunoblotting to detect LPL, HSL, and ALBP proteins

As an adjunct to Northern analysis in the study of the expression of the LPL, HSL, and ALBP genes, the gene products were also analyzed at the protein level. Adipose tissue and LF cytoplasm were isolated, respectively, from adult rat epididymal fat and LF that had been isolated at various postnatal days as previously described. Adipose tissue and LF that had been previously frozen in liquid nitrogen and stored at −75°C were cooled in liquid nitrogen and then fractured, while still frozen, with a Polytron Homogenizer (Kinnematica, Littau, Switzerland). The fractured tissue was then homogenized in 0.25 m sucrose, 50 μm Tris-HCl, pH 7.0, 1 mm EDTA, 1 mm monothioglycerol, 20 μg/ml leupeptin, 1 μg/ml pepstatin. The homogenate was centrifuged at 109,000 *g* for 45 min at 4°C and the infranatant (below the cream layer and above the pellet) was carefully removed. The protein content of the cytoplasm-containing infranatant was assayed using Coomassie brilliant blue G (22). LPL enzymatic activity was analyzed using a [^{3}H]triglyceride substrate (23, 24). Bovine LPL (provided by Dr. David Chappell, University of Iowa) was used as a standard and the incubations with the substrate were conducted at 25°C for 90 min. The [^{3}H]oleic acid product was extracted and an aliquot of the alkalinized aqueous phase was subjected to liquid scintillation spectrometry (23). The cholesterol esterase activity of hormone sensitive lipase was assayed using [^{14}C]cholesterol oleate as a substrate (25). Adipocyte cytosol was used as a positive control and the activity was expressed as pmol of [^{14}C]oleate released per mg or

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protein per min. Western immunoblotting was used to analyze the immunoreactive levels of HSL and ALBP. SDS-PAGE was conducted and immunoblotting was performed as described previously using 10% cross-linked gels for HSL and 15% cross-linked gels for ALBP (26). Antibodies against the two proteins were as follows. Polyclonal anti-rat HSL IgG was a gift from Dr. Fredrick Kraemer (Stanford University) and anti-rat ALBP was a gift from Dr. David Bernlohr (University of Minnesota) (25, 26). Both had been obtained from rabbits and the second antibody was an affinity-purified peroxidase-linked goat anti-rabbit IgG (Sigma Chemical). The peroxidase reaction was developed using the enhanced chemiluminescence (ECL) kit produced by Amersham Corp. (Arlington Heights, IL). The filters were exposed to Kodak X-OMAT AR-5 film in the dark for varying lengths of time to obtain a fluorographic signal that was within the linear detection range of the film.

RESULTS

Characterization of the purity of the LF-cell isolate

Previous studies had shown that macrophages comprised approximately 10% of the cells in the lung digests of lungs removed at postnatal day 12 that sedimented at a density of 1.04 g/ml (and therefore were present in the fraction containing the LF) (16). In this study, we found that macrophages comprised 9.8% of the cell population from lungs at postnatal day 21 that sedimented at the same density in Percoll (LF cell isolate). More than 95% of the LF cell isolate contained vimentin, and the proportion of cells that stained with oil-red O (contained neutral lipid droplets) also exceeded 90% (**Table 1** and **Fig. 1**). The proportion of oil-red O-staining cells decreased at postnatal days 12 and 21, while the proportion of vimentin-positive cells at days 12 and 21 remained similar to that at postnatal days 4 and 8. The proportion of cells bearing the VWF antigen (endothelial cells) increased with age up to postnatal day 12 when it reached approximately 13%. The maintenance of a constant level of vimentin-containing cells with a decrease in oil-red O and concomitant increase in anti-VWF-positive cells is consistent with an increase in endothelial cells. Endothelial cells would be expected to contain vimentin but not neutral lipids. Only a small fraction (<2.4%) of cells contained cytokeratin at any age, consistent with the presence of very few epithelial cells in the LF cell isolate. In summary, at all ages, 90% or more of the cells contained oil-red O-staining droplets. At postnatal day 21, 12.7% and 9.8% of cells in the LF isolate contained VWF or the OX-41-antigen, respectively. Therefore, if one considers that oil-red O-staining is not limited to LF, at least 77% of the cells in the LF isolate have cytochemical characteristics of fibroblasts and all contain lipid droplets.

TABLE 1. Percentage of cells demonstrating a categorizing antigen or cytochemical property

Age	Vimentin	Oil-Red O	Pan Cytokeratin	von Willibrand's
postnatal day			%	
4	98.4	98.2	0.84	3.7
8	97.2	97.7	1.6	7.7
12	99.4	90.2	2.4	13.4
21	95.6	92.9	1.5	12.7

Percentages were based on a differential enumeration of 300 cells and are a single determination at each age.

ALBP, LPL, and FAT mRNA increase at birth in rat lungs

The steady-state levels of ALBP, LPL, FAT, and RP-0 mRNA in a representative Northern analysis of RNA from LF are shown in **Fig. 2**. The fluctuations in the level of RP-0 mRNA did not have a consistent age-related pattern and most likely reflect variations in the amounts of RNA loaded in the different lanes of the gel. Others have used RP-0 (also referred to as 36B4) to normalize for RNA loading differences (9). The results of this and three similar additional analyses of LF mRNA were combined and are shown in **Fig. 3**. A logarithmic ordinate scale was used to facilitate a visual comparison of FAT and LPL with ALBP, which generally had a lower radiographic density, relative to RP-0. **Figure 4** summarizes the results of a comparable analysis of RNA that was isolated from whole lung tissue at various perinatal ages. These data demonstrate that the maximal levels of the expression of these genes occur postnatally. In both the isolated LF and whole lung tissue, the quantity of FAT mRNA increases approximately 3- to 5-fold from gestational day 19 (P−3) to postnatal day 2. LPL mRNA increases 20-fold in LF and 3-fold in lung over the same period. The expression of both FAT and LPL remains relatively constant after postnatal day 2. In whole lung tissue, ALBP mRNA follows a course similar to that of LPL and FAT. The mRNA for ALBP increases approximately 5-fold in LF between gestational day 19 (P−3) and postnatal day 2, decreases by postnatal day 4, and remains relatively constant after that. HSL mRNA could not be detected by Northern analysis when 15 μg of total lung or LF RNA was loaded per lane.

Ontogeny of ALBP, HSL, and LPL proteins

The enzymatic assay for LPL demonstrated that the activity was approximately 3-fold higher in LF at postnatal day 8 than it was at gestational day 21 (P−1) (**Table 2**). HSL enzymatic activity was detected at postnatal day 8 but not at gestational day 21 (P−1) (Table 2). Immunoblotting demonstrated that HSL increased in LF from gestational day 21 (P−1) to postnatal day 8 (**Fig. 5A**, and Table 2). Figure 5B illustrates an immunoblot in which LF cytosolic proteins were subjected to SDS-PAGE and then probed with an anti-rat ALBP polyclonal antibody. ALBP protein was barely detectable at G-21, was maximal at postnatal day 2, and then declined by postnatal day 14. The doublet band pattern was not observed in two other immunoblots, and may have resulted from partial proteolysis in the samples that were used for the analysis shown in Fig. 5B. A second immunoblot using LF cytosolic material isolated from different rats was subjected to densitometry. Densitometric analysis showed that ALBP protein contents increased from 6.1 arbitrary density

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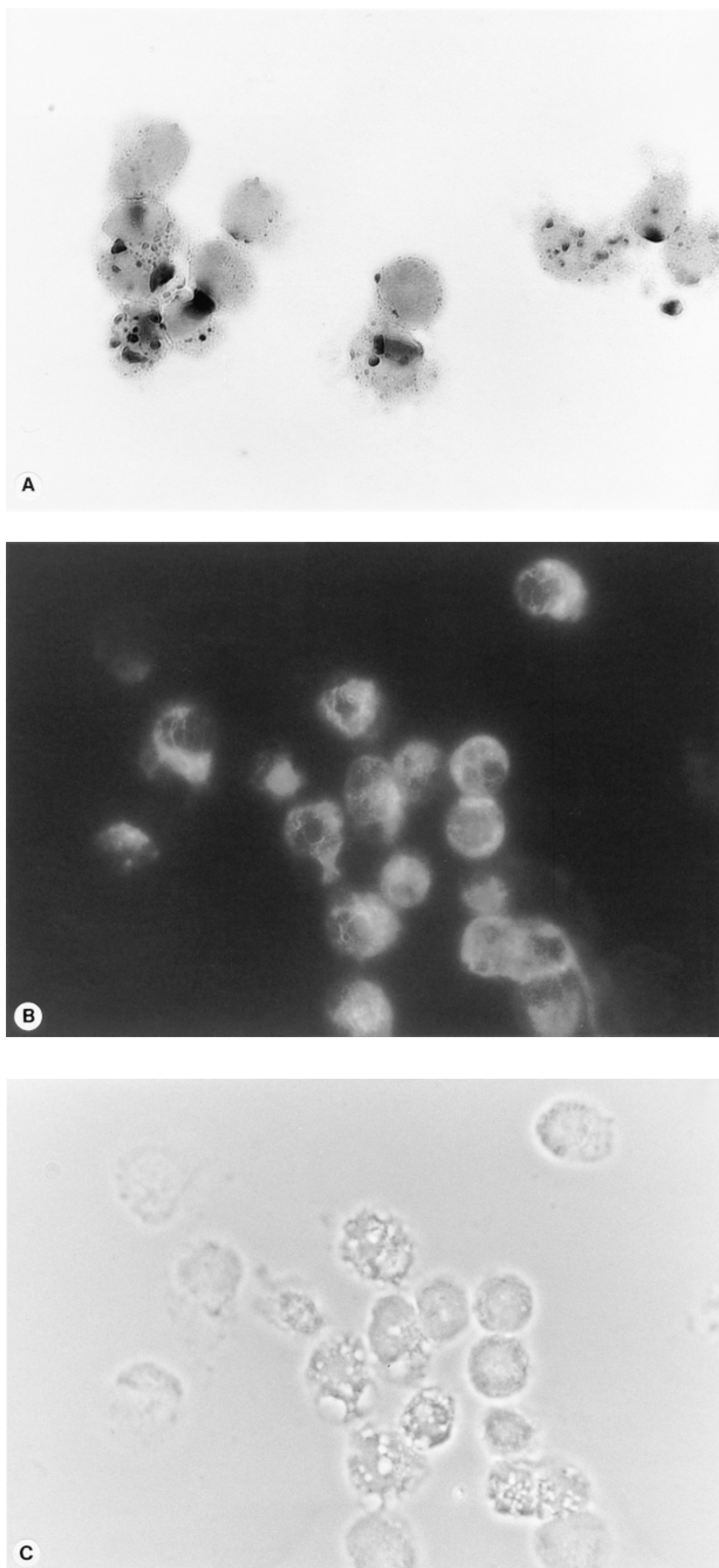

Fig. 1. Lipofibroblasts (LF) have characteristics of lipid storing mesenchymal cells. Photomicroscopy of LF immediately after isolation at postnatal day 4 and sedimentation onto glass slides using a Cytospin centrifuge. (A) Lipid droplets were stained with Oil-red O, followed by counter staining with Gill's hematoxylin. Original magnification, 250×. (B) Vimentin-containing intermediate filaments were stained with mouse monoclonal anti-vimentin, followed by an FITC-labeled sheep anti-mouse IgG $F(ab')_2$ fragment. Photographic exposure time was 4 s for vimentin immunofluorescence. A comparable exposure, after substituting non-immune mouse IgG for anti-vimentin, revealed no fluorescence. (C) Phase contrast micrograph of the same field shown in (B) demonstrates that all of the cells contain vimentin filaments.

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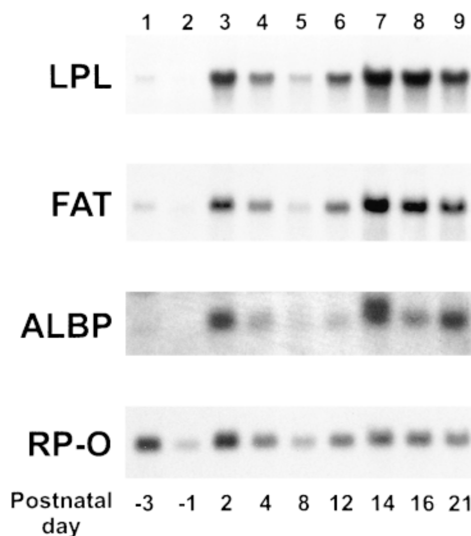

Fig. 2. Representative Northern analysis of fatty acid transporter (FAT), lipoprotein lipase (LPL), adipocyte lipid binding protein (ALBP), and ribosomal phosphoprotein P-0 (RP-0) mRNA in lipofibroblasts (LF). Total RNA was isolated from LF at various perinatal ages and 15 μg was subjected to Northern analysis and probed successively with cDNAs to detect various mRNAs that are involved in lipid hydrolysis, uptake, and transport. Postnatal days −3 and −1 are gestational days 19 (P − 3) and 21 (P − 1), respectively. The data from this and three similar Northern analyses were combined and summarized in Fig. 3. The autoradiograms were exposed for the following durations in hours at −75°C: FAT, 23; LPL, 19.5; ALBP, 45; and RP-O, 7.

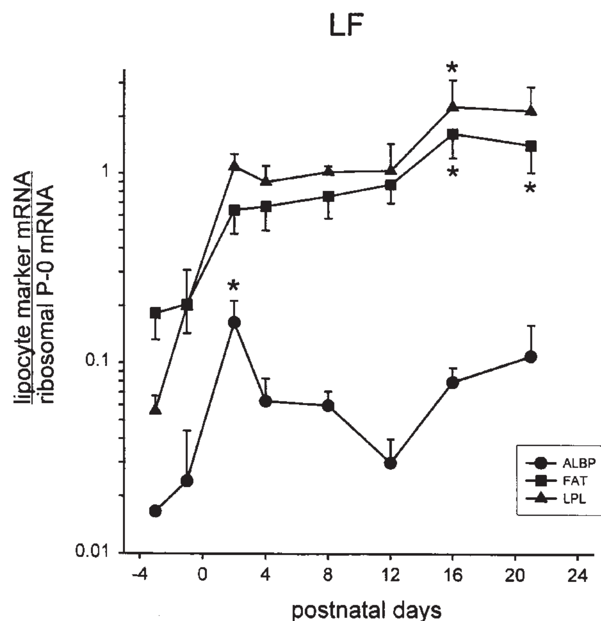

Fig. 3. Steady-state levels of fatty acid transporter (FAT), lipoprotein lipase (LPL), adipocyte lipid binding protein (ALBP), and ribosomal phosphoprotein P-0 (RP-0) mRNAs in the perinatal rat lipofibroblast (LF). RNA was obtained from LF immediately after their isolation from rats at various ages. Northern analysis was performed and the cationic nylon filters were successively probed with cDNAs for ALBP, FAT, LPL, and RP-0. Autoradiograms were exposed for varying times to ensure that the signal was within the linear range of the film. RP-0 served as a control to normalize for inequalities in the amounts of RNA loaded in the various lanes. Autoradiograms were subjected to densitometry and the densities of the bands representing the various mRNAs were normalized to the density of the corresponding band for ribosomal P-0 mRNA for each sample. The error bars are 1 SEM. (*) $P < 0.05$ compared to postnatal day −3 (gestational day 19 (P − 3) for LPL and FAT, or −1 for ALBP), 2-way analysis of variance (ANOVA), Student-Newman-Keuls multiple comparison. The data for P-14 in Fig. 2 are not shown in Fig. 3, as this was the only analysis that included this age; n = 4 for LPL and FAT at all ages and for ALBP at all ages except days −3 and −1, when 1 and 3 analyses, respectively, revealed a detectable signal.

units at gestational day 21 (P−1) to 79.2 at postnatal day 2, then declined to 26.6 at postnatal day 8. These data indicate that the levels of LPL and ALBP proteins follow the same ontogeny as their respective mRNAs, consistent with pretranslational regulation of the expression of these genes.

PPAR-γ mRNA peaks prenatally and precedes the maximum in LPL, ALBP, and FAT expression

Ribonuclease protection analysis was performed to analyze the expression of the PPAR genes that are known to activate the expression of several genes that are involved in lipid metabolism in adipocytes. A representative analysis for PPAR-γ and PPAR-δ in LF is shown in **Fig. 6**. The data for PPAR-α mRNA are not shown because the quantity of this mRNA did not vary significantly during the perinatal period in either lung or isolated LF. The results of the analysis of PPAR-γ and -δ shown in Fig. 6 were combined with those from two additional analyses of PPAR-δ and three analyses for PPAR-γ in LF and are summarized in **Fig. 7**. A summary of a similar group of analyses using RNA that was isolated from whole lung tissue is shown in **Fig. 8**. In LF, the mean steady-state level of PPAR-γ mRNA is maximal at gestational day 21 (P−1) and then decreases by postnatal day 2. The level of PPAR-γ mRNA in LF and lung increases during the third postnatal week and at 21 postnatal days, is again significantly higher than at postnatal day 8. PPAR-δ is significantly higher at postnatal day 2 in LF, but its expression did not change in a consistent pattern in whole lung tissue during the perinatal period.

DISCUSSION

Our findings indicate that the accumulations of triglycerides in the perinatal lung and in LF that reach their maxima postnatally are accompanied by increases in the expression of genes that have traditionally been considered to be adipocyte-specific. Our data extend the findings of others regarding LPL and indicate that the LF are a significant pulmonary source of LPL. The findings regarding ALBP and FAT gene expression in the lung are novel and again illustrate that genes that are involved in triglyceride accumulation by adipocytes are expressed in LF which accumulate triglycerides in the perinatal lung. The increase in PPAR-γ gene expression precedes the increase in the steady-state levels of mRNAs for LPL and ALBP,

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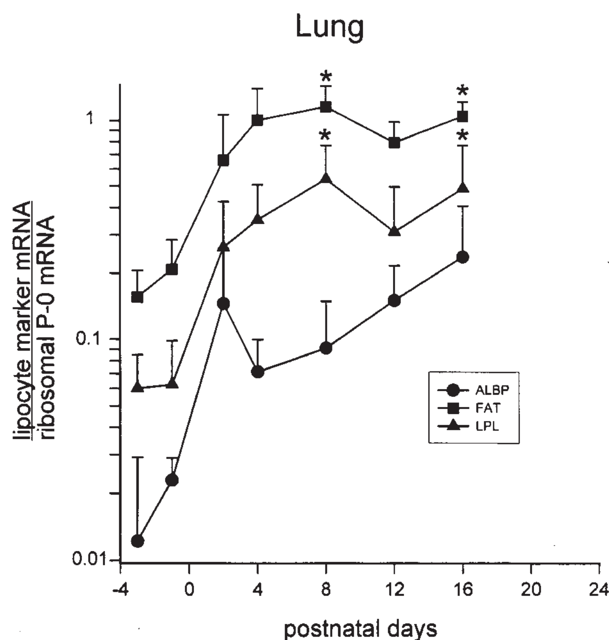

Fig. 4. Steady-state levels of fatty acid transporter (FAT), lipoprotein lipase (LPL), adipocyte lipid binding protein (ALBP), and ribosomal phosphoprotein P-0 (RP-0) RNAs in the perinatal rat lung. The Northern analysis was conducted as described in the legend to Figs. 2 and 3, except that the RNA was obtained at various ages from whole lung tissue, instead of from LF. (*) $P < 0.05$ compared to postnatal day −3 (gestational day 19), 2-way analysis of variance (ANOVA), Student-Newman-Keuls multiple comparison; n = 3 for all ages for LPL and FAT and for all ages for ALBP, except postnatal days −3 and −1, when n = 2 for each.

which are both products of PPAR-γ-responsive genes. Therefore, PPAR-γ could be involved in the regulation of genes that participate in triglyceride accumulation by LF.

We anticipated that we would observe a higher level of expression of LPL, FAT, and ALBP mRNA relative to ribosomal P-0 mRNA in isolated LF than in whole lung tissue. However, we generally observed similar levels of expression in the two RNA preparations. If the LF preparation contained an approximately equal number of cells that were not actually LF, one would expect to observe similar quantities of lipocyte marker mRNAs in RNA isolated from LF and from whole lung tissue. To establish that this was not the case, we examined the purity of the LF preparation. Ninety-five percent or more of the cells contained vimentin, less than 3% of the cells contained cytokeratin, and fewer than 10% of the cells were macrophages. Others have used vimentin as a marker of pulmonary fibroblasts; however, it can be present in activated macrophages including those found in the alveolus (27). The proportion of VWF-containing endothelial cells did increase at postnatal days 12 and 21, to approximately 13%. These data are consistent with those of Brody and Kaplan (4) who have shown that the proportion of endothelial cells in the lung increases from postnatal day 4 to day 11. Therefore, 77% or more of the cells had characteristics of lipid-laden mesenchymal cells that contained lipid droplets and lacked endothelial and macrophage cell markers. As the LF cell isolate contains at least 3-fold more LF than other types of lung cells, alternate explanations must exist for the unanticipated abundance of lipocyte marker mRNA in the whole lung samples. One explanation is that LF comprise a large proportion of the cells in the perinatal lung and, in fact, others have shown that this is the case (28). During the second postnatal week in rats, interstitial fibroblasts comprise 50–52% of all alveolar cells, and two-thirds of these contain lipid droplets.

Others have also demonstrated an increase, at birth, in LPL enzymatic activity and mRNA in the lung and other organs, and a further increase in mRNA concomitant with weaning (3, 29–31). It was known that cultured LF contain LPL, but no information was previously available about either LPL protein or mRNA within LF immediately after isolation from the lung (32). Our data indicate that, like adipocytes, LF contain LPL mRNA and enzymatic activity. LF are not the only lung cells in which LPL is expressed, as macrophages also contain LPL mRNA (30). Although we were unable to detect HSL mRNA at any age, we did identify HSL protein by immunoblotting at gestational day 21 (P−1) and at postnatal day 8, and HSL enzymatic activity at postnatal day 8, but not at gestational day 21 (P−1). We suspect that these discrepancies reflect differences in the sensitivities of the various techniques, and hesitate to speculate about HSL gene regulation in LF. HSL protein appears to be 6- to 7-fold lower in LF than in adipocytes, and RPA may be necessary to reliably detect HSL mRNA in LF.

We have studied FAT because the basal expression of FAT exceeds those of fatty acid transport protein (FATP) and membrane fatty acid binding protein (membrane FABP) in adipocytes of normal adult rats (33). FAT expression in the adult rat is highest in organs that accumulate triglycerides (adipose tissue), heavily use fatty acids as an energy source (heart, skeletal muscle), or transport long chain fatty acids (small intestine) (13). The develop-

TABLE 2. Lipase activities and immunoreactive HSL in LF and adipose tissue

	Enzyme Source		
	LF, Postnatal Day −1	LF, Postnatal Day 8	Adipose Tissue
HSL activity (pmol oleic acid/h/mg protein)	not detected	11.0 ± 0.8 (2)	62.3 ± 1.0 (2)
Immunoreactive HSL (density units/μg protein)	48.9 ± 8.6 (2)	132.2 ± 33.9 (2)	919.0 ± 101.8 (3)
LPL (ng equivalents/mg protein)	11.4 ± 0.9 (3)	32.5 ± 4.4 (3)	108.5 ± 27.3 (3)

Values given as mean ± SEM; numbers of determinations in parentheses. HSL, hormone sensitive lipase; LF, lipofibroblast; LPL, lipoprotein lipase.

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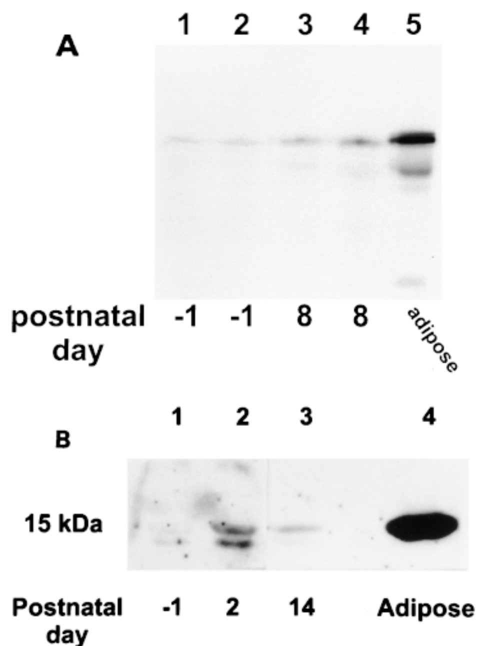

Fig. 5. Immunoreactive adipocyte lipid binding protein (ALBP) and hormone-sensitive lipase (HSL) proteins in LIF and adipose tissue. Cytosolic extract was prepared from LF that were isolated on at gestational day 21 (P − 1) and postnatal days 2, 8, and 14, as described in Materials and Methods. (A) HSL: 75 μg of protein was loaded in each lane, and the gel was subjected to SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-HSL. The immunoblot was developed by incubating with peroxidase-linked anti-rabbit IgG and a chemiluminescent detection system. Lanes 1 and 2 represent LF isolated from two different litters at postnatal day −1, while lanes 3 and 4 represent LF isolated from two different litters at postnatal day 8. Adipose tissue was from an adult epididymal fat pad. HSL co-migrated with molecular mass markers at 85 kDa. (B) ALBP: cytosolic extract was prepared from LF and 75 μg of each sample was subjected to Western immunoblotting. ALBP co-migrated with molecular mass standards at 15 kDa. Lane 4 contained 75 μg of cytosolic protein from adult epididymal fat pad and served as a positive control. The fluorogram was cut between lanes 2 and 3 prior to photography.

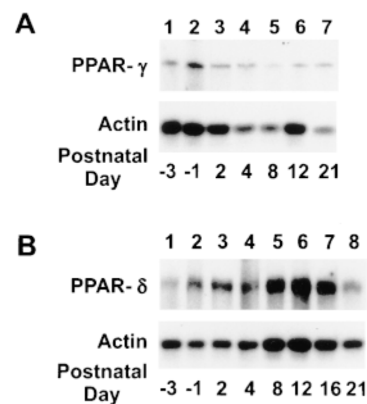

Fig. 6. Representative ribonuclease protection analysis of peroxisome proliferator-activated receptor (PPAR) mRNA in lipofibroblasts. Total RNA was subjected to hybridization in solution with cRNA that was generated by in vitro transcription using cDNAs for rat PPAR-γ and PPAR-δ. After digestion with a mixture of RNase A and RNase T1, the protected mRNA was resolved on a denaturing 5% polyacrylamide gel containing 6 m urea. Postnatal days −3 and −1 are gestational days 19 and 21, respectively. The ratios of the autoradiographic densities of PPAR-γ: actin mRNA, normalized to postnatal day 8, were as follows: postnatal day (P)−3, 0.84; P−1, 2.24; P2, 1.80; P4, 1.93; P8, 1.00; P12, 0.70; P21, 2.95. The autoradiograms were exposed for the following times in hours at −75°C: PPAR-γ 164 h and its respective actin 1.5 h; PPAR-δ, 134 h and its respective actin 1 h.

mental expression of FAT mRNA has been examined in perinatal rat heart tissue and increases approximately 2-fold between gestational day 21 (P−1) and postnatal day 5 and remains high throughout the first 3 postnatal weeks (13). This is somewhat less than the approximately 4-fold increase that we observed in whole lung tissue and isolated LF over the same developmental time frame. A temporal co-regulation of FAT mRNA expression with that of cytoplasmic fatty acid binding protein mRNA expression has been demonstrated in the small intestine, breast tissue, and heart (34, 35). ALBP expression has been most extensively studied in, and has generally been considered to be specific for, adipocytes. The levels of ALBP mRNA and protein increase markedly as preadipocytes convert to adipocytes in vitro. During the second and third postnatal weeks, the level of ALBP mRNA remains more elevated in the lung than it does in the LF. This was unexpected, as we predicted that ALBP mRNA, which is highly expressed in the adipocyte, would also remain high in LF, which is the most prominent neutral lipid storing cell in the lung, at that time. Because the number of LF decreases by postnatal day 16 and the concentration of ALBP mRNA in LF decreases, it is likely that another type of cell, in addition to the LF, contains ALBP mRNA. Two candidate cells, that approximately double in number in the rat lung between postnatal days 7 and 14 are the alveolar type II (AT2) cell and the alveolar macrophage (36, 37). As fatty acid trafficking in the cytoplasm generally occurs while it is associated with a fatty acid binding protein, it is reasonable to expect that these cells may also contain one or more FABP family members, such as ALBP. Others have shown that the alveolar type 2 cell contains PPAR-γ1 gene products and that this gene is coordinately expressed with surfactant protein-A (38). As the surfactant protein A gene was not shown to directly interact with PPAR-γ1, the authors suggested that PPAR-γ1 may have a more general effect on lipid metabolism by alveolar type 2 cells, although the pathways involved were not identified.

It is known that the expression of ALBP and LPL are transcriptionally regulated by PPARs (39, 40). Two isoforms of PPAR-γ (PPAR-γ1 and PPAR-γ2) are derived from alternate promoter usage and differential splicing of the same gene (9). Both are expressed in adipocytes and can induce the expression of the ALBP gene (41). Our RPA probe does not distinguish between the γ1 and γ2 isoforms, as it encompasses a portion of the ligand binding domain and not the 5′-terminus of the cDNA, where the alternative splicing occurs. The expression of PPAR-γ1 is more generalized than that of PPAR-γ2 as the γ1-isoform is also detected by RPA in liver, muscle, and heart but not lung of adult rats (42). Our detection of PPAR-γ

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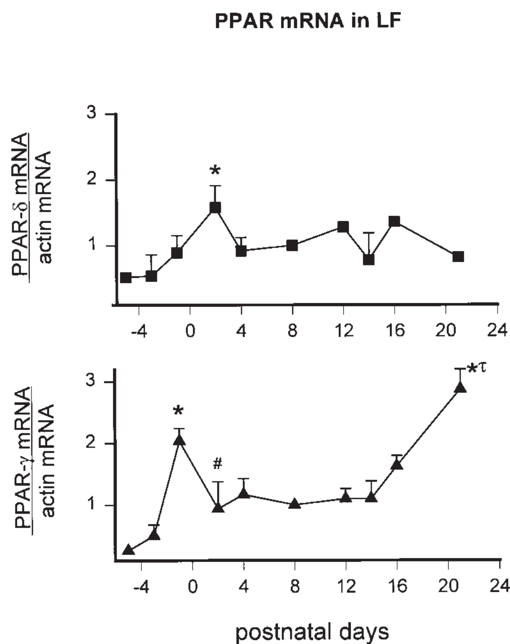


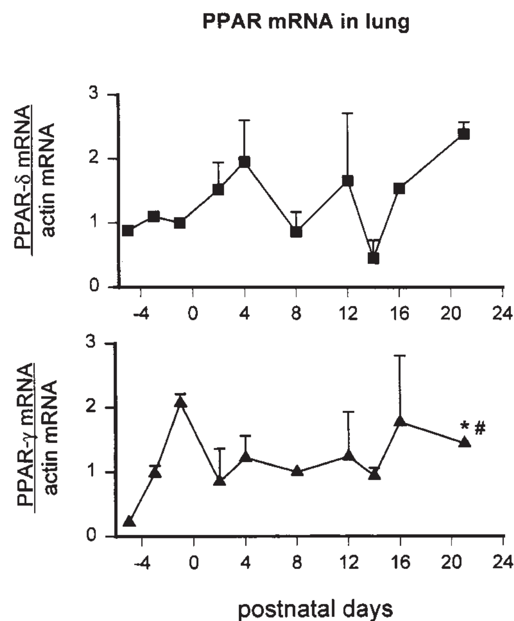
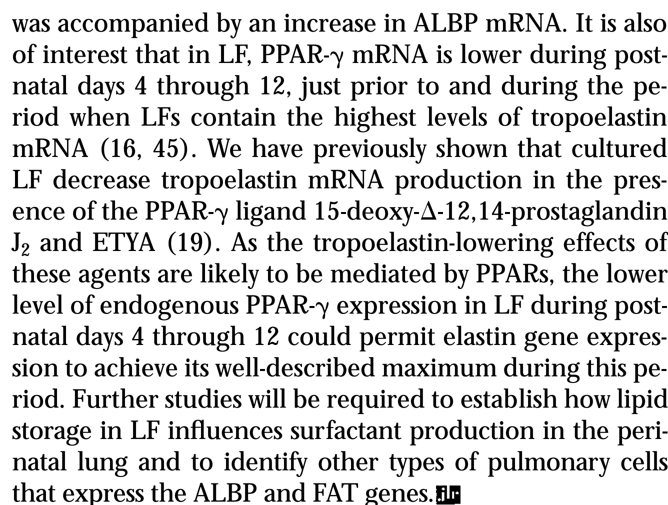


Fig. 7. Steady-state levels of perosixome proliferator-activated receptor (PPAR) mRNAs in lipofibroblasts (LF) were analyzed by ribonuclease protection assays using RNA obtained from lipofibroblasts (LF) that were isolated from rats at various ages. Actin served as a control to normalize for minor inequalities in the amounts of RNA loaded in the various lanes. The ratios of the densities for PPAR: actin for the various ages were normalized to the ratio for the sample of RNA that had been obtained at postnatal day 8 and was analyzed on the same gel. This allowed the data obtained from several analyses to be combined. Postnatal day −5 is gestational day 17. The error bars are 1 SEM. (*) $P < 0.05$ compared to postnatal day −3 (gestational day 19); 2-way ANOVA, Student-Newman-Keuls multiple comparison test. (#) $P < 0.05$ compared to postnatal day −3: (τ) $P < 0.05$ compared to postnatal day 14. PPAR-γ, n = 4 for all ages, except postnatal day (P) −5, n = 1. PPAR-δ , n = 3 for all ages except P−5, and P16 when n = 1. For Ps 12 and 21, the standard error bars for PPAR-δ were too small to be observed in the graph.

Fig. 8. Steady-state levels of peroxisome proliferator-activated receptor (PPAR) mRNAs in lung tissue were analyzed by ribonuclease protection assays using RNA obtained from lungs that were isolated from rats at various ages. The analyses were performed using the same procedure that was summarized in the legend to Fig. 7. Error bars are 1 SEM. (*) $P < 0.05$ compared to postnatal day −3 (gestational day 19). PPAR-γ, n = 3 for all ages except P−5, n = 1 and P14, n = 2. Standard error bar at P21 for PPAR-γ is too small to be observed. PPAR-δ: n = 3 for all ages except P−5, n = 1; P12, n = 2, and P16, n = 1. Standard errors for PPAR-δ at P−5, and −1 were too small to be observed on the graph.

in pre- and postnatal rat lung indicates that the expression of PPAR-γ in the perinatal rat lung is greater than that in the adult lung.

The relationships between PPAR gene expression and the induction of genes that encode proteins that participate in fatty acid transport and metabolism have been most extensively studied in adipocytes (43). The conversion of preadipocytes to adipocytes is accompanied by an increase in PPAR-γ mRNA which precedes the induction of the ALBP gene by approximately 24–48 h (44). A direct link between PPAR-γ and ALBP induction has been demonstrated using transfected NIH-3T3 cells (9). As in the differentiating pre-adipocyte, the transient, approximately 4-fold, increase in PPAR-γ mRNA in pulmonary LF precedes the increase in ALBP expression in LF by approximately 48 h. In LF, PPAR-δ mRNA reaches its maximum at postnatal day 2 and could also contribute to the induction of ALBP mRNA at this time. The decrease in both PPAR-γ and PPAR-δ mRNA by postnatal day 4 temporally corresponds to the decrease in ALBP mRNA. The increase in PPAR-γ mRNA in LF between postnatal days 14 and 21



was accompanied by an increase in ALBP mRNA. It is also of interest that in LF, PPAR-γ mRNA is lower during postnatal days 4 through 12, just prior to and during the period when LFs contain the highest levels of tropoelastin mRNA (16, 45). We have previously shown that cultured LF decrease tropoelastin mRNA production in the presence of the PPAR-γ ligand 15-deoxy-Δ-12,14-prostaglandin J_2 and ETYA (19). As the tropoelastin-lowering effects of these agents are likely to be mediated by PPARs, the lower level of endogenous PPAR-γ expression in LF during postnatal days 4 through 12 could permit elastin gene expression to achieve its well-described maximum during this period. Further studies will be required to establish how lipid storage in LF influences surfactant production in the perinatal lung and to identify other types of pulmonary cells that express the ALBP and FAT genes.

These studies were supported by the Department of Veterans Affairs Research Service and a Grant-in-Aid from the American Heart Association. We thank Drs. David Chappell, Fredrick Kraemer, and David Bernlohr for generously providing antibodies to LPL, HSL, and ALBP, respectively. We also thank Hui-Hui Dai and Jill Grout for their assistance in performing some of the immunohistological studies.

Manuscript received 31 March 1998 and in revised form 30 July 1998.

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