Published in final edited form as:

Acta Physiol (Oxf). 2015 April ; 213(4): 933–946. doi:10.1111/apha.12419.

Alveolar-capillary Adaptation to Chronic Hypoxia in the Fatty Lung

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Abstract

Aim—Obese diabetic (ZDF fa/fa) rats with genetic leptin resistance suffer chronic lipotoxicity associated with age-related lung restriction and abnormal alveolar ultrastructure. We hypothesized that these abnormalities impair adaptation to ambient hypoxia.

Methods—Male fa/fa and lean (+/+) ZDF rats (4-months old) were exposed to 21% or 13% O₂ for 3 weeks. Lung function was measured under anesthesia. Lung tissue was assayed for DNA damage and ultrastructure measured by morphometry.

Results—In normoxia, lung volume, compliance, and diffusing capacity were lower while blood flow was higher in fa/fa than +/+ rats. In hypoxia, fa/fa animals lost weight, circulating hematocrit rose higher, and lung volume failed to increase compared to +/+. In fa/fa, the hypoxia-induced increase in postmortem lung volume was attenuated (19%) vs. +/+ (39%). Alveolar ducts were 35% smaller in normoxia but enlarged two-fold more in hypoxia compared to +/+. Hypoxia induced broad increases (90–100%) in the volumes and surface areas of alveolar septal components in +/+ lungs; these increases were moderately attenuated in fa/fa lungs (58–75%), especially that of type-II epithelium volume (16% vs. 61% in +/+). In fa/fa compared to +/+ lungs, oxidative DNA damage was greater and hypoxia induced efflux of alveolar macrophages. Harmonic mean thickness of the diffusion barrier was higher, indicating higher structural resistance to gas transfer.

Conclusion—Chronic lipotoxicity impaired hypoxia-induced compensatory lung expansion and growth with disproportionate effect on resident alveolar progenitor cells. The moderate structural impairment was offset by physiological adaptation primarily via a higher hematocrit.

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Conflict of Interest

None

Keywords

High altitude adaptation; lung structure; lung function; metabolic syndrome; morphometry; obesity

Introduction

Obesity is associated with chronic lipid infiltration of extra-adipocyte tissues leading to a pro-inflammatory state and end-organ damage (Unger and Zhou, 2001, Unger and Scherer, 2010). In the lung, obesity causes abnormal ventilatory control and restrictive mechanics (O'Donnell et al., 2000); the impairment in lung function is independent of physical activity or fitness (Leone et al., 2009, Lin et al., 2006, Steele et al., 2009, van Huisstede et al., 2013). Obesity often co-exists with type-2 diabetes mellitus; both reflect a state of tissue hypoxia (Pasarica et al., 2009, Girgis et al., 2012) that heightens susceptibility to tissue injury and may aggravate existing organ dysfunction (Watz et al., 2009). Because normal alveolar microvascular reserves are extensive, significant alveolar lipotoxicity remains subclinical under basal conditions and is under-recognized as a pathological entity. However, lipotoxicity diminishes alveolar-capillary reserves, which may interfere with adaptation to additional challenges such as ageing, concurrent lung disease, cardio-renal co-morbidity or hypoxic stress, thereby causing or exacerbating morbidity. For example, obesity increases the risk of acute mountain sickness in human subjects (Ri-Li et al., 2003, Wu et al., 2007), suggesting the failure to acclimatize to a hypoxic environment.

On the other hand, hypoxia is a primitive and universal stimulus for the growth and remodeling of gas exchange organs. While extreme hypoxia suppresses metabolic function and causes cell death, postnatal exposure to moderate hypoxia (equivalent to 3,100–3,800m altitude) accelerates alveolar tissue and capillary growth and acinar remodeling in various mammals (Reinke et al., 2011, Lechner and Banchero, 1980, Burri and Weibel, 1971b, Johnson et al., 1985, Hsia et al., 2005) as a compensatory mechanism for preserving O₂ uptake. The interactions between obesity and hypoxia on the structure and function of the distal lung have not been examined.

To characterize the effects of lipotoxicity on alveolar-capillary structure and function during hypoxia challenge, we utilized the Zucker diabetic fatty (ZDF) rat model (Yilmaz et al., 2010, Foster et al., 2010), which originated in outbred Zucker fatty (*fa/fa*) rats carrying the *fa* mutation, an amino acid substitution in the extracellular domain of the leptin receptor that renders the animal insensitive to leptin, leading to hyperphagia, diet-induced obesity, extra-adipocyte fat infiltration, systemic pro-inflammatory stress, end-organ damage and a shortened life span (Unger and Zhou, 2001, Unger and Scherer, 2010). The type-2 diabetic trait develops in an inbred substrain of obese *fa/fa* males [ZDF/Drt-*fa*] receiving at least 6% dietary fat; hyperglycemia develops by age ~12 weeks as insulin production and peripheral insulin sensitivity decline (Shimabukuro et al., 1997, Shimabukuro et al., 1998). The lungs of *fa/fa* animals show baseline restrictive changes (Yilmaz et al., 2010, Yilmaz et al., 2014) resembling that in clinical metabolic syndrome. The distal lung exhibits age-related thickening of alveolar septa and capillary basement membrane, cellular hyperplasia,

elevation of triglyceride content, infiltration of lipid-laden macrophages, increased connective tissue elements, and altered surfactant protein profiles (Foster et al., 2010). Here we tested the hypothesis that these obesity-associated abnormalities impair alveolar adaptation during exposure to ambient hypoxia.

Materials and methods

Animals and exposure

The Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center approved the protocol. Male ZDF diabetic fatty (fa/fa, n=7) and lean ZDF control (+/+, n=8) rats (4mo old), bred in the Unger laboratory, were fed rodent chow containing 6.5% fat (Formulab diet 5008, Purina, St. Louis, Missouri) *ad libitum* and had free access to water. The diabetic phenotype was confirmed by tail vein blood glucose level (557 ± 50 and 102 ± 13 mg.dL⁻¹ in fa/fa and +/+, respectively). The animals were exposed to 13% ambient O₂ (hypoxia, equivalent to ~3,800m high altitude) in an environmental chamber (Biospherix, Lacona, NY) for 3 weeks. The chamber was opened for no more than 20min each day for animal feeding and maintenance. The buildup of CO₂ and moisture was prevented using soda lime. Body weight was measured each week. Hematocrit was measured from tail vein blood. At the end of exposure, the animal was fasted 3 hr, then deeply anesthetized with an intraperitoneal injection of ketamine (100 mg/kg), xylazine (10 mg/kg) and acepromazine (2.0 mg/kg) to suppress spontaneous respiration. The trachea was cannulated via a midline neck incision, and the cannula tied securely with silk suture. Mechanical ventilation (CIV 101, Columbus Instruments, Columbus, OH) was maintained (tidal volume 10 mL/kg, respiratory rate 90 breaths per min). Heart rate and transcutaneous O₂ saturation were monitored via a tail probe (Vet/Ox G2, West Yorkshire, UK). Cardiopulmonary function was measured followed by euthanasia and lung harvest (*described below*). Results were compared to age- and gender-matched control animals exposed to normoxia (21% O₂, fa/fa n=18, +/+ n=12) (Table 1). All animal procedures were performed within a span of 6 months by the same personnel using the same methods. Postmortem tissue analysis was first completed in the normoxia groups (+/+ and fa/fa); these results (lung function and morphometry) have been published (Foster et al., 2010, Yilmaz et al., 2010). Due to personnel constraints and other laboratory priorities, data analysis of hypoxia-exposed groups (+/+ and fa/fa) was completed later and the results reported here.

Apparatus

As described previously (Yilmaz et al., 2010), the tracheal cannula was attached via a manifold to the ventilator, a 10–20 mL glass syringe, and 2 three-way computer-controlled solenoid valves (GH3315, Hans Rudolph, Kansas City, MO). Total apparatus dead-space was 0.5 mL. Airway pressure was recorded using a pressure transducer (Amplifier 1 Model 1100, Hans Rudolph, Kansas City, MO) calibrated against a manometer before each experiment. Data were acquired (100 Hz) using a laptop computer and LabView software (National Instruments, Austin, TX).

Static airway pressure–lung volume (PV) relationship

A glass syringe containing the desired volume of air was used to inflate the lungs from end-expiration. Each inflation was held for ~6 s before exhalation and resumption of mechanical ventilation for 10 to 15 s before initiating the next inflation step. Mean airway pressure during the 3rd second after reaching peak pressure was recorded. The maneuver was repeated at incremental inflation volumes (10 mL/kg steps) until airway pressure exceeded 30 cmH₂O then repeated in stepwise volume decrements. Duplicate measurements at each volume were averaged.

Rebreathing measurements

A test gas mixture (0.3% CO, 0.5% Ne, 0.8% C₂H₂, 40 or 90% O₂ in balance of N₂) was drawn into a Mylar reservoir bag and the initial gas concentrations measured by a gas chromatograph (CP-4900 Micro GC, Varian Inc, CA) with 2 columns (M5AHIBF and PPUHI) and a thermal conductivity detector using 100% high purity helium as a carrier gas. A volume of the test gas equal to the inflation volume at 30cmH₂O of airway pressure (11.8±1.1 and 15.3±2.3 mL in fa/fa and +/- animals, respectively, mean±SD) was drawn into the glass syringe and attached to the manifold. The ventilator was stopped to allow exhalation and valve switching. Rebreathing was conducted by gently pumping the syringe at 60 strokes per minute to mix the test gas with resident lung gas for 5, 7 or 9 s in separate maneuvers in random order. The precise rebreathing duration was determined from the change in airway pressure signals with valve opening or closing. Final gas concentrations in the syringe were measured immediately. Duplicate measurements were made at each inspired O₂ tension in random order. The interval between successive rebreathing maneuvers was at least 2 min. At the end, CO backpressure was measured by rebreathing 30 mL of 100% O₂ for 60 s.

Euthanasia, open chest PV curves, and tissue harvest

Following the above procedures, the thoracic cavity was opened to expose the lungs and heart, and the PV measurements repeated as described above. Blood was drawn by cardiac puncture to measure hematocrit. The right lung was clamped at the hilum, and the heart stopped by an intracardiac overdose injection of Euthasol™. The left lung was perfused with oxygenated PBS, removed and snap frozen in liquid nitrogen. The clamp was removed and the right lung fixed via tracheal instillation of 2.5% buffered glutaraldehyde at 25cmH₂O of hydrostatic pressure. Liver and kidney were removed as control organs.

Triglyceride content analysis

Lung and liver tissue (50–100mg) were homogenized in 2:1 chloroform-methanol, and lipids were extracted for 1 h at room temperature with intermittent vortexing. Volume of distilled water (2X) was vortexed into the homogenates and the mixture centrifuged (2,000g). The lipid extract was withdrawn, and 50–600µL was dried completely. The dried samples were mixed with 30µL of t-butanol, 20µL of 1:1 Triton X-100-methanol, and 1mL of a 4:1 mixture of Free Glycerol Reagent: Triglyceride Reagent (Sigma-Aldrich, St. Louis, MO), and the absorbance read (540nm). Triglyceride content was calculated from a glycerol

standard curve (Sigma-Aldrich) and expressed in each animal as the mean milligrams triglyceride per gram of tissue in duplicate samples.

DNA damage

Lung 8-hydroxy-2'-deoxyguanosine (8-OHdG) level was measured as a marker of oxidative DNA damage. DNA was extracted using DNAzol (Life Technologies, Grand Island, NY), precipitated in 100% ethanol, washed with 70% ethanol, suspended in 8mM NaOH, and the 8-OHdG concentration determined by ELISA (OxiSelect™ Oxidative DNA damage Cell BioLabs, San Diego, CA) compared with that of a standard curve.

Estimation of lung fluid

Lung tissue (~100mg) was weighed and transferred to a platinum ashing crucible and placed on a hot plate set at 100°C under a heat lamp for 2h. Then, the dry sample-containing crucible was weighed to determine the sample dry weight and the dry-to-wet-weight ratio. The crucible was placed overnight in the ash oven at 600°C, removed and weighed to determine the ash weight. The ash was dissolved in 2 mL of HCl and the sodium content measured by flame photometry. Sodium-to-dry weight ratio serves as a surrogate marker for the relative amount of interstitial fluid (extracellular fluid sodium concentration 135–140 mM; intracellular sodium concentration 10–15 mM) (Ravikumar et al., 2014).

Lung volume estimation and sampling

Volume of the intact fixed right lung was measured by saline immersion (Yan et al., 2003). The lung was serially sectioned (3mm 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 (Yan et al., 2003); this tension-free volume was used in subsequent morphometric calculations. Lung slices were divided into roughly equal cranial and caudal regions. Using a systematic sampling scheme with a random start, 2 tissue blocks were sampled from each region (total of 4 blocks per lung), post-fixed with 1% osmium tetroxide in 0.1M cacodylate buffer, treated with 2% uranyl acetate, dehydrated through graded alcohol, and embedded in Spurr (Electron Microscopy Sciences, Hatfield, PA). Each block was sectioned (1µm) and stained with toluidine blue.

Morphometric analysis

A stratified analytical scheme was used (Foster et al., 2010, Hsia et al., 2010): low-power light microscopy (LM; 275×), high power LM (550×) and transmission electron microscopy (TEM; ~19,000×). One section per block was overlaid with a test grid at the appropriate magnification. From a random start, at least 20 non-overlapping microscopic fields per block (80 per lung) were systematically examined at 275× magnification. Using point counting, conducting structures larger than 20µm in diameter, i.e., bronchioles and blood vessels, were excluded from the reference space to estimate the volume density of fine parenchyma, alveolar ducts and sacs per unit lung volume. At 550× magnification, 15 non-overlapping microscopic fields per block (60 per lung) were systematically imaged to estimate the volume density of alveolar septa per unit lung volume.

For TEM, 2 blocks per region (4 blocks per lung) were sectioned (70nm thickness), mounted on copper grids, and examined at ~19,000× (JEOL EXII). Thirty non-overlapping EM fields per grid were systematically sampled (total 120 per lung). The volume densities of epithelium, interstitium, and endothelium were estimated by point counting. Alveolar epithelial and capillary surface densities per unit septum volume were estimated by intersection counting. About 300 points/intersections were counted per grid with a coefficient of variation <10%. The volume and surface densities at each level were related through the cascade of levels to lung volume measured by the Cavalieri method, to obtain absolute volumes and surface areas. The lengths of test lines (l) that transect the tissue-plasma barrier (from epithelial surface to erythrocyte membrane) were measured to estimate the harmonic mean thickness of the tissue-plasma barrier (τ_{hb}), an index of diffusive resistance.

Data analysis

Lung volumes were expressed at BTPS conditions. The PV curves were analyzed as described by Salazar and Knowles (Salazar and Knowles, 1964). Compliance of the respiratory system and the lung were calculated between airway pressures of 10–20 cmH₂O. Total system volume was calculated from Ne dilution during rebreathing; from this volume the apparatus dead space was subtracted to obtain end-inspiratory lung volume (EILV), then the syringe volume was subtracted to obtain end-expiratory lung volume (EELV). Pulmonary blood flow and DL_{CO} were calculated from the simultaneous logarithmic disappearance of C₂H₂ and CO, respectively, with respect to Ne. Complete gas mixing was achieved rapidly during rebreathing; Ne concentration stabilized within 5 s while C₂H₂ and CO concentrations declined in a log linear fashion with respect to time (Yilmaz et al., 2010). Membrane diffusing capacity (DM_{CO}) and pulmonary capillary blood volume (V_c) were estimated from DL_{CO} measured at two alveolar O₂ tensions (P_{AO2} in mmHg) using the Roughton-Forster relationship (Roughton and Forster, 1957):

$$\frac{1}{DL_{CO}} = \frac{1}{DM_{CO}} + \frac{1}{\theta_{CO} \cdot V_C} \quad \text{Eq. 1}$$

where θ_{CO} is the empirical rate of CO uptake by whole blood at 37°C in [mL CO·(min·mmHg·mL blood)⁻¹] estimated from the mean P_{AO2} during rebreathing. Because the relationship between θ_{CO} and O₂ tension is not available for rodents, we used the values obtained by Holland (Holland, 1969) for dog blood ($\lambda=1.6$):

$$\frac{1}{\theta_{CO}} = (0.929 + 0.00517 \cdot P_{AO2}) \cdot \frac{0.45}{Hematocrit} \quad \text{Eq. 2}$$

DM_{CO} and V_c were used to calculate the DL_{CO} at a constant P_{AO2} (120 mmHg) and hematocrit (0.45). Estimates of DL_{CO}, DM_{CO} and V_c were plotted with respect to blood flow. The timing of the C₂H₂ disappearance curve was corrected by assuming that the CO disappearance curve intercepted at unity at time zero (Sackner et al., 1980). Septal tissue volume, which includes extravascular tissue and microvascular blood in the gas exchange region, was estimated from the extrapolated intercept of C₂H₂ disappearance curve.

Because body weight differences between fa/fa and +/+ animals predominantly reflected the amount of adipose tissue, we expressed the data (mean $\pm$ SD) in absolute values without normalizing by body weight. Statistical comparisons were performed with respect to genotype and O₂ exposure by factorial ANOVA. The PV relationships were compared by repeated measures ANOVA. To assess relative responses, the measurements in hypoxia-exposed animals were also expressed as ratios to the corresponding mean values in normoxia-exposed controls of the same genotype, and the ratios compared between genotypes by factorial ANOVA. A p value of 0.05 was considered significant.

Results

Physiological measurements

Physiological and morphometry data in normoxia-exposed animals (fa/fa and +/+) were taken from our previous publications (Foster et al., 2010, Yilmaz et al., 2010). Both normoxia and hypoxia studies were performed within a span of 6 months using the same experimental set up and methodology. The level of hypoxia was well tolerated without complications. Cardiopulmonary function is summarized in Table 1 and Figures 1–2. Following hypoxia exposure compared to normoxia, body weight decreased significantly more in fa/fa than +/+ animals (16% and 7%, respectively). Systemic hematocrit increased significantly more in fa/fa than +/+ animals (26% and 12%, respectively). Mean P_{AO2} (measured during rebreathing while rebreathing 40% inspired O₂) was mildly (5–8%) lower in fa/fa than +/+ animals. Mean lung volumes were consistently lower in fa/fa than +/+ animals. In hypoxia compared to normoxia, EILV was unchanged in +/+, but significantly (~10%) lower in fa/fa animals while EELV increased in +/+ but remained unchanged in fa/fa animals. The PV relationships (Figure 2A) showed mildly depressed (closed chest) to unchanged (open chest) lung volume at a given airway pressure in fa/fa compared to +/+ animals in normoxia, but consistently lower lung volume in fa/fa animals following hypoxia (open and closed chest). Lung compliance was not altered by hypoxia in +/+, but declined significantly (11–16%) in fa/fa animals. Lung compliance was 15–26% lower in fa/fa than +/+ animals following hypoxia exposure (Figure 2B). Pulmonary blood flow was (24%) higher in fa/fa than +/+ animals in normoxia, and declined similarly (34–36%) in both genotypes in hypoxia (Figure 1). DL_{CO} in normoxia was modestly lower (14%) in fa/fa than +/+ animals. In hypoxia DL_{CO} was unchanged in +/+ animals but 29% higher in fa/fa animals. DL_{CO} per unit of blood flow (DL_{CO}/Q_c) was lower in fa/fa than +/+ animals but increased following hypoxia exposure in both genotypes. DM_{CO} and V_c were not significantly altered by hypoxia exposure in either genotype (Table 1).

Triglyceride content

Triglyceride content was significantly higher in liver than lung tissue (liver/lung ratio of 8.3 in +/+ and 23.5 in fa/fa, Figure 3). In normoxia, triglyceride content was elevated in fa/fa animals (by 136% in lung and 494% in liver above the corresponding +/+ level); the increase was further exaggerated in hypoxia (219% and 791% higher in lung and liver, respectively, above +/+ levels). In +/+ animals, hypoxia exposure reduced triglyceride content in both liver and lung (by 61% and 30%, respectively) compared to normoxia. In

fa/fa animals, hypoxia significantly reduced triglyceride content of the liver (by 41%, $p<0.05$) but not the lung (5%, $p>0.05$).

DNA damage and lung fluid

In both normoxia and hypoxia, the 8-OHdG content in lung tissue was elevated by 30–31% in fa/fa compared to +/+ controls, indicating greater oxidative DNA damage (Figure 4). In both genotypes, 8-OHdG content was ~192% higher in hypoxia than in normoxia. The dry/wet weight ratios were not significantly different between +/+ and fa/fa animals in normoxia (0.06 ± 0.02 and 0.09 ± 0.04 , respectively) or hypoxia (0.10 ± 0.02 and 0.11 ± 0.02). Similarly, the sodium/dry weight ratios ($\text{mEq.L}^{-1}.\text{g}^{-1}$) were not significantly different between +/+ and fa/fa animals in normoxia (0.83 ± 0.27 and 0.63 ± 0.24 , respectively) or hypoxia (1.02 ± 0.17 and 0.99 ± 0.16).

Structural analysis

Volume of the fixed right lung was similar between genotypes in normoxia, but the expected increase following hypoxia exposure was less in fa/fa than +/+ animals (19% vs. 39%, respectively, Table 2). Distal lung morphology (Figure 5) showed smaller airspaces in fa/fa animals in normoxia. The airspaces enlarged following hypoxia exposure in both genotypes. Significant cellular influx was observed in the airspace of fa/fa but not +/+ lungs. Following hypoxia exposure, the increase in alveolar duct volume was greater in fa/fa than +/+ animals (3.0 vs. 2.0 fold, respectively) whereas alveolar sac volume increased less (1.3 vs. 1.8 fold, respectively) (Figure 6). Morphometric capillary hematocrit and arithmetic mean septal thickness were not significantly different among exposures or genotypes (Table 2). The index of diffusive resistance of the blood-gas barrier, τ_{hb} , was elevated in both genotypes following hypoxia exposure, and was higher in fa/fa than +/+ animals in both normoxia and hypoxia.

In hypoxia compared to normoxia, the volume densities of fine parenchyma, alveolar ducts and sacs, interstitial cells and matrix, endothelium, and alveolar and capillary surface areas per unit of lung volume increased (by 20–25%, 33–199%, 75–103%, 51–58%, 38–39%, 48–50%, respectively) in both genotypes. Volume density of alveolar septum was not different between genotypes in normoxia, but 30% higher in fa/fa animals following hypoxia (Table 3). Similarly, the volume density of interstitium was not different between genotypes in normoxia, but 40% higher in fa/fa animals following hypoxia. Volume density of type II epithelium was unchanged in both genotypes. Alveolar surface density per unit of lung volume was not different between genotypes in normoxia but increased significantly in hypoxia in both genotypes. Capillary surface density was higher in fa/fa than +/+ animals in normoxia and hypoxia (Table 3).

The absolute volumes of most septal components, with the notable exception of type II epithelium in fa/fa animals, increased significantly (43–140%) following hypoxia exposure in both genotypes (Table 4). Compared to the corresponding values in normoxia, the relative increases of most parameters in hypoxia were mildly but consistently attenuated in fa/fa than +/+ animals ($p<0.05$ by repeated measures ANOVA, Figure 7). In both genotypes the volume of interstitial cells and matrix exhibited the largest hypoxia-induced increase

(~140%). In contrast, the volume of type II epithelium increased 61% in +/+ animals but non-significantly (16%) in fa/fa animals after hypoxia exposure.

Discussion

Summary of results

This study examined alveolar-capillary adaptation to hypoxia in the ZDF rat model of lipotoxicity. As expected, lung triglyceride content was elevated in fa/fa than +/+ lungs. Hypoxia exposure reduced triglyceride content in the liver of both +/+ and fa/fa animals (61 and 41% respectively) and in the +/+ lung (30%), but not the fa/fa lung. Oxidative DNA damage increased ~190% following hypoxia exposure in both genotypes, and was ~30% higher in fa/fa compared to +/+ lungs regardless of O₂ exposure. These results show ectopic lipid infiltration and heightened oxidative damage in fa/fa lungs in normoxia with added oxidative stress damage in hypoxia exposure.

The expected hypoxia-induced loss of body weight and increase in circulating hematocrit were more pronounced in fa/fa than +/+ animals. Following hypoxia, lung volume and compliance were increased or unaltered in +/+ animals whereas they were reduced in fa/fa animals. Owing to a significantly higher hematocrit and a slightly higher blood flow, resting DL_{CO} was normal in fa/fa animals following hypoxia exposure.

The expected hypoxia-induced increase of postmortem lung volume was blunted in fa/fa lungs. The smaller alveolar ducts in fa/fa lungs in normoxia enlarged more in hypoxia compared to +/+ lungs, suggesting greater airway smooth muscle relaxation. Hypoxia exposure induced accumulation of extra-septal alveolar macrophages in fa/fa lungs, consistent with a pro-inflammatory state. The expected hypoxia-induced gain in tissue-capillary volumes and surface areas were modestly though consistently blunted in fa/fa compared to +/+ lungs (Figure 7); in particular, the volume increase in type II epithelium was disproportionately impaired. Results suggest that lipotoxicity impairs hypoxia-induced lung expansion and growth of alveolar septal components especially that of the resident epithelial progenitor cells. The dry/wet and sodium/dry lung weight ratios and the gain in interstitial cell and matrix volumes following hypoxia were not different between +/+ and fa/fa genotypes, which argue against significant interstitial edema in fa/fa animals.

Obesity and the lung

Lung function is inversely related to body mass index (McClean et al., 2008). Obesity broadly alters respiratory chemosensitivity (Buyse et al., 2003) and predisposes to hypoventilation, sleep disordered breathing, airway hyperreactivity and obstruction (Lam et al., 2010, Agrawal et al., 2011), among other complications. Obesity impacts the distal lung in at least two ways: Fat accumulation restricts thoracic and lung expansion via mechanical loading while ectopic lipid infiltration of tissue produces inflammation and oxidative stress. Infiltrative lipotoxicity is ascribed to leptin resistance causing excess fatty acid spillover into non-adipocytes, leading to the generation of reactive oxygen species, oxidative and endoplasmic reticulum stress, and a pro-inflammatory phenotype with disruption of cellular homeostasis, impairment of insulin signaling and blunting of progenitor cell proliferation

(Dali-Youcef et al., 2013, Unger and Scherer, 2010). Lipotoxicity derives mainly from long-chain nonesterified fatty acids and their products such as diacylglycerols and ceramides that downregulate mitochondrial and peroxisomal enzymes of beta oxidation (Cutz et al., 2013). The net result is cellular dysfunction and cumulative tissue injury.

Adaptation to hypoxia

In the metabolic syndrome, moderate hypoxia exposure has no adverse effect and may even improve glucose metabolism (Schobersberger et al., 2003, Schobersberger et al., 2005, Marquez et al., 2013). Active sojourn at a modest altitude (1,500–2,500m) improves hypertension and dyslipidemia, probably due to weight loss (Schobersberger et al., 2010). However, at higher altitudes (3,658 m) obesity increases the risk of acute mountain sickness (Ri-Li et al., 2003, Wu et al., 2007), suggesting failure to acclimatize. In addition, ambient hypoxia inhibits lipogenesis and induces lipolysis as well as fatty acid oxidation in adipocytes with the net effect of impairing adipocyte buffering capacity (O'Rourke et al., 2013), which in turn may exacerbate systemic lipotoxicity.

Ambient hypoxia stimulates the growth and remodeling of gas exchange organs throughout the animal kingdom as a compensatory mechanism for augmenting oxygen uptake. Non-extreme chronic hypoxia accelerates postnatal alveolo-capillary growth and remodeling in mice and rats (Burri and Weibel, 1971b, Reinke et al., 2011, Burri and Weibel, 1971a), guinea pigs (Hsia et al., 2005, Lechner and Banchero, 1980) and dogs (Johnson et al., 1985). Chronic hypoxia induces VEGF expression (Tuder et al., 1995) and Rho-kinase dependent angiogenesis in adult rat lung (Hyvelin et al., 2005, Howell et al., 2003), indicating plasticity of the mature alveolar microvasculature. The mechanisms underlying hypoxia-induced alveolar growth and remodeling are not well understood but likely involve the hypoxia-inducible factor pathways (Asikainen et al., 2006, Zhang et al., 2006).

In fa/fa but not +/- rats exposed to hypoxia, we observed prominent weight loss similar to that in obese people (Lippl et al., 2010). In hypoxia, the +/- animals exhibited significant acinar airway dilatation as well as increased lung air and tissue volumes and surface areas, consistent with airway smooth muscle relaxation and compensatory alveolar growth, respectively. Hypoxia reduces airway smooth muscle tone partly via limiting calcium entry into cells (Vannier et al., 1995, Wetzal et al., 1992, Julia-Serda et al., 1993); the bronchodilation effect extends throughout the bronchial tree down to the distal intra-acinar airways as seen in fa/fa lungs where the normally smaller alveolar ducts became more dilated in hypoxia. The hypoxia-induced gains in septal volumes and surface areas were consistently lower in fa/fa compared to +/- lungs, especially for type II epithelium volume. Taken together, the data indicate a modestly blunted parenchyma response to hypoxia in fa/fa lungs. In addition, the elevated 8-OHdG level and prominent efflux of alveolar macrophages into the alveolar airspace in hypoxia-exposed fa/fa animals signal heightened oxidative DNA damage and pro-inflammatory response. The blunted response of resident alveolar progenitor cells to hypoxia is consistent with reports of greater loss of neuronal progenitor cells (Park et al., 2011) and apoptosis of endothelial progenitor cells (Lembo et al., 2012) associated with lipotoxicity, suggesting that fa/fa lungs may be more susceptible to injury or slower to repair.

The +/+ animals exhibit the expected physiological responses to hypoxia – elevated systemic hematocrit and lung volumes (Barer et al., 1978), unchanged to increased lung compliance (Raymond and Severinghaus, 1971), and downregulated cardiac output (Yilmaz et al., 2007). In fa/fa animals, the baseline lung and thoracic restriction in normoxia is aggravated by hypoxia. These animals are also known to exhibit blunted hypoxic ventilatory response (Lee et al., 2005, Nakano et al., 2001). Both factors could aggravate arterial hypoxemia and polycythemia during hypoxia exposure. The possibility of mild hypoxia-induced interstitial pulmonary edema cannot be ruled out, although we did not find significant differences between +/+ and fa/fa genotypes in dry/wet and sodium/dry lung weight ratios in normoxia or hypoxia, suggesting that fa/fa animals are not more prone to interstitial edema at this moderate level of hypoxia. Furthermore, interstitial edema cannot explain the large hypoxia-induced increases in cellular volumes and surface areas in +/+ animals.

The fa/fa animals consistently exhibit a higher pulmonary blood flow than +/+ animals; this observation is consistent with that seen in obese humans (Vasan, 2003). Following hypoxia exposure, pulmonary blood flow is reduced in both genotypes due to a lower stroke volume. We observed a similarly reduced pulmonary blood flow in conscious non-sedated guinea pigs following 12 mo of residence at 3,800m altitude (Yilmaz et al., 2007), a level of hypoxia comparable to that in the present study. In chronic hypoxia, maximal heart rate, stroke volume, and cardiac output are downregulated (Hsia, 2001). Resting cardiac output is generally preserved in chronic hypoxia, although studies in rodents have shown a significantly lower resting stroke volume with a tendency towards a lower resting cardiac output (Favret et al., 2001, Clancy et al., 1997). It is likely that similar factors, e.g., compartmental fluid shifts and right ventricular pressure overload (Bartsch and Gibbs, 2007), mediate the reduction in resting and maximal stroke volumes in chronic hypoxia. However, hypoxia downregulation of cardiac output is prominent at peak exercise because maximal heart rate is also constrained. In contrast, resting cardiac output may be better preserved because a lower resting stroke volume can be offset by an increase in resting heart rate.

Structurally, membrane gas conductance varies directly with epithelial and endothelial surface areas and inversely with the harmonic mean thickness of the diffusion barrier (Hsia, 2002). In hypoxia-exposed rats, both surface areas and harmonic mean barrier thickness increase concurrently (Tables 2 and 4) and exert opposing effects on membrane conductance, which could explain the lack of a significant change in resting DM_{CO} .

In conclusion, hypoxia challenge in adult fa/fa rats that suffer chronic lipotoxicity accentuates weight loss, aggravates lung restriction, further reduces lung compliance, and moderately impairs compensatory alveolar septal growth. The alveolar resident progenitor (type II) cells are disproportionately affected. These findings, along with the efflux of alveolar macrophages and the elevated tissue oxidative DNA damage, indicate heightened inflammation-injury and possibly impaired repair mechanisms in fa/fa lungs, consistent with the pathophysiology of lipotoxicity reported in other organs (Lee et al., 2007, Unger and Zhou, 2001). To compensate for a suboptimal structural response, fa/fa animals develop greater polycythemia and maintain a slightly higher pulmonary blood flow to protect

alveolar O₂ transfer at rest. It remains to be seen whether these physiological sources of compensation are sufficient to maintain alveolar O₂ uptake during exertion, and whether fa/fa animals are more susceptible to pulmonary edema upon exposure to severe hypoxia. While we examined only a single time point, longer hypoxia exposure may induce overt manifestations of chronic mountain sickness in fa/fa animals. These results are significant in underscoring the lung parenchyma as a target of metabolic derangement, and highlighting the interactions between lipotoxicity and hypoxic stress as additive factors that erode alveolar-capillary reserves, which in turn impacts adaptation to other challenges such as ageing, primary lung disease, or systemic pathology that impair distal lung function. Further studies should elucidate the cellular-molecular basis of these structural abnormalities and establish their translational implications to human obesity.

Acknowledgment

The authors thank Dennis J. Bellotto and Rebecca Camille Brewer for technical assistance, and the staff of the Animal Resources Center at the University of Texas Southwestern Medical Center for veterinary assistance. This research was supported by grants from the American Lung Association (RG-170442-N), the National Institute of Diabetes and Digestive and Kidney Diseases (DK-063242), and the National Heart, Lung and Blood Institute Grants RO1 HL40070 and UO1 HL111146. The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the National Heart, Lung, and Blood Institute or of the National Institutes of Health.

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Figure 1. Systemic hematocrit, end-expiratory lung volume (EELV), pulmonary blood flow, and DL_{CO}, in +/+ and fa/fa animals exposed to normoxia or hypoxia. Mean±SD. P<0.05: * vs. +/+ at the same O₂; § vs. normoxia in the same genotype, by factorial ANOVA.

Figure 2.

Airway pressure–lung volume curves (**A**) and lung compliance between 10 and 20 cmH₂O of airway pressure (**B**) were measured with the chest closed (*upper row*) and open (*lower row*). Mean±SD. P<0.05: * vs. +/+ at the same O₂ level; § vs. normoxia in the same genotype, by repeated measures ANOVA (**A**) and factorial ANOVA (**B**).

Figure 3.

Triglyceride content in the lung (*left*) and liver (*right*) of +/+ and fa/fa animals exposed to normoxia or hypoxia. Mean±SD. $P<0.05$: * vs. +/+ at the same O_2 ; § vs. normoxia in the same genotype, by factorial ANOVA.

Figure 4. 8-hydroxy-2'-deoxyguanosine (8-OHdG) level in the lung of +/+ and fa/fa animals exposed to normoxia or hypoxia. Mean±SD. P<0.05: * vs. +/+ at the same O₂; § vs. normoxia in the same genotype, by factorial ANOVA.

Figure 5. Representative micrographs of normoxia and hypoxia exposed lungs in +/+ and fa/fa animals stained with toluidine blue. Bar= 100µm.

Figure 6.

Postmortem total volumes of the right lung, alveolar ducts, alveolar sacs, and septal interstitial cells and matrix, in +/+ and fa/fa animals exposed to normoxia or hypoxia. Mean $\pm$ SD. * $p < 0.05$ vs. +/+ at the same O_2 ; § $p < 0.05$ vs. normoxia in the same genotype by factorial ANOVA.

Figure 7.

Fold changes in the volumes and surface areas of individual alveolar septal components following hypoxia exposure in +/+ and fa/fa animals, expressed as ratios to the corresponding mean value in the normoxia control group. Int: Interstitial collagen or cells/matrix. For each parameter: * $p < 0.05$ fa/fa vs. +/+, § $p < 0.05$ vs. normoxia (mean 1.0) in the same genotype, by factorial ANOVA. For all parameters: † $p < 0.001$ fa/fa vs. +/+ by repeated measures ANOVA.

Table 1

Physiological measurements

Genotype	fa/fa			
Exposure	Normoxia	Hypoxia	Normoxia	Hypoxia
n	12	8	18	7
Body Weight (g)	383 ± 27	355 ± 38	498 ± 58	416 ± 27 *§
Hematocrit (%)	45.7 ± 1.0	49.6 ± 4.4	44.1 ± 1.9	55.7 ± 5.7 *§
Heart rate (beats·min ⁻¹)	196 ± 39	151 ± 37	175 ± 34	170 ± 22
Mean P _{AO2} (mmHg)				
Rebreathing 40% O ₂	206 ± 12	214 ± 7	195 ± 12	197 ± 19 *
Rebreathing 90% O ₂	584 ± 12	597 ± 11	581 ± 9	568 ± 31 *
Lung compliance (mL·cmH ₂ O ⁻¹)	0.607 ± 0.060	0.664 ± 0.075	0.548 ± 0.065	0.461 ± 0.109 *§
End-expiratory lung volume (mL)	3.89 ± 0.50	5.03 ± 0.52	3.39 ± 0.50	3.26 ± 0.84 *
Total lung capacity (mL)	19.9 ± 1.7	20.8 ± 1.3	17.6 ± 1.5	15.2 ± 2.9 *§
Pulmonary blood flow (mL·min ⁻¹)	45.0 ± 15.0	28.6 ± 11.1	55.9 ± 12.4	37.0 ± 9.7 §
Stroke volume (mL)	0.232 ± 0.054	0.192 ± 0.052	0.346 ± 0.061	0.216 ± 0.017 *§
DL _{CO-std} (mL·[min ⁻¹ ·mmHg] ⁻¹)	0.259 ± 0.075	0.253 ± 0.072	0.224 ± 0.065	0.270 ± 0.096 §
DL _{CO-std} /Q _C (mL·[min ⁻¹ ·mmHg·L] ⁻¹)	6.1 ± 1.6	9.5 ± 3.2	4.1 ± 0.7	7.8 ± 2.9 §
DM _{CO} (mL·[min ⁻¹ ·mmHg] ⁻¹)	0.655 ± 0.323	0.413 ± 0.082	0.597 ± 0.332	0.455 ± 0.125
Pulmonary capillary blood volume (mL)	0.630 ± 0.202	0.512 ± 0.081	0.613 ± 0.221	0.663 ± 0.131
Septal volume (mL)	2.90 ± 1.31	2.19 ± 0.89	2.54 ± 1.22	1.90 ± 0.75 §

Mean±SD. DL_{CO-std}: DLCO expressed under standardized conditions (PAO₂ = 120mmHg and hematocrit = 0.45).

* p 0.05 vs. +/- at the same O₂.

§ p 0.05 (a, p=0.06) vs. normoxia in the same genotype by factorial ANOVA.

Morphometric results

Table 2

Genotype	+/+		fa/fa	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Exposure				
Number of animals	9	8	9	8
Terminal body weight (g)	363 ± 29	355 ± 38	489 ± 57	412 ± 28 * §
Right lung volume (mL)				
Intact (immersion method)	6.09 ± 0.37	8.26 ± 0.89	6.25 ± 0.54	7.40 ± 1.21 * §
Sectioned (Cavalieri method)	5.19 ± 0.29	7.23 ± 0.79	5.42 ± 0.45	6.46 ± 1.02 * §
Morphometric capillary hematocrit (%)	53.8 ± 3.4	55.1 ± 1.6	54.5 ± 6.3	55.4 ± 3.2
Arithmetic mean thickness of septum (µm)	4.75 ± 0.63	4.59 ± 0.31	5.05 ± 0.47	4.80 ± 0.49
Harmonic mean thickness of blood–gas barrier (t_{bg} , µm)	0.575 ± 0.037	0.645 ± 0.035	0.653 ± 0.052	0.716 ± 0.030 * §
Mean±SD.				
* p 0.05 vs. +/+ at the same O ₂ .				
§ p 0.05 vs. normoxia in the same genotype by factorial ANOVA.				

Table 3

Volume and surface densities of septal structures in the right lung

Genotype	fa/fa			
Exposure	+/+		fa/fa	
	Normoxia	Hypoxia	Normoxia	Hypoxia
<i>Volume density per unit lung volume</i>				
Fine parenchyma to lung	0.7888 ± 0.0204	0.9830 ± 0.0345	0.8328 ± 0.0145	0.9974 ± 0.0035
Septum to lung	0.1257 ± 0.0163	0.1663 ± 0.0265	0.1457 ± 0.0184	0.1910 ± 0.0343
Alveolar sac	0.4285 ± 0.0399	0.6810 ± 0.0463	0.5043 ± 0.0244	0.6733 ± 0.0267
Alveolar duct	0.0684 ± 0.0121	0.1201 ± 0.0151	0.0437 ± 0.0116	0.1307 ± 0.0194
Total epithelium	0.0278 ± 0.0047	0.0359 ± 0.0055	0.0305 ± 0.0043	0.0361 ± 0.0077
Type 1 epithelium	0.0137 ± 0.0020	0.0194 ± 0.0028	0.0146 ± 0.0028	0.0204 ± 0.0036
Type 2 epithelium	0.0141 ± 0.0031	0.0166 ± 0.0033	0.0159 ± 0.0018	0.0157 ± 0.0042
Interstitial	0.0250 ± 0.0070	0.0348 ± 0.0058	0.0306 ± 0.0060	0.0428 ± 0.0093
Collagen fibers	0.0202 ± 0.0051	0.0260 ± 0.0044	0.0244 ± 0.0046	0.0303 ± 0.0073
Cells and matrix	0.0049 ± 0.0020	0.0085 ± 0.0016	0.0062 ± 0.0018	0.0126 ± 0.0021
Endothelium	0.0152 ± 0.0023	0.0228 ± 0.0036	0.0169 ± 0.0033	0.0267 ± 0.0066
Extravascular tissue	0.0680 ± 0.0108	0.0929 ± 0.0138	0.0786 ± 0.0103	0.1057 ± 0.0230
Capillary blood	0.0576 ± 0.0089	0.0734 ± 0.0138	0.0671 ± 0.0147	0.0853 ± 0.0126
<i>Surface density per unit lung volume (cm⁻¹)</i>				
Alveolar surface	532 ± 59	732 ± 145	579 ± 78	803 ± 172
Capillary surface	564 ± 60	846 ± 151	646 ± 68	959 ± 189

Mean±SD.

* p 0.05 vs. +/+ at the same O₂.

§ p 0.05 vs. normoxia in the same genotype by factorial ANOVA.

Table 4

Absolute volumes and surface areas in the right lung

Genotype	fa/fa			
	+/+			
Exposure	Normoxia	Hypoxia	Normoxia	Hypoxia
Volume (mL)				
Fine parenchyma	4.10 ± 0.31	7.11 ± 0.81 §	4.51 ± 0.37	6.44 ± 1.02 §
Alveolar ducts	0.447 ± 0.067	0.883 ± 0.135 §	0.286 ± 0.084 *	0.856 ± 0.247 §
Alveolar sacs	2.822 ± 0.326	5.011 ± 0.660 §	3.278 ± 0.293	4.362 ± 0.727 *§
Septum	0.653 ± 0.106	1.199 ± 0.182 §	0.788 ± 0.107	1.222 ± 0.226 §
Epithelium				
Type I	0.071 ± 0.011	0.139 ± 0.019 §	0.079 ± 0.016	0.131 ± 0.026 §
Type II	0.074 ± 0.019	0.119 ± 0.025 §	0.086 ± 0.012	0.100 ± 0.024
Interstitial	0.131 ± 0.045	0.251 ± 0.045 §	0.165 ± 0.033	0.273 ± 0.056 §
Collagen fibers	0.106 ± 0.033	0.188 ± 0.034 §	0.132 ± 0.026	0.193 ± 0.043 §
Cells and matrix	0.026 ± 0.012	0.061 ± 0.012 §	0.033 ± 0.009	0.080 ± 0.013 *§
Endothelium	0.079 ± 0.014	0.164 ± 0.026 §	0.092 ± 0.021	0.170 ± 0.040 §
Extravascular tissue	0.355 ± 0.074	0.674 ± 0.108 §	0.426 ± 0.065	0.674 ± 0.139 §
Capillary blood	0.299 ± 0.046	0.526 ± 0.082 §	0.362 ± 0.077	0.547 ± 0.100 §
Surface area (cm ²)				
Alveolar surface	2773 ± 453	5251 ± 937 §	3131 ± 442	5143 ± 1144 §
Capillary surface	2939 ± 451	6080 ± 1075 §	3504 ± 527	6146 ± 1336 §

Mean±SD.

* p 0.05 vs. +/+ at the same O₂.

§ p 0.05 vs. normoxia in the same genotype by factorial ANOVA.