Fatty diabetic lung: altered alveolar structure and surfactant protein expression

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Am J Physiol Lung Cell Mol Physiol 298: L392–L403, 2010. First published January 8, 2010; doi:10.1152/ajplung.00041.2009.—Pulmonary dysfunction develops in type 2 diabetes mellitus (T2DM) in direct correlation with glycemia and is exacerbated by obesity; however, the associated structural derangement has not been quantified. We studied lungs from obese diabetic (fa/fa) male Zucker diabetic fatty (ZDF) rats at 4, 12, and 36 wk of age, before and after onset of T2DM, compared with lean nondiabetic (+/+) rats. Surfactant proteins A and C (SP-A and SP-C) immunoexpression in lung tissue was quantified at ages 14 and 18 wk, after the onset of T2DM. In fa/fa animals, lung volume was normal despite obesity. Numerous lipid droplets were visible within alveolar interstitium, lipofibroblasts, and macrophages, particularly in subpleural regions. Total triglyceride content was 136% higher. By 12 wk, septum volume was 21% higher, and alveolar duct volume was 36% lower. Capillary basement membrane was 29% thicker. Volume of lamellar bodies was 45% higher. By age 36 wk, volumes of interstitial collagen fibers, cells, and matrix were respectively 32, 25, and 80% higher, and capillary blood volume was 18% lower. ZDF rats exhibited a strain-specific increase in resistance of the air-blood diffusion barrier with age, which was exaggerated in fa/fa lungs compared with +/+ lungs. In fa/fa lungs, SP-A and SP-C expression were elevated at age 14–18 wk; the normal age-related increase in SP-A expression was accelerated, whereas SP-C expression declined with age. Thus lungs from obese T2DM animals develop many qualitatively similar changes as in type 1 diabetes mellitus but with extensive lipid deposition, altered alveolar type 2 cell ultrastructure, and surfactant protein expression patterns that suggest additive effects of hyperglycemia and lipotoxicity.

diabetes mellitus; lung morphometry; lipid deposition; collagen; surfactant-associated proteins

THE LUNG IS A RECOGNIZED TARGET of diabetic microangiopathy, manifested by modest reductions of ventilatory capacity, lung volume, and diffusing capacity (12, 24, 41). Because alveolar microvascular reserves are extensive, pulmonary dysfunction is usually not the presenting complaint in diabetes mellitus, although modest pulmonary dysfunction may become overtly debilitating under physiological stress (e.g., high-altitude exposure or exercise) or following the loss of alveolar microvascular reserves brought on by aging or disease (23, 24). In patients with type 1 diabetes mellitus (T1DM), we have found a significant restrictive defect associated with decreased lung diffusing capacity for carbon monoxide (DLCO) at a given pulmonary blood flow, mainly due to a reduction of membrane conductance compared with age-matched nondiabetic healthy subjects (41, 52). Structural abnormalities observed at autopsy in diabetic human lungs include thickened epithelial and capillary basement membranes (72, 77), alveolar septal destruction, and enlarged air spaces (32). In streptozotocin-induced T1DM, volumes of basal laminae, extracellular matrix, and interstitial connective tissue are increased in diabetic lungs compared with control lungs (31, 50, 51); the increases are partly attributed to diminished degradation of connective tissue proteins (43). Morphology of alveolar type 2 (AT2) epithelial cells was altered (49), accompanied by enlarged alveolar air spaces, diminished alveolar surface area, and reduced alveolar subdivisions (42). Experimental T1DM is associated with diminished phospholipid content (61) and increased triglyceride deposits in lung tissue (54); surfactant biosynthesis and secretion in isolated AT2 cells were also reduced. Expression of surfactant proteins SP-A and SP-B mRNA were higher, whereas expression of SP-C mRNA and SP-A protein were lower in lungs of streptozotocin-treated rats compared with controls (62, 63). Many of these in vivo changes were reversible following insulin treatment (8, 53, 67).

Pulmonary dysfunction also develops in type 2 diabetes mellitus (T2DM), consisting of restriction of lung volume and DLCO; the latter is exacerbated by obesity (12). However, unlike T1DM, the reduced DLCO in T2DM is mainly due to impaired alveolar microvascular recruitment; pulmonary perfusion is also impaired (12). Alveolar septal structure and surfactant protein expression are not well-documented in T2DM. The Zucker diabetic fatty (ZDF) rat, a genetic model of obesity and T2DM, originated in a colony of outbred Zucker fatty (fa/fa) rats (13) that carry the fa mutation, an amino acid substitution in the extracellular domain of the leptin receptor that renders fa/fa rats insensitive to leptin (26, 47), leading to hyperphagia and obesity (71). Leptin regulates esterification and oxidation of intracellular triglycerides so that loss of leptin signaling results in excessive fat accumulation in adipocytes and nonadipocytes (58). The diabetic trait develops in an inbred substrate of obese fa/fa males (ZDF/Drt-fa; Ref. 45); these animals develop hyperglycemia at 10–12 wk of age as insulin production by pancreatic islet β-cells and peripheral insulin sensitivity decline (33, 44). As the number of β-cells declines, the remaining islet cells are unable to compensate for the increased insulin demand brought on by the growing adipocyte mass and insulin resistance (59), leading to decompensated β-cell function and overt diabetes by age 12–14 wk (68).

We reasoned that insulin resistance and lipotoxicity could exert differential and possibly additive effects on lung structure and function. We tested the null hypothesis that changes in alveolar structure and in surfactant protein expression are similar in T2DM as in T1DM. To examine this issue, we analyzed the lungs from fa/fa and +/- male rats at different ages, before and after onset of overt diabetes, to quantify alveolar ultrastructure and immunoexpression of SP-A and SP-C.
MATERIALS AND METHODS

Animals. The Institutional Animal Care and Use Committee approved all procedures. ZDF fa/fa male rats were bred from ZDF/Drt-fa animals in the Unger laboratory. For lung morphology, male Zucker wild-type (+/+ ) and diabetic fatty (fa/fa) animals at ages 4 wk (n = 3), 12 wk (n = 9), and 36 wk (n = 5) were used. For triglyceride analyses, male ZDF fa/fa rats (n = 11, 13–17 wk old), fa/+ heterozygotes (n = 6, 13–17 wk old), and Zucker +/+ rats (n = 4, 33–34 wk old) were used. Additional animals were used for studies of surfactant protein expression: +/+ animals at 14, 18, 36, and 52 wk (n = 3–5) and fa/fa animals at 14, 18, 36–38, and 52 wk of age (n = 3–7).

Lung fixation, sampling, processing, and analysis. The animals were deeply anesthetized with an intramuscular injection of xylazine and ketamine and euthanized by an overdose of sodium pentobarbital. The trachea was cannulated with a 15-gauge catheter and tied securely. The chest was opened, and the lungs were air-inflated to 15 mHg vol. In some animals, the trachea was tied, and the lungs were removed; samples were cut into 2- to 3-mm3 pieces and snap-frozen.

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Lung fixation, sampling, processing, and analysis. The animals were deeply anesthetized with an intramuscular injection of xylazine and ketamine and euthanized by an overdose of sodium pentobarbital. The trachea was cannulated with a 15-gauge catheter and tied securely. The chest was opened, and the lungs were air-inflated to 15 mHg vol. In some animals, the trachea was tied, and the lungs were removed; samples were cut into 2- to 3-mm3 pieces and snap-frozen in liquid nitrogen. In other animals, the left lung was removed and processed as above, whereas the right lung was fixed in situ by tracheal instillation of 2.5% buffered glutaraldehyde (pH 7.4) at a constant airway pressure (25 cmH2O). Following instillation, the tracheal cannula was closed to maintain airway pressure. The fixed right lung was removed and immersed in 2.5% buffered glutaraldehyde for at least 3 wk before further processing.

Volume of the fixed right lung was measured by saline immersion (78). The lung was serially sectioned at 3-mm intervals starting with a random orientation; the cut surfaces were imaged using a digital camera. Volume of the sectioned lung was estimated from the images using the Cavalieri principle, i.e., measuring the slice area by point counting, multiplying by slice thickness, and summing the volume of all slices (78). The slices were divided into roughly equal cranial and caudal regions, placed side by side, and overlaid with a transparent grid. Using a systematic sampling scheme with a random start, 2 tissue blocks were sampled from each region (total of 4 blocks per lung) for further analysis. A previously established stratified analytical scheme was employed: low-power light microscopy (LM; ×275), high-power LM (×550), and transmission electron microscopy (EM; ×19,000; Ref. 75).

For low-power LM, each block was embedded in glycol methacrylate, sectioned (4-μm thick), and stained with toluidine blue. One section per block was overlaid with a test grid. From a random start, 30 nonoverlapping fields per grid were systematically sampled (total of 60 fields per animal). Using point counting, the volume density of fine parenchyma relative to lung volume (Vv(fp,L)) was measured from the reciprocal of the lengths of all intercepts with the test grid extending from the air-epithelial interface to the erythrocyte membrane (25, 76). This scheme resulted in at least 300 points or intersections per block for each parameter of interest, yielding a coefficient of variation of 5 to 10%. Data from 2 blocks of each region were averaged; a volume-weighted average for the entire lung was then obtained for each animal. Absolute volume or surface area was calculated by relating the respective volume and surface densities at each level back through the cascade of levels to the lung volume measured by the Cavalieri principle (78).

Triglyceride assay. Animals were perfused with 300 ml of warm, oxygenated PBS via the right ventricle at 8 ml/min. Well-perfused tissue was flash-frozen in liquid nitrogen. For analysis, 50–100 mg of lung and liver tissue was homogenized in 2:1 chloroform-methanol, and lipids were extracted for 1 h at room temperature with intermittent vortexing. Volume of distilled H2O (2×) was vortexed into the homogenates, and the mixture was centrifuged at 2,000 g. The lipid extract was withdrawn, and 50–600 μl were dried completely, depending on the expected lipid content. The dried samples were mixed with 30 μl of t-butanol, 20 μl of 1:1 Triton X-100/methanol, and 1 ml of a 4:1 mixture of Free Glycerol Reagent: Triglyceride Reagent (Sigma-Aldrich, St. Louis, MO). Absorbance was read at 540 nm, and triglyceride was calculated from a Glycerol Standard curve (Sigma-Aldrich). Results were expressed as mean milligrams triglyceride per gram tissue analyzed in duplicate samples.

Immunoblotting. Lung tissue (25–50 mg wet wt) was minced on ice and homogenized by Polytron in a buffer containing 300 mM sucrose, 20 mM Tris, pH 8.0, 10 mM HEPES, and 2 mM 2-mercaptoethanol, and protease inhibitors aprotinin (5 μg/ml), leupeptin (1 μg/ml), pepstatin A (1.5 μM), trypsin inhibitor (5 μg/ml), p-aminobenzamide (200 μM), and PMSF (1 mM). Homogenates were cleared of nuclei and debris by centrifugation at 15,000 g. Protein content of the lysate was quantified by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, blocked in Blotto-Tween solution (5% nonfat dry milk, 0.05% Tween in PBS) for 1 h, and incubated in Blotto-Tween with primary antibody for at least 2 h. SP-A was detected using goat polyclonal anti-human antiserum (0.5 μg/ml; clone N-19; Santa Cruz Biotechnology, Santa Cruz, CA). SP-C was immunolabeled using rabbit polyclonal antiserum raised against full-length human SP-C (1 μg/ml; clone FL-197; Santa Cruz Biotechnology). Labeled protein was visualized by chemiluminescence (Renaissance Chemiluminescence Reagent Plus; PerkinElmer, Wellesley, MA). Blots were immediately washed in PBS and immunostained with mouse monoclonal anti-β-actin (clone AC-15; Sigma-Aldrich) to assess lane loading.

At least three replicate immunoblots, prepared using separate tissue samples, were analyzed to compare SP-A and SP-C expression (79) with respect to genotype (+/+ and fa/fa) at 14 and 18 wk of age, and 2) with respect to age (14–52 wk) in +/+ and fa/fa rats. To adjust for protein loading, signal intensities were divided by the corresponding β-actin signals and then expressed as a percentage of the mean signal intensity of the respective control group quantified on the same blot (+/+ for genotype comparisons, 52-wk-old animal group for developmental comparisons). Percentage values for each animal were determined by point counting, with total septum volume as the reference space. The septum consists of tissue and blood. Septal tissue includes the epithelium, endothelium, and interstitium. Within the epithelium, type 1 and type 2 cell volumes were quantified separately. Within AT2 cells, the volumes of nuclei, cytoplasm, lamellar bodies (LB), mitochondria, and protein pools were quantified separately. Within the interstitium, cells and matrix, collagen fibers and lipid droplets were quantified separately.

The surface-to-volume ratios of alveolar epithelial and endothelial surface area to septal volume were determined by intersection counting. Harmonic mean diffusion length of the tissue-blood barrier (L393ALVEOLAR STRUCTURE AND SURFACTANT PROTEINS IN OBESE DIABETIC RATS

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averaged across replicates, and the mean values were used for statistical analysis.

Data analysis. Results (means ± SD) were analyzed by unpaired t-test or ANOVA with post hoc Fisher multiple comparisons tests using StatView (version 5.0; SAS Institute, Cary, NC). A P value of 0.05 or less was considered significant.

RESULTS

Morphometric results. At 4 wk of age, body weight did not differ between fa/fa and +/+ groups (Table 1). By 12 wk, fa/fa animals were 35% heavier than +/+ animals (P < 0.05) due to noticeable accumulation of body fat and remained 13% heavier at 36 wk (P = 0.07). Absolute lung volume in 4-wk-old fa/fa animals was significantly (28%) less than in corresponding +/+ animals measured by immersion and Cavalieri methods, but the difference disappeared by 12 and 36 wk of age. Morphometric hematocrit did not differ between geno-
types at any time point. As expected, capillary basement membrane thickening was evident in the fa/fa lung and was increased at 12 and 36 wk by 29.1 and 25.7%, respectively (Table 1). Resistance of \( \tau_{ab} \) increased with age in both +/+ and fa/fa lungs and was modestly but significantly higher in fa/fa lungs than in +/+ lungs at 12 and 36 wk (by 12 and 5%, respectively; Table 1 and Fig. 4).

In fa/fa but not +/+ lungs, numerous lipid deposits were present within septal interstitium and the abundant alveolar macrophages at 12 and 36 wk, visible with Oil Red O staining (Fig. 1, top right and bottom left). Lipid-laden alveolar septa and macrophages were prominent near the pleural surface (Fig. 1, bottom right). Under EM, numerous lipid droplets were present within interstitial cells and matrix, and their electron opacity was enhanced by imidazole contrast (Fig. 2). AT2 cell morphology was altered in fa/fa lungs, with deposition of irregular

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Table 1. Basic morphometric data

<table>
<thead>
<tr>
<th>Age</th>
<th>4 wk</th>
<th>12 wk</th>
<th>36 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>+/+</td>
<td>fa/fa</td>
<td>+/+</td>
</tr>
<tr>
<td>Diabetes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Number of animals</td>
<td>3</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>96 ± 4</td>
<td>97 ± 3</td>
<td>363 ± 29</td>
</tr>
<tr>
<td>Right lung volume, ml</td>
<td>4.5 ± 0.2</td>
<td>3.2 ± 0.3*</td>
<td>6.1 ± 0.4</td>
</tr>
<tr>
<td>Intact (immersion method)</td>
<td>3.7 ± 0.2</td>
<td>2.7 ± 0.3*</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>Sectioned (Cavalieri method)</td>
<td>57.1 ± 2.1</td>
<td>53.8 ± 4.8</td>
<td>53.8 ± 3.4</td>
</tr>
<tr>
<td>Morphometric hematocrit, %</td>
<td>60.3 ± 1.0</td>
<td>60.9 ± 1.3</td>
<td>62.2 ± 2.0</td>
</tr>
<tr>
<td>Arithmetric septal thickness, ( \mu m )</td>
<td>0.52 ± 0.01</td>
<td>0.55 ± 0.03</td>
<td>0.58 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05, †P = 0.07 obese diabetic (fa/fa) vs. corresponding lean nondiabetic (+/+) by ANOVA. \( \tau_{ab} \), harmonic mean length of diffusion barrier.

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Fig. 1. Representative micrographs of lean nondiabetic (+/+: top left) and obese diabetic (fa/fa) lungs (remaining panels) from 36-wk-old animals stained with Oil Red O. Top left: in +/+ lung, lipid deposits are absent (bar = 50 \( \mu m \)). Top right and bottom left: in fa/fa lung, numerous lipid deposits are visible within the septum and alveolar macrophages (bar = 50 \( \mu m \)); sections were counterstained with hematoxylin. Bottom right: the counterstain was omitted in a section from a fa/fa lung under lower magnification to demonstrate the preferential distribution of lipid deposits and alveolar macrophages near the pleural surface (bar = 100 \( \mu m \)).
intracellular pools that exhibit protein-like electron opacity; some pools were enclosed with a limiting membrane shown at high magnification to be that of the endoplasmic reticulum (Fig. 3).

**Volume and surface densities.** The relative volume and surface area of septal components are shown in Table 2. Volume ratios of fine parenchyma and alveolar septa with respect to total lung volume were significantly higher in fa/fa animals at 12 wk. Volume density of alveolar ducts increased from 4 to 12 wk in +/+ lungs, but the increase was attenuated in fa/fa lungs, resulting in significantly lower values at 12 wk compared with corresponding +/+ lungs. Volume density of AT2 cells per unit septum volume was unchanged at all ages. The volume of irregular intracellular protein pools comprised 3 and 23% of total AT2 cell volume in 12- and 36-wk-old diabetic fa/fa lungs, respectively; these pools were not present in 4-wk prediabetic fa/fa or in wild-type lungs of any age (Table 2). Volume density of LB per unit of AT2 cell volume increased by ~21% in both 12- and 36-wk fa/fa lungs, whereas volume density of cytoplasm diminished 8.0–9.3% at these time points. Volume density of mitochondria did not differ between genotypes.

Intracellular (within lipofibroblasts) and extracellular interstitial lipid droplets comprised 0.1 and 0.6% of total septum volume in 12- and 36-wk fa/fa lungs, respectively (Table 2). By 36 wk, volume densities of extravascular septal tissue per unit septum volume were 19% higher in fa/fa compared with +/+ lungs due to a higher volume density of the interstitium (32%), including collagen fibers (25%), cells, and matrix (81%). Capillary blood volume density per unit septum volume was 18% lower in fa/fa lung compared with +/+ lung. Surface densities of alveoli and capillaries were not different between genotypes at any time point. Thus fa/fa lungs demonstrate a higher septal volume fraction of interstitial tissue and a lower volume of irregular intracellular protein pools that exhibit protein-like electron opacity; some pools were enclosed with a limiting membrane shown at high magnification to be that of the endoplasmic reticulum (Fig. 3).

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**Fig. 2.** Representative electron micrographs of +/+ and fa/fa lungs from 36-wk-old animals are shown. Top left: an alveolar type 2 (AT2) cell from +/+ lung is shown (bar = 5 μm). Top right and bottom left: in fa/fa lung, uncontrasted lipid droplets within a lipofibroblast (arrows) and irregular protein pools within AT2 cells (*) are shown (bar = 5 μm). Bottom right: interstitial lipid droplets (arrow) are contrast-enhanced by treatment with imidazole (bar = 1 μm).

**Fig. 3.** Representative high-magnification electron micrographs from 36-wk-old animals show protein accumulation within dilated rough endoplasmic reticulum in fa/fa lungs but not in +/+ lungs. Bar = 1 μm.
volume fraction of alveolar capillary blood; these differences developed with age following the onset of diabetes.

Absolute volumes and surface areas. Because lung volume was similar between groups despite a difference in body size, we did not normalize these parameters by body weight. Total volumes of fine parenchyma and alveolar septa were lower in 4-wk fa/fa lungs and then increased 10–20% above control levels at 12 wk before returning to control levels by 36 wk. Absolute volumes of septal tissue and capillary blood were significantly (20%) higher in 12-wk fa/fa lungs (Table 3). By 36 wk, septal tissue volume remained higher in fa/fa lungs, but the increase no longer reached statistical significance. In contrast, between 12 and 36 wk, alveolar capillary blood volume in fa/fa lungs declined to 18% below that in +/+ lungs (Fig. 4). At 36 wk, absolute volume of the interstitium, including collagen fibers, cells, and matrix, was significantly elevated over +/+ levels (Fig. 4). Volume of intracellular and extracellular lipid droplets progressively increased between 12 and 36 wk in fa/fa lungs.

Absolute AT2 cell volume did not differ between fa/fa and +/+ lungs at all ages. Absolute volumes of LB and mitochondria in AT2 cells were significantly higher in fa/fa lungs at 12 wk; the differences subsided by 36 wk. Volume of irregular protein pools within AT2 cells increased fivefold from 12 to 36 wk in fa/fa lungs, whereas volume of AT2 cytoplasm and nuclei did not differ between genotypes at any age.

Absolute capillary surface area in fa/fa lung was significantly higher (19%) above +/+ value at 12 wk but not at other time points. Absolute alveolar surface area was not different between genotypes at any point. Thus, following the development of diabetes at 12 wk in fa/fa lungs compared with corresponding +/+ lungs, AT2 cell morphology was persistently altered, with a transient burst of LB formation followed by progressive accumulation of intracellular proteinaceous material within organelles. There was progressive accumulation of interstitial lipid, cells, matrix, and connective tissue fibers associated with thickened capillary basement membrane and reduced alveolar capillary blood volume.

Triglyceride content. Triglyceride content was determined in perfused lung and liver from fa/fa animals, lean fa/+ heterozygotes, and +/+ controls (Fig. 5). The lung or liver triglyceride contents were similar between fa/+ and +/+ animals, so the two groups were combined as “lean animals” for this analysis. Triglycerides were elevated by 136% in lung and 494% in liver from fa/fa animals compared with lean controls. Triglyceride content was also significantly higher in liver compared with lung tissue by 9- and 24-fold in lean and fa/fa animals, respectively. Thus the liver as a major site of triglyceride synthesis exhibits a greater increase in lipid content in fa/fa animals, but perturbation of fat metabolism affects lung as well.
ALVEOLAR STRUCTURE AND SURFACTANT PROTEINS IN OBESE DIABETIC RATS

Table 3. Absolute volumes and surface areas of alveolar structures

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age 4 wk</th>
<th>12 wk</th>
<th>36 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume, ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine parenchyma</td>
<td>3.254 ± 0.271</td>
<td>4.097 ± 0.313</td>
<td>4.508 ± 0.365*</td>
</tr>
<tr>
<td>Alveolar duct</td>
<td>0.189 ± 0.035</td>
<td>0.447 ± 0.067</td>
<td>0.286 ± 0.084*</td>
</tr>
<tr>
<td>Septum</td>
<td>0.534 ± 0.116</td>
<td>0.653 ± 0.106</td>
<td>0.788 ± 0.107*</td>
</tr>
<tr>
<td>Epithelium</td>
<td>0.115 ± 0.039</td>
<td>0.145 ± 0.028</td>
<td>0.165 ± 0.026</td>
</tr>
<tr>
<td>Type 1</td>
<td>0.056 ± 0.022</td>
<td>0.071 ± 0.011</td>
<td>0.079 ± 0.016</td>
</tr>
<tr>
<td>Type 2</td>
<td>0.059 ± 0.017</td>
<td>0.074 ± 0.019</td>
<td>0.086 ± 0.012</td>
</tr>
<tr>
<td>Nuclei</td>
<td>0.018 ± 0.004</td>
<td>0.021 ± 0.008</td>
<td>0.024 ± 0.005</td>
</tr>
<tr>
<td>Lamellar bodies</td>
<td>0.006 ± 0.001</td>
<td>0.011 ± 0.003</td>
<td>0.016 ± 0.004*</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>0.029 ± 0.010</td>
<td>0.036 ± 0.010</td>
<td>0.035 ± 0.004</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.005 ± 0.002</td>
<td>0.006 ± 0.001</td>
<td>0.008 ± 0.001*</td>
</tr>
<tr>
<td>Protein pools</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.003 ± 0.001*</td>
</tr>
<tr>
<td>Intercstitium</td>
<td>0.136 ± 0.051</td>
<td>0.131 ± 0.045</td>
<td>0.165 ± 0.032</td>
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<tr>
<td>Collagen fibers</td>
<td>0.111 ± 0.045</td>
<td>0.106 ± 0.033</td>
<td>0.132 ± 0.026</td>
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<tr>
<td>Cells and matrix</td>
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<td>0.026 ± 0.012</td>
<td>0.032 ± 0.009</td>
</tr>
<tr>
<td>Lipid</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.00131 ± 0.000107*</td>
</tr>
<tr>
<td>Extracellular</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.000087 ± 0.000067*</td>
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<tr>
<td>Lipidoproteinblasts</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0.00045 ± 0.00048*</td>
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<tr>
<td>Endothelium</td>
<td>0.062 ± 0.010</td>
<td>0.097 ± 0.014</td>
<td>0.092 ± 0.021</td>
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<tr>
<td>Septal tissue</td>
<td>0.314 ± 0.094</td>
<td>0.355 ± 0.074</td>
<td>0.426 ± 0.065*</td>
</tr>
<tr>
<td>Capillary blood</td>
<td>0.220 ± 0.051</td>
<td>0.299 ± 0.046</td>
<td>0.362 ± 0.077*</td>
</tr>
<tr>
<td>Alveoli</td>
<td>2.229 ± 1.056</td>
<td>2.773 ± 453</td>
<td>3.131 ± 442</td>
</tr>
<tr>
<td>Capillaries</td>
<td>2.245 ± 781</td>
<td>2.939 ± 451</td>
<td>3.504 ± 527*</td>
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Surface area, cm²

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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine parenchyma</td>
<td>1,657 ± 261</td>
</tr>
<tr>
<td>Alveoli</td>
<td>2,773 ± 453</td>
</tr>
<tr>
<td>Capillaries</td>
<td>3,504 ± 527*</td>
</tr>
</tbody>
</table>

Values are means ± SD. *P < 0.05 †P < 0.07 vs. corresponding +/+ by ANOVA.

Surfactant protein expression. SP-A immunolabeling, detected at ~32 and 38 kDa, is shown in Fig. 6. Another previously reported band, ~26 kDa (74), was variably detected. All bands were blocked by 5 × blocking peptide (data not shown). Expression of total SP-A in fa/fa lungs was quantified relative to mean total SP-A in +/+ animals separately at 14 and 18 wk. Immunoreactive SP-A was elevated by ~60% in fa/fa lungs compared with +/+ lungs at 14 wk, and the difference diminished to ~14% by 18 wk (both P < 0.05; Fig. 6), suggesting that development of the diabetic phenotype was associated with an initial increase in SP-A immunolabeling that subsided with time.

Immunoreactive SP-C was detected at ~21 kDa (Fig. 7), likely the initial proprotein translation product (3), and at 16 kDa, a possible processing intermediate. A strongly immunoreactive protein was present at ~13 kDa and may represent multimers of mature SP-C (2). At 14 wk, expression of the 21- and 16-kDa proprotein bands was 39 and 75% greater in fa/fa lungs relative to +/+ lungs, respectively, and the 16-kDa protein remained elevated at 18 wk (P < 0.05). Expression of the 13-kDa SP-C did not differ between genotypes at either time point. Thus SP-C proprotein forms increased in fa/fa lungs after onset of diabetes and the increase subsided with time.

In +/+ lungs, total SP-A increased with age, consistent with its known ontogeny in rats (56). In fa/fa lungs, the mature level of SP-A expression was achieved at 14 wk and then did not change with age up to 52 wk (Fig. 8). In +/+ lungs, SP-C protein expression was either lower or unchanged in younger animals compared with 52-wk-old animals. In contrast, in fa/fa lungs, the 21- and 16-kDa SP-C proteins were respectively elevated by 51 and 179% at 14 wk and then declined with age, whereas expression of the 13-kDa protein did not change with age (Fig. 9). Thus normal age-related expression profile of both SP-A and SP-C was altered in fa/fa lungs. Age-related SP-A expression was accelerated, whereas SP-C proprotein expression was elevated transiently with the onset of T2DM and then subsided with age.

DISCUSSION

Summary of findings. The obese diabetic rat lung exhibits numerous structural changes in the gas-exchange region as well as disturbance of surfactant protein expression. Lung volume was reduced in 4-wk-old fa/fa animals before the onset of diabetes but preserved after the onset. Volume density of fine parenchyma per unit lung volume declined less rapidly with age in fa/fa lungs, whereas alveolar duct density increased slowly and to a lesser extent. From 12 to 36 wk of age, fa/fa lungs exhibited multifocal lipid deposition within septal lipofibroblasts, interstitial matrix, and the abundant alveolar macrophages, associated with increased amounts of interstitial cells, matrix, and collagen fibers, diminishing capillary blood volume as well as thickened capillary basement membrane. Reciprocal changes in the interstitium and capillary offset one another so that the volume and average arithmetic thickness of...
the septum remained normal. Resistance of the barrier, indexed by $\tau_{hb}$, increased with age in both genotypes but was significantly higher in $fa/fa$ lungs compared with $+/+$ lungs. AT2 cell morphology was altered following the onset of diabetes in $fa/fa$ lungs; an early increase in LB volume subsided with age, whereas the deposition of proteinaceous material within AT2 organelles progressively increased with age.

In $fa/fa$ lungs compared with $+/+$ lungs, triglyceride content increased twofold, which was less than the sixfold elevation seen in the liver. Protein expression of SP-A and SP-C were significantly higher in $fa/fa$ lungs at 14 and 18 wk, respectively, compared with $+/+$ lungs. In $fa/fa$ lungs, the expected age-related increase in SP-A expression was accelerated; the mature (52-wk) level of SP-A was reached at 14 wk. In addition, a transient burst of SP-C expression was seen at 14 wk, which subsided with age.

In these $fa/fa$ lungs, the accumulation of interstitial cells, fibers, and matrix is similar to that observed in T1DM, consistent with the effect of hyperglycemia on connective tissue metabolism. The lipid infiltration and altered surfactant protein expression patterns are distinct from that seen in T1DM, suggesting that they are related to the state of heightened adipogenesis.

**Structural changes related to diabetes.** Diabetic microangiopathy affects all organs, brought on by generalized protein glycosylation that results from either insulin insufficiency or insulin resistance. T1DM is associated with reduced lung capacities, elastic recoil, and dynamic lung compliance (55, 57), likely a consequence of increased collagen cross-linking in the lung and thorax (9). Autopsy of diabetic lung tissue shows basement membrane thickening (32, 73) including alveolar epithelium and capillary endothelium basal lamina (72, 77). In streptozotocin-induced T1DM, the volume of alveolar wall is increased due to higher contents of collagen, elastin, and basal laminae (31, 42, 51), which partially result from slower degradation of connective tissue (43).

Functional derangement in T2DM includes reduced lung volume, diffusing capacity, and capillary blood volume as well as impaired alveolar-capillary recruitment, consistent with diminution of alveolar microvascular reserves (5, 12, 19, 37); the extent of impairment correlates inversely with glycosylated hemoglobin level (12). Alveolar-capillary permeability may be reduced (35, 46). Obesity further aggravates pulmonary dysfunction in T2DM (12). Obese nondiabetic Zucker rats exhibit reduced lung volume and respiratory system compliance (15), and overnutrition in young rats results in hyperplastic lung growth (29) associated with excessive lipid deposition, enlarged alveoli, reduced respiratory surface area, and diminished lung compliance (27, 28). Thus obesity alone impairs lung function; however, in obese diabetic patients, pulmonary dysfunction may or may not correlate with truncal or abdominal fat mass (34, 36), suggesting that obesity alone cannot fully explain the changes observed in T2DM.
Obese fa/fa rats represent a convenient model of T2DM because the animals uniformly develop T2DM and other lipotoxic complications by 12–14 wk of age (70). The structural changes in fa/fa lungs—accumulation of interstitial components, including collagen fibers, cells, and matrix, the reduction in capillary blood volume, and the increase in \(^{\text{\text{H}270}}\text{hb}\)—are qualitatively similar to that reported in clinical and experimental T1DM. The finding of similar connective tissue abnormalities in both insulin insufficiency and insulin resistance states suggests that these abnormalities reflect the effects of chronic hyperglycemia.

**Structural changes related to obesity.** The basic disturbance in obesity is excessive lipid accumulation. Increasing evidence suggests that lipid accumulation within adipocytes is harmless, whereas fat infiltration in nonadipose tissue such as that resulting from leptin insensitivity induces a proinflammatory response that is associated with accretion of connective tissue elements and eventual fibrosis in various organs, notably the pancreas islet cells, liver, muscle, and heart (69). The same mechanism likely operates in the fa/fa lung, with extraadipose lipid deposits, elevated triglyceride content, alveolar macrophage accumulation, connective tissue accretion, thickened capillary basement membrane, and increased \(^{\text{\text{H}270}}\text{hb}\), which are additive to the changes induced by hyperglycemia. The increase in \(^{\text{\text{H}270}}\text{hb}\) may underlie the clinical observation of greater reduction in lung diffusing capacity at a given pulmo-

![Fig. 6. Surfactant protein A (SP-A) expression in wild-type (+/+, \(n = 7\)) and fatty diabetic (fa/fa, \(n = 6\)) lungs. Representative immunoblots of SP-A expression at age 14 and 18 wk are shown. Blots were reprobed for β-actin. Signal intensities at 38, 32, and 26 kDa were totaled, normalized to β-actin intensity, and expressed as percentage of the mean normalized total +/+ signal quantified on the same blot. Triplicate assays used separate lung tissue samples; results were averaged for individual animals. Means ± SD, unpaired \(t\)-test. \(*P < 0.05\) fa/fa vs. corresponding +/+.

![Fig. 7. SP-C expression in +/+ (\(n = 5–7\)) and fa/fa (\(n = 6\)) lungs. Representative immunoblots at 14 and 18 wk are shown. Blots were reprobed for β-actin expression. Signal intensities of proteins at 21, 16, and 13 kDa were scored separately, normalized to β-actin, and expressed as percentage of the mean normalized +/+ signal for each protein quantified on the same blot. Triplicate assays used separate lung tissue samples; results were averaged for individual animals. Means ± SD, unpaired \(t\)-test. \(*P < 0.05\) fa/fa vs. corresponding +/+.
nary blood flow in obese diabetic than in nonobese diabetic subjects (12), which also supports additive impairment by obesity and hyperglycemia. Cell proliferation and tissue growth and turnover are more active in subpleural alveoli than in deeper alveoli (17, 38). The preferential lipid deposition and macrophage aggregation in subpleural alveoli may reflect subclinical alveolar inflammation, which, in turn, could alter surfactant protein and phospholipid levels (below) and has important implications for lung function, growth, maintenance, repair, and aging.

Age-related changes in $\tau_{hb}$. $\tau_{hb}$ is measured as the reciprocal of the mean of the reciprocal of random intercept lengths from the air-tissue interface to the capillary erythrocyte membrane and is proportional to the diffusive resistance of the tissue-plasma barrier. Previous reports in Wistar rats (11), hamsters (10), and guinea pigs (22) show an age-related decline in $\tau_{hb}$ from birth to adulthood; the decline reflects alveolar septal remodeling that progressively reduces net barrier resistance to gas diffusion. In both +/+ and fa/fa ZDF rats, $\tau_{hb}$ increased with age. To determine whether the paradoxical increase in $\tau_{hb}$ with age is specific to the ZDF strain, we analyzed additional right lungs from Sprague-Dawley rats inflation-fixed in 2.5% glutaraldehyde at ages 8, 12, and 47 wk using the same methods. In Sprague-Dawley rats, body weight increased 2-fold from the youngest to oldest animals; lung volume increased 13%, whereas $\tau_{hb}$ declined 20% (unpublished data), consistent with that reported in maturing lungs from other species and rat strains mentioned above. Thus an age-related increase of $\tau_{hb}$ is unique to the ZDF strain, consistent with inborn variations in postnatal alveolar remodeling. In fa/fa rats

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**Fig. 8. SP-A protein expression during maturation in +/+ and fa/fa lungs.** Representative immunoblots showing SP-A expression with increasing age. Total SP-A signal intensity per lane was normalized to the corresponding $\beta$-actin signal and expressed as percentage of the mean total normalized signal for 52-wk animals quantified on the same blot. Triplicate assays used separate lung tissue samples; results were averaged for individual animals. +/+ ($n = 3, 5, 3, and 3$) and fa/fa ($n = 5, 5, 6, and 3$) at 14, 18, 36, and 52 wk, respectively. Means ± SD, 1-way ANOVA: *$P < 0.05$ vs. 52 wk.

**Fig. 9. SP-C protein expression during maturation in +/+ and fa/fa lungs.** Representative immunoblots show SP-C expression with increasing age. Signal intensities of proteins at 21, 16, and 13 kDa were scored separately, normalized to $\beta$-actin, and expressed as percentage of the mean normalized signal for 52-wk animals quantified on the same blot. Triplicate assays used separate lung tissue samples; results were averaged for individual animals. +/+ ($n = 3, 5, 3, and 3$) and fa/fa ($n = 5, 5, 6, and 3$) at 14, 18, 36, and 52 wk, respectively. Means ± SD, 1-way ANOVA: *$P < 0.05$ vs. 52 wk.
genotype, the age-related increase is further exaggerated by a thickened interstitial barrier to produce significantly elevated $\tau_{ha}$ at 12 and 36 wk compared with corresponding $+/+$ lungs. Whether $\tau_{hb}$ continues to increase throughout the life span of diabetic animals or whether physiological diffusing capacity is reduced in these animals requires further investigation.

**AT2 cell morphology and surfactant protein expression.** Insulin regulates surfactant phospholipid content and protein expression. In streptozotocin-treated rats, triglyceride deposits were observed in the pulmonary artery and lung tissue, the relative phospholipid content was reduced compared with control lungs (54); insulin therapy normalized these parameters (53). Ultrastructure of AT2 cells was disrupted in streptozotocin-diabetic rats, with dilated rough endoplasmic reticulum (49), reduced biosynthesis and impaired stimulated secretion of surfactant (8, 67), which were restored by in vivo insulin treatment. Fetal lung maturation was delayed in offspring of streptozotocin-diabetic rats (66), associated with decreased activity of phosphatidylcholine synthetic enzymes (60), reduced phosphatidylglycerol synthesis in diabetic rat offspring (4, 6), and delayed degradation of glycogen stores (14, 18). In human pregnancy, poorly controlled maternal diabetes is associated with low amniotic levels of lung phospholipids (65, 79) and delayed appearance of phospholipid markers of fetal lung maturity (40, 48).

Dilated endoplasmic reticulum in AT2 cells has been reported in rat models of T1DM in association with an apparently reduced number of LB that are thought to reflect surfactant protein dysregulation (49, 64). In our fa/fa lungs, we observed irregular protein pools lacking imidazole contrast enhancement that are similarly located within massively dilated endoplasmic reticulum of AT2 cells. The identity of the pooled substance is currently unknown. Surfactant mRNA and protein expression are differentially regulated in T1DM. SP-A mRNA transcription was considerably enhanced in lungs of streptozotocin-treated diabetic rats, but immunohistochemical labeling of SP-A in AT2 cells was weak compared with control lungs (63). SP-B mRNA expression was also increased in AT2 cells of diabetic rats, whereas SP-C transcription declined (62). Surfactant protein and message were either reduced (39) or delayed (20, 21) in fetal and neonatal lungs of offspring of streptozotocin-treated rats. In contrast to T1DM, circulating SP-A level was significantly higher in patients with insulin resistance compared with normal subjects (16). Surfactant dysregulation also occurs in diet-induced obesity. Disaturated phosphatidylcholine, SP-A, and SP-B protein were elevated in surfactant aggregates from obese animals along with increased levels of phospholipid and triglyceride in lung tissue (27). In our study, the elevated SP-A and SP-C expression following onset of T2DM in fa/fa lungs correlated with increased tissue triglyceride content, LB volume, and protein pooling in AT2 organelles, suggesting possible overactive surfactant production. With longer diabetic duration, SP-C expression and LB volume normalized, but intracellular AT2 protein pooling increased, indicating partial adaptation.

In addition to a strain-specific developmental increase in $\tau_{hb}$, strain-specific differences in surfactant protein expression were also observed. In normal lung tissue from $+/+$ Zucker animals, SP-A level progressively increased from 14 to 52 wk, in contrast to the reported SP-A ontogeny in Sprague-Dawley rats where adult SP-A levels were achieved by postnatal day 28 (56). In fa/fa lungs, the maturational increase in SP-A level was accelerated, with maximal SP-A expression achieved at 14 wk. In $+/+$ animals, SP-C expression profile showed age-related increases between 14 and 52 wk, but in fa/fa lungs the expression of proprotein processing intermediates were elevated at 14–18 wk and then declined with age. The elevation of SP-A and SP-C immunoreactivity and increased LB volume in obese T2DM contrasts with their correspondingly reduced expression and volume in T1DM; differences reflect the divergent pathogenesis in T1DM and T2DM as well as the adipogenicity of the fa/fa genotype. In T1DM, surfactant translation appears defective and/or protein degradation accelerated, whereas in fa/fa animals a burst of overactive surfactant protein expression following the onset of T2DM suggest responses to increased substrate stores and a proinflammatory state related to lipooxidative stress (7, 16, 30). These mechanisms will require further examination.

In conclusion, ultrastructural abnormalities in the distal lung of obese diabetic fa/fa animals reflect additive effects of hyperglycemia and lipid loading; some ultrastructural features are similar to those reported in experimental T1DM, whereas others and the changes in SP-A and SP-C expression are distinct. The major abnormalities are intra- and extracellular lipid accumulation, increased interstitial cells, connective tissue fibers, and matrix content within alveolar septa, increased resistance of the air-blood barrier to diffusion, as well as age-specific increases in SP-A and SP-C protein expression. These perturbations could contribute to the impairment of lung mechanics and alveolar gas exchange reported in human T2DM and could accelerate functional decline with ageing or in the presence of primary pulmonary disease.

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**DISCLOSURES**

No conflicts of interest are declared by the author(s).

**REFERENCES**