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Authors

Rehan, Virender K
Torday, John S

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FORUM REVIEW ARTICLE

The Lung Alveolar Lipofibroblast: An Evolutionary Strategy Against Neonatal Hyperoxic Lung Injury

Virender K. Rehan and John S. Torday

Abstract

Significance: Oxygen, the main mode of support for premature infants with immature lungs, can cause toxicity by producing reactive oxygen species (ROS) that disrupt homeostasis; yet, these same molecules were entrained to promote vertebrate lung phylogeny. By providing a deeper understanding of this paradox, we propose physiologically rational strategies to prevent chronic lung disease (CLD) of prematurity. ***Recent Advances:*** To prevent neonatal hyperoxic lung damage biologically, we have exploited the alveolar defense mechanism(s) that evolutionarily evolved to combat increased atmospheric oxygen during the vertebrate water to land transition. ***Critical Issues:*** Over the course of vertebrate lung evolution, ROS promoted the formation of lipofibroblasts, specialized adepithelial cells, which protect the alveoli against oxidant injury; peroxisome proliferator-activated receptor gamma (PPARγ), the master switch for lipofibroblast differentiation, prevents such oxidant lung injury, both by directly promoting mesodermal differentiation and its antioxidant defenses, and indirectly by stimulating the developmental epithelial–mesenchymal paracrine interactions that have physiologically determined lung surfactant production in accord with the lung's phylogenetic adaptation to atmospheric oxygen, preventing Respiratory Distress Syndrome at birth. ***Future Directions:*** The molecular strategy (PPARγ agonists) to prevent CLD of prematurity, proposed by us, although seems to be robust, effective, and safe under experimental conditions, it awaits detailed pharmacokinetic and pharmacodynamic studies for its safe and effective clinical translation to human infants. *Antioxid. Redox Signal.* 21, 1893–1904.

> ''I have procured air [oxygen]…between five and six times as good as the best common air that I have ever met with.''
>
> —Joseph Priestley, 1775

Introduction

OXYGEN THERAPY IS one of the main supportive modalities that is instituted to keep premature infants alive. In view of the compelling experimental and clinical evidence for the involvement of oxidant and antioxidant imbalance in many morbidities associated with prematurity, for example, the chronic lung disease (CLD) of prematurity or bronchopulmonary dysplasia (BPD), retinopathy of prematurity, intra/periventricular hemorrhage, and necrotizing enterocolitis, the safety of this practice has recently been called into question yet again (69). The mechanistic basis for free radical involvement in these disorders is that free oxidant radicals are formed too rapidly to be detoxified by the limited antioxidant defenses of the premature infant, with resultant tissue-specific damage. In contrast to the conventional approach to this problem, we will focus on the dyshomeostasis of the preterm alveolus as a failed evolutionary mechanism (83), which allows us to consider interventions based on phylogenetic adaptations of the lung to atmospheric oxygen.

The fetus develops in a relatively hypoxic environment *in utero*, its adaptive responses well suited to the relatively low fetal oxygen saturation state, but ill-suited for a smooth and safe transition of the prematurely delivered infant to

Department of Pediatrics, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, California.

extrauterine life in a relatively hyperoxic environment. Although there are plenty of animal data suggesting the developmental increases in the expression of antioxidant enzymes during fetal lung development, with the exception of limited data supporting a developmental increase in the expression of lung catalase activity, there are very few human studies that have directly examined the expression of antioxidant enzymes during human lung development (52). In fact, some studies have failed to demonstrate any late fetal surges in pulmonary superoxide dismutase and glutathione peroxidase activities in human tissues (71). On the other hand, there is no doubt that significantly lower levels of antioxidant enzymes have been observed in many studies in premature infants, and their impaired ability to adequately upregulate antioxidant enzymes in response to oxidant stresses, making them highly susceptible to oxidant injury. Therefore, premature infants are almost certainly developmentally unprepared for extrauterine life in an oxygen-rich environment, and exhibit a unique sensitivity to oxidant injury. Furthermore, the greater the prematurity, the higher is the associated risk of oxidant injury.

Antioxidant Defenses and Neonatal Hyperoxic Lung Injury

CLD of prematurity, or BPD, although multifactorial in its pathogenesis, is strongly linked to the exposure of the premature infant to supraphysiologic concentrations of oxygen (59, 65). Although it is amply clear that through evolution organisms have adapted to reactive oxygen species (ROS) for various physiologic intracellular and intercellular signals, high oxygen concentrations induce oxidative stress by promoting superoxide anion ($O_2^{\bullet -}$) generation in mitochondria and peroxisomes, the organelles specialized in the oxidation of fatty acids (25), as well as a consequence of phagocyte activation. Superoxide, in turn, initiates a chain reaction of radicals that damages macromolecules and leads to fluid accumulation, inflammation, and cell death, eventually leading to pulmonary failure. In support of this, a number of studies have reported high concentrations of metabolites reflecting increased peroxidation products such as pentane, ethane, protein carbonyl, *o*-tyrosine, allantoin, and F2-isoprostanes, as well as low levels of glutathione and sulfhydryl/total protein ratios, also reflecting an increased oxidative load, in premature infants at risk for developing CLD. Furthermore, although some animal and human studies have suggested a protective benefit of antioxidant strategies (22), surprisingly, elimination of antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase, by developing knockout mouse models, does not seem to sensitize them to hyperoxia-mediated lung injury (35–37). Likewise, high levels of exogenous antioxidants also do not seem to provide adequate protection against hyperoxia (38). Moreover, in the lung, these antioxidant enzymes appear during late gestation (29) and are stimulated by glucocorticoids (30), making them good candidates for a developmentally dependent and hormonally regulated antioxidant mechanism. However, the empiric data on their responses to oxygen under a variety of biologic conditions render doubts about the central significance of antioxidant enzymes in combating neonatal hyperoxic lung injury. For example, there is a sex difference in the incidence of BPD, but there is no sex difference in the rates of antioxidant enzyme maturation (75). Similarly, there are age and species (28) differences in susceptibility to oxidant injury that are unaccounted for on the basis of the antioxidant enzyme activities. These data suggest a role for alternate regulatory cellular/molecular pathways that must have evolved over the eons in combating hyperoxic neonatal lung injury (83). As reviewed below, regarding the lipid-enriched alveolar interstitial fibroblast, that is, the lipofibroblast, peroxisome proliferator-activated receptor gamma (PPARγ) signaling, the determinant of this fibroblast phenotype seems to be of fundamental significance in modulating the mammalian lung's antioxidant response.

From a physiologic perspective, it is well known that newborn animals are more resistant to hyperoxia than their adult counterparts (28, 101). This is likely due to the presence of lipofibroblasts in the newborn lung, which protect the lung against oxidant injury (11, 90); the number of lipofibroblasts decreases rapidly following the newborn period (10). Several lines of evidence suggest that lipids are cytoprotective against oxygen free radical lung injury both *in vitro* and *in vivo* (28, 90, 101). Therefore, it has been suggested that the greater oxygen tolerance of newborn rats and mice, compared with their adult counterparts, relates, in part, to the higher number of lipofibroblasts, and the greater amount of triglyceride in the lipid fraction of the newborn lung compared with the adult lung (1, 72–74). Feeding pregnant rats a high triglyceride diet results in increased triglyceride content in the lungs of their offspring, increased survival, and improved pathological status after prolonged hyperoxic exposure (92). In contrast, newborn offspring of rats fed low-polyunsaturated fatty acid diets are more susceptible to pulmonary oxygen toxicity, and early lethality in hyperoxia (94), bearing in mind that only unsaturated fatty acids undergo lipid peroxidation, whereas saturated fats do not.

Mechanism of Mammalian Lung Development

The development of the lung is mediated by coordinately integrated, mutually regulated networks of transcription factors, growth factors, matrix components, and physical forces, which play important roles in determining the lung structure and function. Lung development is divided into two major phases—the branching of the airways, followed by the formation of the alveoli. The lung begins as an out-pouching of the primitive foregut at 4–6 weeks of gestation (term gestation = 40 weeks) in man—the proximal portion generating the larynx and trachea, while cells located at the distal end of the trachea give rise to the left and right main stem bronchi. Branching morphogenesis of the left and right bronchi forms specific lobar, segmental, and lobular branches. This process extends through the canalicular stage of lung development up to midgestation. Saccularization starts at midgestation, leading to alveolarization, which continues up to 8–10 years of age to generate the 300 million alveoli of the mature lung, providing an enormous gas exchange surface, paired with an equally large and efficient alveolar capillary network. This sequence of biologic events has been positively selected evolutionarily over biologic time and space (87), resulting in optimal gas exchange mediated by alveolar homeostasis (85). We have previously suggested that CLD causes simplification of the lung alveoli in a manner, as if reversing the evolutionary process (81, 88).

Theorizing that by identifying those mechanisms that have evolved under selection pressure for optimal gas exchange (90), for example, evolution of lipofibroblasts, we can effectively reverse the deleterious effects of CLD by mimicking the evolutionarily adaptive mechanism (82), rather than by superficially treating the symptoms (64).

Epithelial–Mesenchymal Paracrine Model of Alveolar Development

Under the influence of Sonic Hedgehog, the developing endoderm expresses parathyroid hormone-related protein (PTHrP) and its cognate receptor on the adjoining mesenchyme. PTHrP binding to its receptor on the mesenchyme activates the protein kinase A second messenger pathway, which actively downregulates the default wingless/int (Wnt) pathway and upregulates the adipogenic pathway through a key nuclear transcription factor, PPARγ, and its downstream target genes, such as adipocyte differentiation-related protein (ADRP) and leptin (Fig. 1), reviewed in Refs. (64, 82). ADRP is necessary for the transit of neutral lipid from the lipofibroblast to the alveolar type II (ATII) cell for surfactant phospholipid synthesis. Leptin secreted by lipofibroblasts acts on its receptor on ATII cells, stimulating both surfactant phospholipid and protein synthesis. Therefore, epithelial (PTHrP)–mesenchymal (PPARγ) signaling provides a complete paracrine loop for the synthesis of pulmonary surfactant, maintaining alveolar homeostasis. Overall, PTHrP signaling, by inhibiting Wnt signaling, prevents the default myogenic phenotype, and by stimulating PPARγ signaling, induces the lipogenic phenotype, which is necessary for maintaining alveolar homeostasis through its paracrine effects on interstitial fibroblasts and ATII cells. Specifically, the interstitial lipofibroblast phenotype provides protection against oxygen free radicals (*i.e.*, protection against oxotrauma) (90), traffics neutral lipid substrate to ATII cells for surfactant phospholipid synthesis (*i.e.*, protection against atelectrauma) (78, 79), or causes ATII cell proliferation (*i.e.*, protection against any insult causing epithelial injury), thereby promoting alveolar growth, development, and injury/repair (91). Homeostatically, this stabilizes the alveolus, preventing its collapse, maintaining adequate gas exchange, and reducing energy expenditure by decreasing the work of breathing. On the other hand, although myofibroblasts (MYFs) may also be important for normal mammalian lung development (18, 56), these cells are the hallmark of all CLDs in both the neonate and adult. In the developing lung, MYFs are fewer in number and localize to the periphery of the alveolar septa, where they participate in the formation of new septa. However, in CLDs MYFs not only increase in number, but are also abnormally located in the center of the alveolar septum in great abundance. Figure 2 contrasts marked lipid staining (red fluorescence) in cultured human lung lipofibroblasts *versus* marked alpha-smooth muscle actin (α-SMA) staining (green fluorescence) in MYFs.

Evolutionary Origin Lipofibroblasts in the Mammalian Lung

There is strong circumstantial and molecular evidence suggesting that the increased atmospheric oxygen tension over evolutionary time might have led to the formation of lipofibroblasts in the evolving lung since it is the first anatomic site where increased atmospheric oxygen would have exerted selection pressure for an evolutionary change. In this regard, the physiologic significance of oxygen in the atmosphere has long been recognized as the selection pressure behind vertebrate evolution (68). The role of oxygen in vertebrate adaptation has more recently been reprised by Berner (6), who has found that oxygen tensions during the last 500 million years (mya) did not rise gradually, but instead fluctuated between 15% and 35%. Since on the one hand hypoxia is the most potent affector of vertebrate physiology (49), and on the other, mammals have evolved to gestate under hypoxic conditions, begs the question as to what the evolutionary strategy constitutes. Therefore, how animals might have

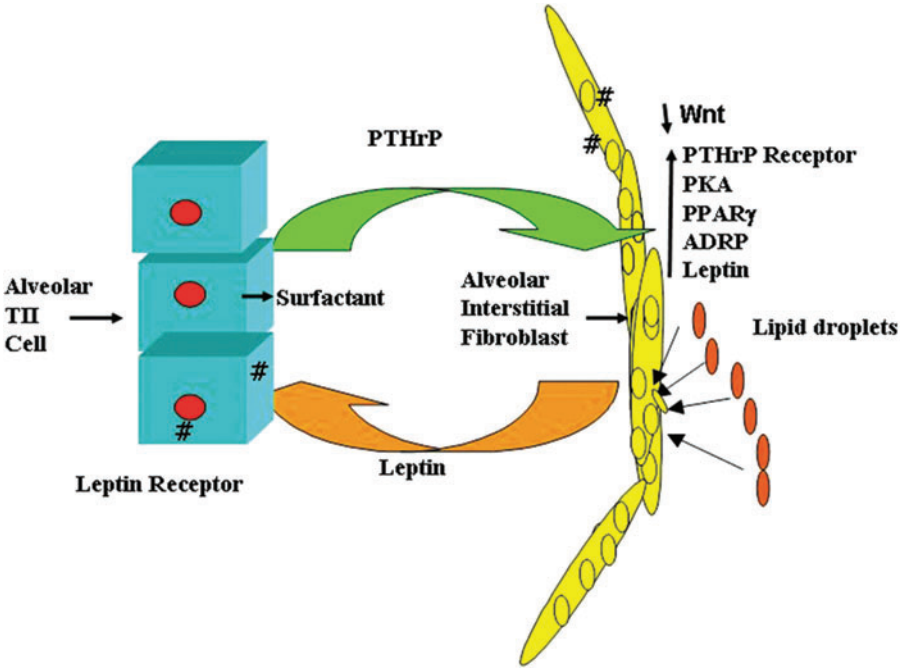

FIG. 1. PTHrP, secreted by the ATII cell, binds to its receptor on the adjoining alveolar interstitial fibroblast, activating the PKA pathway, which actively downregulates the default Wnt pathway and upregulates the adipogenic pathway through the key nuclear transcription factor, PPARγ, and its downstream regulatory genes ADRP and leptin. Lipofibroblasts in turn secrete leptin, which acts on its receptor on ATII cell, stimulating surfactant synthesis. ADRP, adipocyte differentiation-related protein; ATII, alveolar type II; PPARγ, peroxisome proliferator-activated receptor gamma; PTHrP, parathyroid hormone-related protein; Wnt, wingless/int. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

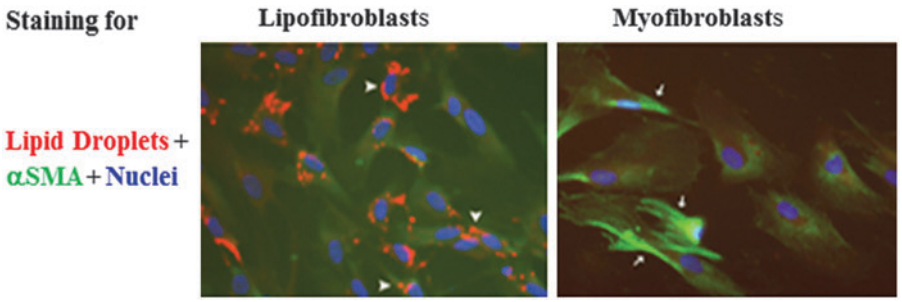

FIG. 2. Representative immunofluorescence staining for lipid droplets (red staining), α-SMA (green staining), and nuclear (blue staining) in cultured human lung lipofibroblasts and MYFs is shown. Lipid droplets were stained using Oil Red O, α-SMA staining using a specific monoclonal antibody, and nuclear staining using DAPI. Lipofibroblasts show marked lipid staining (absent α-SMA staining), whereas in contrast, MYFs show marked α-SMA staining. DAPI, 4′,6′-diamidino-2-phenylindole; MYF, myofibroblast; α-SMA, alpha-smooth muscle actin.

adapted to such episodes of hyperoxia followed by hypoxia is highly relevant and has been readdressed recently (12).

Evolutionarily, the lipofibroblast is absent from the vertebrate lung until shortly before the appearance of land mammals (87), suggesting that these cells facilitated the adaptation to atmospheric oxygen. Lipofibroblasts are homologous with adipocytes, which differentiate from MYFs through the activation of PPARγ, the gene that determines adipogenesis (39). Direct evidence for oxygen sensing affecting the expression of this gene has shown that hypoxia inducible factor (HIF)-1 signals through DEC1/Stra13 to inhibit PPARγ expression; conversely, hyperoxia upregulates PPARγ. Csete *et al.* (19) have shown that muscle satellite cells in culture will spontaneously become adipocytes in room air (21% O_2), but not in 6% oxygen, suggesting that the episodic rise and fall in atmospheric oxygen over the last 500 mya have caused the evolution of fat cells both in the lung (~lipofibroblast) and in the periphery (54). Such a mechanism provides a selection advantage since the lipofibroblast protects the alveolus against oxidant injury (90), and its production of leptin (89) may have fostered modern day stretch regulation of alveolar surfactant (80), mediating ventilation perfusion matching (96), the physiologic principle for alveolar gas exchange, thus facilitating the evolution and homeostasis of the lung (84). The concomitant production of oxygen free radicals, lipid peroxides, and other oxidative products likely generated eicosinoids as a balancing selection for endogenous PPAR ligands. The improved alveolar gas exchange, with the resultant increased reactive oxygen generation also likely led to the emergence of an increasing number of NADPH oxidase (NOX) homologs, the oxygen-reducing enzymes dedicated to ROS in more complex metazoans (42, 76). It is also interesting to note that NOX4 is required for the differentiation and activation of MYFs (20), the key cellular mediators of alveolar septation and lung injury and repair, perhaps representing antagonistic pleiotropy, that is, the paradoxical selection of genes that are beneficial during early/reproductive life, but may also mediate deleterious effects in later life (99).

The Evolution of Peroxisome Biology

Peroxisomes were discovered by Christian de Duve, whose laboratory was the first to isolate peroxisomes from rat liver and determine their biochemical properties (23). The basic mechanisms involved in peroxisome biology are shared by a variety of organisms, suggesting a common evolutionary origin. Speculation regarding peroxisome evolution began almost immediately after their discovery. Photomicrographic images suggested that there might be interactions between peroxisomes and the endoplasmic reticulum (ER), leading to speculation that peroxisomes were derived from the endomembrane system (77). Alternatively, the view that peroxisomes were independent organelles originating by endosymbiosis was subsequently proposed upon the observation that peroxisomes formed from the division of existing peroxisomes and that they imported proteins (Fig. 3) (31), both aspects resembling bacterially derived organelles such as mitochondria and chloroplasts. The most flamboyant hypothesis regarding the evolutionary origin of the peroxisome was that from de Duve himself (24), proposing a metabolic scenario for the endosymbiosis mechanism entailing the role of peroxisome enzymes in the detoxification of highly ROS

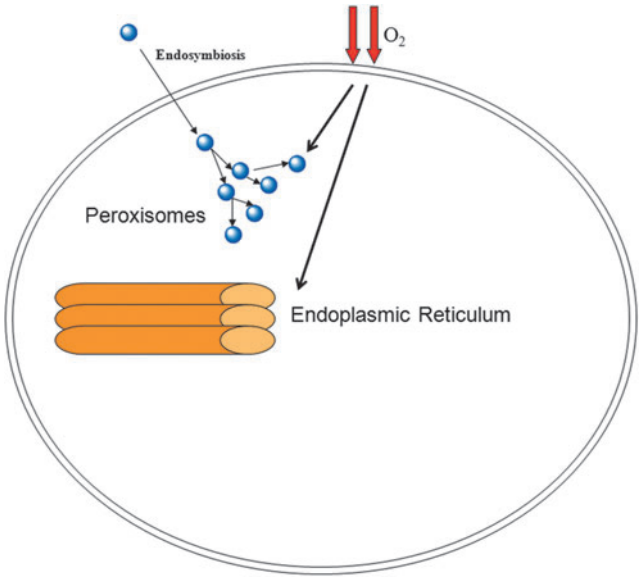

FIG. 3. Endosymbiosis theory of peroxisome evolution. Peroxisomes evolved in eukaryotes in response to rising atmospheric oxygen. One theory for their origins, based on the observation that peroxisomes divide to generate more peroxisomes, is that they originated by endosymbiosis. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

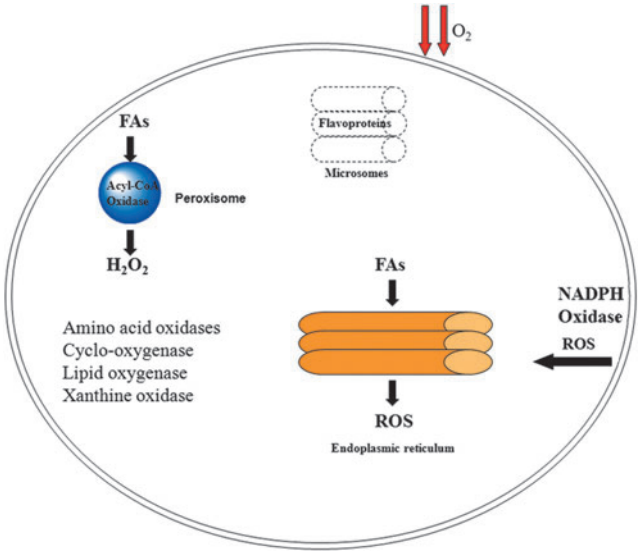

FIG. 4. Metabolic origin of peroxisomes. Peroxisomes detoxify highly ROS. Proto-peroxisomes evolved from the endoplasmic reticulum during the period when atmospheric oxygen levels were rising, generating toxic oxidation products as a by-product of various metabolic pathways. For example, during the FA oxidation that occurs in peroxisomes, high-potential electrons are transferred to O_2, which yields H_2O_2, the initial reaction being catalyzed by acyl-CoA oxidase. Microsomal ω-oxidation of fatty acids is catalyzed by cytochrome P450 enzymes, which form ROS through flavoprotein-mediated donation of electrons to molecular oxygen. Several enzymatic systems in the cytosol also generate H_2O_2, such as amino acid oxidases, cyclo-oxygenase, lipid oxygenase, and xanthine oxidase. H_2O_2 generated in the cytoplasm has the potential to perform signaling functions as it may diffuse to various organelles, including the nucleus. Notable sources of ROS at the plasma membrane are the NOXs, which are associated with cell signaling rather than with a metabolic pathway. ROS, reactive oxygen species; FA, fatty acid; NOX, NADPH oxidase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(Fig. 4). Bolstered by the popularity of the serial endosymbiosis theory (16), this view has been the most widely accepted among biologists. Based on this scenario, the proto-peroxisome was acquired at a time when the atmospheric oxygen levels were increasing, representing a toxic compound for most living organisms. This concept is consistent with the evolution of the lung lipofibroblast (83), an example of the way in which vertebrates entrain otherwise highly toxic substances from the environment and adopt them as physiologic mechanisms (13, 93).

More recently, the endosymbiosis theory for the origin of the peroxisome has been challenged. Experimentally, there is an interrelationship between peroxisome formation and the ER—specific peroxisomal membrane proteins must first be targeted to the ER before reaching the peroxisome (2), and peroxisome-less mutant yeast can form new peroxisomes from the ER upon introduction of the wild-type peroxisome gene (2). Phylogenetic studies have substantiated an evolutionary link between peroxisomes and the ER, showing homologous relationships between components of the peroxisome import machinery and those of the endoplasmic reticulum decay (ERAD) pathway (9). Such data have led to the conclusion that the peroxisome originates from the ER (45, 57), but they have not obviated the possibility that the peroxisome originated as an endosymbiont (47).

Based on the sequence homology with previously identified members of the nuclear hormone receptor superfamily, discovery of peroxisomes was followed by the identification of three PPAR isotypes (PPARα, β/δ, and γ), first in frogs and mice, and later in man, rat, fish, hamster, and chicken (27). These isoforms were initially shown to be activated by substances able to induce peroxisome proliferation. Various endogenous and exogenous PPAR ligands were later identified, namely, fatty acids and eicosanoids, as well as synthetic hypolipidemic and antidiabetic agents (43); however, the physiologic relevance of each of the endogenous PPAR ligands can be questioned. Nitrated fatty acids (NFAs), produced by nonenzymatic reaction of NO and its products with unsaturated fatty acids, have been suggested as the newest endogenous PPARγ ligands. Although the total amount of NFAs in the bloodstream significantly exceeds their EC_{50} for PPARγ activation, whether concentrations of free endogenous NFAs are sufficient for efficacious PPARγ activation remains unknown (57). This is particularly relevant since recently, critical roles for reactive nitrogen species such as NO and peroxynitrites have been suggested in both physiologic intracellular signaling as well as in mediating oxygen toxicity (51). Overall, although PPARs are involved in rodent development, most importantly, they are involved in lipid metabolism and energy homeostasis, PPARγ playing a role in adipogenesis and lipid storage and PPARα playing a role in fatty acid catabolism, with the liver being the best characterized (98).

PPARγ Mediates the Evolutionary History of the Lipofibroblast: When Homologies Run Deep

During the Phanerozoic phase of vertebrate evolution (the last 550 mya), atmospheric oxygen rose to its current level of 21%, but it did not increase linearly.

Rather, it increased and decreased several times, reaching concentrations as high as 35%, and falling to as low as 15% over this time period (Fig. 5) (6). As mentioned previously, the oxygen increase may have induced the differentiation of muscle cells into lung lipofibroblasts, since the first place where increased atmospheric oxygen would have affected selection pressure for evolutionary change would be in the alveolar wall, as the lipids stored in these lipofibroblasts protect the lung against oxidant injury (90), consistent with this hypothesized adaptive response to the rising oxygen tension in the atmosphere. PPARγ must be upregulated for lipofibroblast differentiation to occur (70). Subsequently, leptin is secreted by the lipofibroblasts, binding to its receptor on the alveolar epithelial cells lining the alveoli, stimulating surfactant synthesis (89) and reducing alveolar surface tension, resulting in a more deformable and efficient gas exchange surface. Such positive selection pressure could have led to the stretch-regulated coregulation of surfactant and microvascular perfusion (32) by PTHrP, recognized physiologically as the mechanism of alveolar ventilation perfusion matching. The evolution of these molecular mechanisms could ultimately have given rise to the definitive mammalian lung alveolus, with maximal gas exchange resulting from

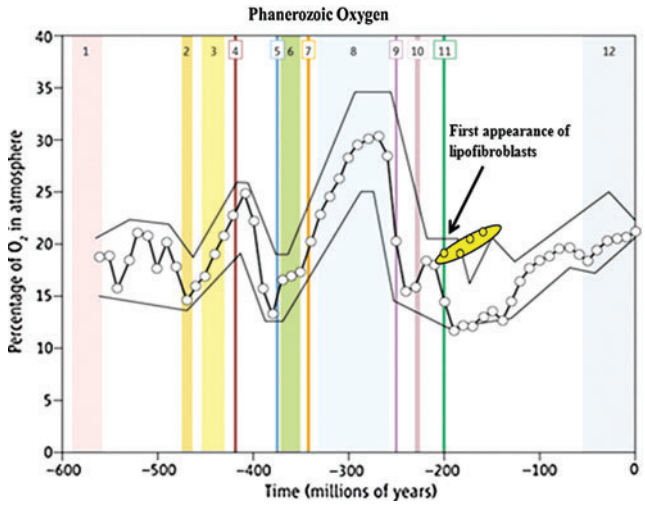

FIG. 5. Atmospheric oxygen level *versus* time for the Phanerozoic period (past 550 million years): the *upper* and *lower* boundaries are estimates of error in modeling atmospheric O_2 concentration. The numbered intervals denote important evolutionary events that may be linked to changes in oxygen concentration [for details, see Ref. (39)]. *Arrow* points to the first appearance of lipofibroblasts in the mammalian lung. (Modified from Ref. 39). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

coordinate stretch-regulated surfactant production and alveolar capillary perfusion, thinner alveolar walls due to PTHrP's apoptotic or programmed cell death effect on fibroblasts (17), and a blood–gas barrier buttressed by type IV collagen (5). We speculate that this last feature may have contributed generally to the molecular bauplan for the peripheral microvasculature of evolving vertebrates, given its effect on angiogenesis (26, 40). One physiologic consequence of the increased oxygenation may have been the concomitant induction of fat cells in the peripheral circulation, which led to endothermy or warm bloodedness (54). The increase in body temperature synergized increased lung oxygenation because the lung surfactant is 300% more active at 37°C than at ambient atmospheric temperature (*i.e.*, the body temperature for cold blooded organisms). For example, map turtles (*Graptemys geographica*) show different surfactant compositions depending on the ambient atmospheric temperature (46). Therefore, the advent of thermogenesis would have facilitated the physical increase in the lung surfactant surface tension-lowering activity. These synergistic selection pressures would have been further functionally enhanced by the coordinate physiologic effects of epinephrine on the heart, lung, and fat depots (49), underpinned structurally by the increased production of leptin by fat cells, which is known to promote the formation of blood vessels (44) and bone (100), accommodating the infrastructural changes necessitated by the evolution of complex physiologic traits (97).

Evolutionary Knowledge Explains the Benefits of Continuous Positive Airway Pressure

Parenthetically, the argument outlined above is not a ''Just So Story''—the cited cell/molecular events that evolutionarily determine alveolar homeostasis follow a sequence that is consistent with the phylogeny and ontogeny of the vertebrate lung in both the forward and pathologically reverse directions (79, 80, 86), allowing us to suggest an approach to lung biology and pathophysiology consistent with Evolutionary Medicine (87). This is abundantly exemplified by the failure to explain the reduction in CLD by surfactant replacement in the surfactant-deficient premature infant when traditional wisdom would predict its reduction due to improvement in oxygenation and ventilation following provision of the deficient substance, namely, pulmonary surfactant. This is because CLD is not simply due to the lack of surfactant in the alveoli, but more fundamentally, it is due to the lack of fully established epithelial lipofibroblast communications in the alveolar wall, which leads to surfactant insufficiency. Therefore, unless these homeostatic communications are established, regardless of what treatment is provided, it will not prevent or reverse CLD. This principle is likely the basis for the success of continuous positive airway pressure (CPAP), specifically, providing just the right amount of alveolar distension, capitalizing on billions of years of lung evolutionary phylogeny and development, stimulates the epithelial lipofibroblast cross talk induced by PTHrP, leading to a more physiologic milieu, and that is why premature infants supported on CPAP are less likely to develop CLD (14, 33).

Hyperoxia, Peroxisomes, and ROS

As outlined above, it is likely that to cope with the rising levels of oxygen in the atmosphere during the Phanerozoic Era (99), generation of ROS emerged as a by-product of various metabolic pathways (34). For example, during the fatty acid oxidation that occurs in peroxisomes, high-potential electrons are transferred to O_2, which yields H_2O_2, the initial reaction being catalyzed by acyl-CoA oxidase. Microsomal ω-oxidation of fatty acids is catalyzed by cytochrome P450 enzymes, which form ROS through flavoprotein-mediated donation of electrons to molecular oxygen. Sulfhydryl oxidases in the ER catalyze oxidative protein folding, with the generation of disulfides and the reduction of oxygen to H_2O_2. Several enzymatic systems in the cytosol also generate H_2O_2, such as amino acid oxidases, cyclo-oxygenase, lipid oxygenase, and xanthine oxidase. H_2O_2 generated in the cytoplasm has the potential to perform signaling functions, as it may diffuse to various organelles, including the nucleus. Notable sources of ROS at the plasma membrane are the NOXs, which are associated with cell signaling rather than with a metabolic pathway [reviewed in Ref. (93)].

Presence of Lipofibroblast in the Human Lung

Given the evolutionary significance of lipofibroblasts in lung biology, it is rather surprising that their presence in the human lung was unequivocally demonstrated only recently, even though this cell type has been extensively documented and studied in many other species for decades (50, 53). Based on their adepithelial localization, morphological (lipid staining), molecular (presence of characteristic lipogenic and absence of myogenic markers), and functional (triglyceride uptake) characteristics that are the hallmarks of the rodent lung lipofibroblast, the presence of lipofibroblasts in both neonatal and adult human lung autopsy specimens has now been confirmed (63).

Innovation

Oxygen support for preterm infants can paradoxically have toxic effects due to lung immaturity. Through phylogenetic insights to the role of lipofibroblasts in lung homeostasis, we have devised a biologically rational means of promoting lung development using PPARγ agonists.

Lipo-to-MYF Transdifferentiation Is a Cardinal Feature in BPD Pathogenesis

Using a variety of cellular and animal models, in a series of studies from our laboratory, we have shown that in the presence of deranged mesenchymal–epithelial signaling, for example, on exposure to hyperoxia, infection, volutrauma, and other insults that lead to BPD, pulmonary lipofibroblasts rapidly lose their lipogenic phenotype and transdifferentiate into a myogenic phenotype, that is, MYFs (10, 21, 58–62, 65–67, 95). Transdifferentiated lipofibroblasts (*i.e.*, MYFs) are unable to maintain pulmonary epithelial cell growth and differentiation, resulting in failed alveolarization, seen characteristically in BPD and other CLDs, unequivocally signifying the importance of lipofibroblasts in lung development and injury repair. Using oxotrauma as a prototype, as detailed below, in these models, using PPARγ agonists, we have effectively abrogated specific alveolar molecular changes following oxotrauma, barotrauma, and infection that are known to lead to BPD (Fig. 6).

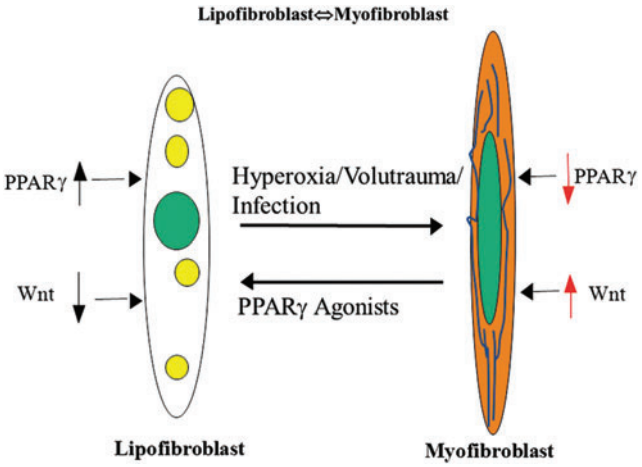

FIG. 6. Blockage of lipofibroblast to MYF transdifferentiation by using PPARγ agonists. In the presence of deranged mesenchymal–epithelial signaling, for example, on exposure to hyperoxia, infection, volutrauma, and other insults that lead to BPD, pulmonary lipofibroblasts rapidly lose their lipogenic phenotype (high PPARγ and low Wnt signaling) and transdifferentiate into a myogenic phenotype, that is, MYFs (low PPARγ and high Wnt signaling). Transdifferentiated lipofibroblasts are unable to maintain pulmonary epithelial cell growth and differentiation, resulting in failed alveolarization, seen characteristically in BPD and other CLDs, signifying the importance of lipofibroblasts in lung injury repair. Using a variety of cell and animal models, we have shown that PPARγ agonists can not only block, but can also reverse lipofibroblast to MYF transdifferentiion, thereby preventing and potentially reversing CLD. BPD, bronchopulmonary dysplasia; CLD, chronic lung disease. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Prevention of Oxotrauma by PTHrP/PPARγ Signaling Pathway Agonists

We initially studied the effects of hyperoxia on the fibroblast phenotype in immature [embryonic day (e) 18; term = day 22] and relatively mature (e21) rat lungs, and found that exposure to hyperoxia downregulated PTHrP/PPARγ signaling, augmenting the transdifferentiation of pulmonary lipofibroblasts to MYFs (58). Cells were maintained either in normoxia (21% O_2) or subjected to hyperoxia for 24 h (95% O_2) at passages (P)1 and P5. Serial passaging and maintenance of cells in normoxia resulted in a significant spontaneous decrease in the expression of the lipogenic markers, based on molecular (reverse transcription–polymerase chain reaction [RT-PCR] for PTHrP receptor, PPARγ, and ADRP), triglyceride uptake, and leptin assay criteria, from P1 to P5. This decrease was greater for relatively immature (e18) than for more mature e21 fibroblasts. Exposing cells to hyperoxia augmented the loss of the lipogenic markers and gain of the myogenic marker α-SMA from P1 to P5 in comparison to cells maintained in normoxia. This augmentation was also greater for e18 *versus* e21 lipofibroblasts. These data suggested that exposure to hyperoxia augmented the transdifferentiation of pulmonary lipofibroblasts to MYFs. Importantly, we also found that pretreatment with an endogenous PPARγ signaling pathway agonist, prostaglandin J_2 (PGJ_2), at least partially attenuated the hyperoxia-augmented lipofibroblast-to-MYF transdifferentiation.

In a follow-up series of experiments, using [1,2-$^{13}C_2$]-D-glucose tracer and gas chromatography/mass spectrometry, we then metabolically profiled e18 and e21 rat lung fibroblasts with and without hyperoxia exposure at passages 1, 4, 7, and 10 (10). For this series of studies, glucose carbon redistribution between the nucleic acid ribose, lactate, and palmitate synthetic pathways, and ADRP expression by RT-PCR were examined. Exposure to hyperoxia at each passage caused a decrease in ADRP mRNA expression. This passage-dependent transdifferentiation was accompanied by a moderate increase in the synthesis of nucleic acid ribose from glucose through the nonoxidative steps of the pentose cycle. E18 fibroblasts showed over an 85% decrease in the *de novo* synthesis of palmitate from glucose, whereas e21 fibroblasts showed a less pronounced 32% to 38% decrease in *de novo* lipid synthesis in hyperoxia-exposed cultures, suggesting that (i) there is a maturation-dependent sensitivity to hyperoxia; (ii) transdifferentiation of the fibroblast is accompanied by metabolic enzyme changes affecting ribose synthesis from glucose, and (iii) hyperoxia specifically inhibits lipogenesis from glucose. These molecular and metabolomic data were further complemented by genomewide microarray analysis of RNA extracted from P1 and P10 e19 rat lung fibroblasts, with or without exposure to hyperoxia (95% O_2 for 24 h) (59). The rat chip *RAE230*, which had nearly 16,000 RNA transcripts, including genes of our interest, for example, PTHrP, PPARγ, and adipophilin (moderate homology with ADRP), was used. In accord with our molecular and metabolomic data, cluster analysis of the microarray data confirmed the downregulation of cholesterol and fatty acid synthetic genes,

and upregulation of fatty acid degradation and Wnt signaling pathway genes upon passaging e19 fibroblasts from P1 to P10, and on exposure to hyperoxia at passages 1 and 10, thereby confirming lipofibroblast-to-MYF transdifferenation under these conditions.

Following these *in vitro* studies, we determined whether *in vivo* exposure to hyperoxia also resulted in pulmonary alveolar lipofibroblast-to-MYF transdifferentiation, and whether treatment with a potent PPARγ signaling pathway agonist, rosiglitazone (RGZ), would prevent this process (65). Newborn Sprague Dawley rat pups were exposed to normoxia (21% O_2), hyperoxia alone (95% O_2 for 24 h), or hyperoxia with RGZ (95% O_2 for 24 h + RGZ, 3 mg/kg, administered intraperitoneally). Hyperoxia-exposed lungs demonstrated arrest of alveolarization, characterized by large air spaces, thinned interstitial septa, and decreased secondary septal crest formation compared with air-exposed controls. Accompanying these morphometric changes, there was a significant decrease in the expression of lipogenic markers, and a significant increase in the expression of myogenic markers in the hyperoxia-alone group. The hyperoxia-induced morphologic, molecular, and immunohistochemical changes were almost completely prevented by pretreatment with RGZ, providing the first evidence for the *in vivo* effectiveness of exogenously administered PPARγ agonists in preventing hyperoxia-induced neonatal lung injury.

Subsequently, we demonstrated that *in vivo* neonatal lung injury, even following a 7-day exposure to hyperoxia (95% O_2), characterized by the arrest in alveolarization and the equally characteristic molecular and cellular alterations of hyperoxia-induced lung damage, that is, upregulation of TGFβ pathway transducers such as ALK-5, pSmad3, and Smad7, upregulation of canonical Wnt signal transducers such as β-catenin and Lef-1, upregulation of myogenic mesenchymal proteins such as α-SMA and calponin, downregulation of PPARγ and alveolar luminal and interstitial neutrophil influx (21), which were all blocked by the concomitant administration of a PPARγ agonist, RGZ. These findings were confirmed using TOPGAL mice. These data further highlighted the potential role for PPARγ agonists in preventing BPD.

In addition to the protection against hyperoxia-induced neonatal lung injury with PPARγ agonists when used postnatally, we have also determined if this strategy can be instituted prophylactically antenatally. Recognizing that PPARγ can stimulate intrinsic developmental mechanisms (82, 95) that protect the lung against oxidant injury, that is, by stimulating the lung lipogenic phenotype, we have tested the hypothesis that, by targeting the accelerated maturation of the lipofibroblast during development, we can protect against postnatal oxidant injury by administering PPARγ agonists to pregnant dams before delivery. To this end, we observed that antenatal administration of RGZ enhanced fetal lung maturation (Table 1) and virtually blocked 24-h hyperoxia-induced lung molecular and morphometric changes postnatally, suggesting a novel antenatal intervention to protect against hyperoxia-induced neonatal lung injury (61). Although there was some heterogeneity in the cytoprotective effects of antenatal RGZ administration on the different alveolar cell-type markers affected by hyperoxia, ATII cell (surfactant protein A and C, cholinephosphate cytidylyltransferase-α, leptin receptor), fibroblast (PTHrP receptor and PPARγ), vascular (VEGF and FLK-1), and functional differentiation (triolein uptake and [^{3}H]choline incorporation into saturated phosphytidylcholine) markers, which have previously been shown to indicate lung alveolar development and homeostasis (10) and prevent oxidant lung injury (10, 21, 58, 65) clearly increased following antenatal RGZ administration, providing an alternative approach to the standard contemporary antenatal steroid administration for enhancing fetal lung maturation.

Table 1. Rosiglitazone—Responsive Biomarkers

	RGZ: reponsive biomarker
Lung cell type	Surfactant proteins (A, B, and C) Choline phosphate cytidylyltransferase α Leptin receptor ^{3}H-choline incorporation into saturated phosphatidylcholine
Fibroblast	Parathyroid hormone-related protein receptor Peroxisome proliferator activator receptor γ Adipocyte differentiation-related protein Leptin Triolein uptake
Vascular	Platelet endothelial cell adhesion molecule Vascular endothelial growth factor Vascular endothelial growth factor receptors (Flk-1 and Flt-1)

RGZ, rosiglitazone.

Mother Nature Opts for Lipofibroblasts to Maintain Homeostasis Too

Experimentally, Besnard *et al.* (7) found that when they deleted a gene necessary for the synthesis of cholesterol specifically in ATII cells, the lungs appeared to function normally even though cholesterol is necessary for effective surfactant surface activity (4). On further examination, it was found that the lung developmentally compensated for this deficiency by overexpressing the lipofibroblast population in the alveoli, suggesting that by sensing alveolar dyshomeostasis due to cholesterol-less surfactant, which is of poorer surface-active quality, alveoli invoke an evolutionary strategy to facilitate surfactant production, both ontogenetically and phylogenetically (83), that is, by increasing the alveolar lipofibroblast population.

PPARγ Agonists Turn on a "Master Switch" for Normal Lung Development, Universally Preventing Neonatal Lung Injury

It is clear from the work reviewed above that lipofibroblast PPARγ signaling plays a central role in epithelial–mesenchymal interactions, maintaining alveolar homeostasis and aiding lung injury repair. The lipofibroblast expresses PPARγ in response to PTHrP signaling from the ATII cell, resulting in both the direct protection of the mesoderm against oxidant injury (90), and protection against atelectasis by augmenting surfactant protein and phospholipid (21, 61, 82, 95) synthesis. Molecular injury to either the ATII cell or the lipofibroblast downregulates this molecular signaling pathway, causing MYF transdifferentiation—MYFs cannot

promote ATII cell proliferation and differentiation (91), leading to the failed alveolarization characteristic of BPD (15, 41). In contrast, maintaining the alveolar interstitial fibroblasts' lipofibroblastic phenotype supports ATII cell proliferation and differentiation even under the influence of factors implicated in the pathogenesis of BPD. This scenario is validated by a plethora of *in vitro* (10, 58–60, 62, 66, 89, 91) and *in vivo* (21, 48, 63, 65, 67) studies. Importantly, these studies show that exogenously administered PPARγ agonists can prevent or reverse MFY transdifferentiation, potentially preventing the inhibition of alveolarization in the developing lung, the hallmark of CLD of the newborn (3, 8, 41, 55).

In summary, by identifying deep homologous mechanisms that have determined both the phylogeny and ontogeny of the lung, we have experimentally used exogenously administered PPARγ agonists to exploit the lung's evolved cellular strategy to combat hyperoxia and prevent neonatal lung injury leading to the CLD of prematurity. We rationalize that a diagnostic and therapeutic approach predicated on mechanisms that have resulted in the evolution of human lung under the selection pressure of increased atmospheric oxygen can be exploited to understand homeostasis, representing health, and dyshomeostasis, representing disease. However, on a cautionary note, although the above outlined approach appears to be robust, effective, and safe in promoting lung maturity and injury repair under experimental conditions, its clinical translation awaits further detailed pharmacokinetic and pharmacodynamic studies with specific PPARγ agonists for their safe and effective use in human neonates.

''Life is that which can mix oil and water.''

—Robert Frost

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Address correspondence to:
Dr. Virender K. Rehan
Department of Pediatrics
Los Angeles Biomedical Research Institute
at Harbor-UCLA Medical Center
1124 W. Carson Street
Torrance, CA 90502-2006

E-mail: vrehan@labiomed.org

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Abbreviatons Used

α-SMA = alpha-smooth muscle actin
ADRP = adipocyte differentiation-related protein
ATII = alveolar type II
BPD = bronchopulmonary dysplasia
CLD = chronic lung disease
CPAP = continuous positive airway pressure
ER = endoplasmic reticulum
ERAD = endoplasmic reticulum decay
FLK-1 = fetal liver kinase 1
HIF = hypoxia inducible factor
Mya = million years ago
MYFs = myofibroblasts
NFAs = nitrated fatty acids
NOX = NADPH oxidase
PGJ_2 = prostaglandin J_2
PPARγ = peroxisome proliferator-activated receptor gamma
PTHrP = parathyroid hormone-related protein
RGZ = rosiglitazone
ROS = reactive oxygen species
RT-PCR = reverse transcription–polymerase chain reaction
TGFβ = transforming growth factor β
VEGF = vascular endothelial growth factor
Wnt = wingless/int