ABSTRACT

Extremely preterm infants are at high risk for a form of chronic lung disease called bronchopulmonary dysplasia (BPD), which is characterized by impaired alveolar development. Exposure to hyperoxia and deficiency of vitamin A (VA) contribute to the development of BPD in preterm infants. Randomized controlled trials indicate VA supplementation decreases BPD and/or death. However, 25% of preterm infants continue to remain VA deficient despite supplementation, probably due to impairment in VA transport, VA is stored in the liver as retinyl esters which are transported to the lung and irreversibly metabolized into all-trans-retinoic acid (ATRA), the active metabolite. A combination of VA and ATRA increases lung retinyl esters more than either VA or ATRA alone in neonatal rats. We hypothesized that this increase in lung retinyl ester content would reduce hyperoxia-induced lung inflammation and improve lung development. In order to test our hypothesis, we developed models of short and longer-term hyperoxia exposure in newborn C57Bl/6J mice that were administered either vehicle, VA, ATRA, or 10:1 molar combination of VA and ATRA (VARA). Animals exposed to 4 days of hyperoxia had increased lung retinoids with VARA more so than by either VA or ATRA alone. VARA attenuated the hyperoxia-induced increases in macrophage inflam-
matory protein (MIP)-2α, suggesting that VARA may influence pro-inflammatory mediators. The 4 day exposure did not lead to observable differences in alveolar development among groups. Newborn mice that were administered VARA and exposed to 14 days of hyperoxia had increased lung retinoids accompanied by improved alveolar development and lung function. Furthermore, VARA attenuated hyperoxia-induced increases in DNA damage, protein oxidation, and specific pro-inflammatory mediators including MIP-2α. Taken together, these findings demonstrate that VARA is effective in improving lung VA content during short and longer-term hyperoxia exposures and that increased lung VA improves hyperoxia-induced impaired alveolar development and lung function, possibly through modulation of oxidative stress and pro-inflammatory pathways.

Keywords: Bronchopulmonary dysplasia, Hyperoxia, vitamin A, All-trans-retinoic acid
DEDICATION

This dissertation is dedicated to my family and friends whose love and unwavering support I am forever grateful for.
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I would like to acknowledge and thank the individuals who made this possible.

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INTRODUCTION

Bronchopulmonary Dysplasia

Premature delivery occurs in approximately 12% of births and contributes to more than 85% of perinatal morbidity and mortality (1). The premature newborn transitions from the hypoxic fetal environment to a state of relative hyperoxia *ex utero*. Preterm infants born before 28 weeks of gestation are in the late canalicular (formation of primary acini and development of first air-blood barriers) to saccular (formation of alveolar sacs and expansion of air-blood barriers) stage of lung development and are at high risk for the development of bronchopulmonary dysplasia (BPD), a chronic lung disease of prematurity. BPD affects over 10,000 preterm infants every year in the US alone, and is associated with significant morbidity, mortality, and healthcare costs (2). BPD is characterized by impaired alveolar development (fewer and larger alveoli) and reduced microvascular development (3, 4). The etiology of impaired alveolar development is multifactorial and includes exposure to mechanical ventilation, infections, and reactive oxygen species production from prolonged oxygen use and hyperoxia exposure which result in inflammation, damage, and attenuation of normal growth and repair of the developing neonatal lung (5, 6) (*Figure 1*).

Extremely low birth weight (ELBW; birth weight ≤1000g) infants are most susceptible to the development of BPD (7). Low plasma and tissue concentrations of vitamin A (VA) are often observed in ELBW infants and are associated with a higher
Figure 1: The Etiology of Impaired Alveolarization. Exposure of preterm infants to mechanical ventilation, postnatal infection, poor nutrition, glucocorticoids (such as dexamethasone), and prolonged oxygen supplementation leads to the development of bronchopulmonary dysplasia (BPD) in which impaired alveolar development is a key feature.
incidence of BPD (8). Despite supplementation, 25% of infants remain vitamin A
deficient (9). This deficiency may be due to the route of delivery (i.e. intramuscular
injections or enteral administration), dosing regimen, or impaired transport via oxidative
stress and/or inflammation due to prolonged oxygen use (10).

Vitamin A in Normal and Abnormal Alveolar Development

Vitamin A in Normal Alveolar Development

Prenatal lung development involves the formation of the primordial lung from the
foregut and sequential branching morphogenesis into small airways, which are followed
by three maturation phases: pseudoglandular, with continued airway branching;
canalicular, with thinning of the epithelium and cell differentiation; and the terminal
saccular stage, with rapid proliferation of interstitial fibroblasts, alveolar budding,
septation and differentiation of type II and type I epithelia (11, 12). All-trans-retinoic
acid (metabolite of vitamin A) is known to be one of the primary morphogens that
regulates the temporal and spatial expression of many growth factors and matrix
molecules such as transforming growth factor (TGF-β), epidermal growth factor (EGF),
collagen, and elastin (13, 14) in the epithelium and mesenchyme throughout lung
development.

Postnatal development primarily involves alveolarization overlapped with
microvascular maturation. Alveolarization entails thinning of the alveolar walls, growth
of a capillary network, and extensive subdivision of gas exchange units. This period
involves interstitial fibroblast proliferation, while epithelial cells flatten and decrease in
number, resulting in a net thinning of distal airspace walls. Concurrently, the alveolar
capillary network becomes more complex (15). Alveolar septation begins as secondary crests that extend from primary alveolar walls. Development of these crests or septae occurs through deposition of new basement membrane, outgrowth of epithelial cells and myofibroblasts at the tips of septae, and elastin deposition (16). In humans, alveolar septation occurs approximately from 36 weeks of gestation until 1 to 2 years of age (17). Alveolarization in rodents occurs from postnatal day 5 until day 30 (17-19).

All-trans-retinoic acid storage granules are most abundant in the fibroblastic mesenchyme surrounding the alveolar walls, where levels peak prior to alveolar septation (20-24). Depletion of retinyl ester (main storage form of vitamin A) stores parallels the deposition of a new elastin matrix and septation. In neonatal rats fed a VA deficient diet or treated with dexamethasone (glucocorticosteriod), alveolar septation is significantly reduced. At the molecular level, the expression of cellular retinol binding protein and all-trans-retinoic acid receptor-β messenger RNA is diminished in the lungs of VA deficient pups (25, 26). In contrast, the treatment of neonatal pups with all-trans-retinoic acid improves lung alveolarization in dexamethasone exposed rats (27).

Lung lipid interstitial cells (LICs) have been proposed to be the storage site of VA within the lung (23). Dirami et al. has demonstrated that lung LICs are the site for retinol oxidation and the production of biologically active metabolites such as all-trans-RA, 9-cis-RA, and 13-cis-RA which, with the inhibition of RAR/RXR, may produce downstream effects on CRBP (downregulation) in neighboring cells such as pulmonary microvascular cells (24). Transgenic animal models have shown that the deletion of RAR α and γ receptors decreases alveolar development (28) but the deletion of the all-trans-retinoic acid-β receptor leads to the early onset of alveolar septation, indicating the role
of all-trans-retinoic acid receptors in modulating alveolarization (29).

**Vitamin A in Abnormal Alveolar Development**

The fetus accumulates VA in the third trimester, and therefore infants born premature have reduced VA stores (30, 31). Extremely low birth weight infants are most susceptible to the development of BPD (7). Low plasma and tissue concentrations of VA are often observed in ELBW infants and are associated with a higher incidence of BPD (8).

Hyperoxia-induced inhibition of alveolar development in newborn rodents is often used as an animal model of BPD (32-34). Veness-Meehan et al. (34) demonstrated that all-trans-retinoic acid treatment of newborn rats improves survival and alveolarization during hyperoxia exposure. The exact mechanisms by which all-trans-retinoic acid attenuates the effects of hyperoxia-induced impaired alveolar development in the newborn lung are uncertain, but modulation of the expression of antioxidants (35-40), pro-inflammatory mediators (41), or growth factors (42, 43) may play a role.

**Vitamin A**

**Biochemistry of vitamin A**

Vitamin A is a subclass of a family of lipid-soluble compounds referred to as retinoids, obtained from the diet (44). There are essentially three forms of vitamin A: retinols, beta-carotenes, and carotenoids. Retinol, also known as preformed vitamin A, is the most active form and is mostly derived from animal sources of food in the human diet (44).
Retinoids are compounds that possess both a β-ionone ring and a polyunsaturated side chain. The side chain may contain one of the following functional groups: an alcohol, aldehyde, a carboxylic acid group or an ester group. The side chain contains four isoprenoid units, with a conjugated double bond system which may exist in trans or cis configuration. Many reversible isomers of retinol, retinal and retinoic acid (RA) are possible as a result of either a trans or cis isomerization of four of the five double bonds found in the polyene chain, though the instability of the cis isomers causes them to readily convert to the all-trans-configuration.

All-trans-retinol (Figure 2), a primary alcohol (R-OH (hydroxyl)), is a low molecular weight fat-soluble compound which is stored as all-trans-retinyl esters (R-OR) (O-(R=fatty acid)) (Figure 2). The oxidation of all-trans-retinol into all-trans-retinal (R-H) (aldehyde) (Figure 2) is a reversible reaction that is catalyzed by retinol alcohol dehydrogenases (RolDH), short-chain dehydrogenases and several members of the cytochrome P450 (CYP) family (45, 46). This reaction is rate-limiting, while the conversion of all-trans-retinal into all-trans-retinoic acid (Figure 2) (R-OOH (carboxyl)) is irreversible and is catalyzed by retinaldehyde dehydrogenase (RALDH) as well as members of the cytochrome P450 family (46). All-trans-retinoic acid can then undergo cis-trans isomerization of the conjugated double-bond system (movement of atoms around a C-C double bond) (Figure 3) into 9-cis-retinoic acid and 13-cis-retinoic acid. Isomerization of all-trans-retinol yields 9-cis-retinol which can also be metabolized into 9-cis-retinoic acid which can undergo isomerization into 13-cis-retinoic acid. 13-cis-retinoic acid is considered less biologically active than the other isoforms (all-trans-retinoic acid and 9-cis-retinoic acid) and may require isomerization to all-trans-retinoic...
**Figure 2: Retinoid Metabolism.** All-trans-retinol can readily convert to all-trans-retinyl ester (the major storage form of vitamin A). The oxidation of all-trans-retinol into all-trans-retinal is a reversible reaction catalyzed by retinol alcohol dehydrogenases and the members of the cytochrome P450 family. This reaction is rate-limiting, while the conversion of retinal into all-trans-retinoic acid is irreversible and is catalyzed by retinaldehyde dehydrogenase (RALDH) as well as members of the cytochrome P450 family.
Figure 3: Isomerization of Retinoic Acid Isoforms. All-\textit{trans}-retinoic acid can undergo \textit{cis}-\textit{trans} isomerization of the conjugated-double bond system into 9-\textit{cis}-retinoic acid and 13-\textit{cis}-retinoic acid.
acid in order to exert biologic function (47, 48). Several other catabolic products of all-
trans-retinoic acid are produced from decarboxylation and glucuronidation reactions,
though many are not functionally active (46). Cytosolic retinoid-binding proteins such as
cellular retinoid binding protein (CRBP) and cellular retinoid acid binding protein
(CRABP) are thought to participate in controlling oxidation and isomerization of the
retinoid structures. By sequestering retinoids, these cellular binding proteins also serve to
prevent excessive levels of retinoids from disrupting the structure and function of the
plasma membrane and organellar membranes (49-52).

**Vitamin A Transport**

Retinyl esters are hydrolyzed by lipoprotein lipase (LPL) in the intestinal lumen
to free retinol, which (along with carotenoids and other lipids) is absorbed by facilitated
diffusion into the enterocytes of the gut (51-54) ([Figure 4](#)). The dietary retinol in the
enterocytes is bound to cellular (cytosolic) retinol-binding protein (CRBP II), reesterified
with a fatty acid by the enzymes lecithin:retinolacyltransferase (LRAT) (reesterifies
retinol bound to CRBP II) and acyl CoA:retinol transferase (ARAT) (re-esterifies free
cellular retinol), and incorporated into lipoprotein particles (chylomicrons) for delivery to
peripheral tissues via the lymphatic system (51, 52, 55-59) ([Figure 4](#)). The binding of
retinol to CRBP II allows interaction of retinol with the appropriate enzymes for
metabolism and protects it from undesired oxidation or other chemical transformations
(59). CRBP II also protects the plasma membrane from disruption and damage due to
free retinol (51, 52). A small part of the retinol is also metabolized to its active
metabolite, all-trans-RA, in the intestinal cells, which is then transferred to the
Figure 4: Overview of vitamin A Transport and Storage. Retinyl Esters (RE) are hydrolyzed to retinol (ROH) by lipoprotein lipase (LPL) in the intestinal lumen to free retinol which is absorbed by facilitated diffusion into the enterocytes of the gut. ROH is then bound to CRBP II and reesterified into retinyl esters by lecithin:retinol acyltransferase (LRAT) and any free retinol can be reesterified with acylCoA:retinol transferase (ARAT) (ARAT not shown). RE are then incorporated into chylomicrons (CM) which are delivered to peripheral tissues such as the liver via the lymphatic system. In the liver, RE is hydrolyzed into retinyl esters via retinyl ester hydrolases. The hepatic retinol may then have several fates, depending on the nutritional, developmental, or health status of the organism such as transfer to hepatic stellate cells for storage, or mobilization into the circulation as a complex with plasma retinol-binding protein (RBP) which is then complexed with transthyretin (TTR).
circulation bound to albumin (60, 61) (Figure 5).

In the liver, retinyl esters are hydrolyzed by retinyl ester hydrolases (REH) and the retinol product interacts with CRBP I (51, 52, 57, 62) (Figure 4). The hepatic retinol may then have several fates, depending on the nutritional, developmental, or health status of the organism such as transfer to hepatic stellate cells for storage, or mobilization into the circulation as a complex with plasma retinol-binding protein (RBP). The liver is the main tissue for control of retinol homeostasis, but other tissues such as kidney or adipose tissue may also contribute to regulation of retinol levels (63, 64). Stellate cells are also found in the intestine, heart, large blood vessels, ovaries, and testes, and these cells store retinyl esters when large doses of vitamin A are consumed (65).

Circulatory RBP (21kD) is a component of a specific extracellular transport system for retinol (66). RBP serves to solubilize the lipid-soluble vitamin in the aqueous serum and to protect it from oxidative destruction (67). In the plasma, RBP is also associated with transthyretin (TTR) (55kD). In the plasma of some animals, the molar ratio of TTR to RBP is approximately two to one; and virtually all of the RBP is complexed to TTR, thereby making retinol-RBP less susceptible to glomerular filtration (68). The RBP-TTR interaction is important for establishing or maintaining normal plasma RBP and retinol levels (69, 70) (Figures 4 and 5).

Vitamin A Extra-hepatic Cellular Uptake

The mechanisms for cellular uptake of free all-trans-retinol bound to RBP/TTR, or all-trans-retinoic acid are not well defined and may be tissue dependent. Also, the method of transport from the vasculature to the tissue is unknown at present. It is possible
Figure 5: Overview of vitamin A Uptake in Extra-hepatic Cells from the Blood.

Retinol bound to retinol binding protein (RBP) and complexed to transthyretin (TTR) is the predominant form of vitamin A present in the circulation. The uptake of retinoids into cells may be receptor dependent (i.e. the uptake of retinol via the binding of RBP to STRA6), chylomicron dependent receptor-mediated endocytosis (facilitating the uptake of retinyl esters), or by facilitated diffusion (i.e. the uptake of all-trans-retinoic acid by channel proteins). Once within the cell, retinol can either be oxidized to retinal by the actions of retinol dehydrogenases (RoLDH) or esterified to retinyl ester by the action of lecithin:retinol acyltransferase (LRAT). The retinyl esters present within cells can be hydrolyzed to retinol via the actions of retinyl ester hydrolase (REH). Retinol is subsequently oxidized by a retinal dehydrogenase (RALDH) to all-trans-retinoic acid (ATRA). ATRA and 9-cis-retinoic acid are then transported through the cytoplasm to the nucleus by CRABP, where it is thought to passively diffuse into the nucleus or enter the nucleus through receptor dependent mechanisms. ATRA and 9-cis-retinoic acid bind retinoic acid receptor (RAR) and retinoic X receptor (RXR), respectively within the nucleus. ATRA/RAR and 9-cis RA/RXR can then heterodimerize at retinoic acid response elements (RARE) regulating retinoid–responsive genes.
that all-\textit{trans} -retinol uptake into the tissue may involve cell membrane receptors for RBP or TTR (receptor mediated endocytosis or delivery of retinol via a pore-like receptor), or may be independent of any specific receptor for these proteins (partitioning in cell membrane and/or metabolism into all-\textit{trans}-retinoic acid and further sequestration by internal cellular retinoic acid binding protein (CRABP), or via lipid vesicle (chylomicrons) transfer, and evidence for all three of these possibilities have been obtained in different animal model systems (71-77) (\textbf{Figure 5}). It has also been suggested that retinol spontaneously dissociates from its binding site on RBP outside the cell, and is passively transferred through the plasma membrane (73) or that the RBP receptor acts as a channel, delivering retinol bound to extracellular RBP to intracellular CRBP (78).

Kawaguchi et al. reported through the use of mouse and human cell lines that STRA6, a multi-transmembrane domain receptor, takes up retinol with high affinity to its extracellular carrier protein (RBP) and to its intracellular binding protein (CRBP I)(79).

Pasutto et al. determined that loss of STRA6 can lead to multiple organ abnormalities including alveolar capillary dysplasia and lung hypoplasia in mice (80). Ross et al. found that administering a 10:1 molar combination of VA and all-\textit{trans}-retinoic acid (VARA) induced STRA6 (retinol binding protein receptor) expression in newborn rats accompanied by a synergistic increase in lung retinyl esters (81).

Finally, all-\textit{trans}-retinoic acid, which circulates in the blood in nanomolar amounts (in normal states) bound to albumin (\textbf{Figure 5}), can be transferred into specific tissues, such as the brain and the liver, by passive diffusion (59). All-\textit{trans}-retinoic acid is far less hydrophobic than all-\textit{trans}-retinol or all-\textit{trans}-retinyl ester, and it is not absorbed in chylomicrons but rather transported via the portal system bound to albumin because
binding to RBP hinders its interaction with TTR (82). It is possible that all-trans-retinoic acid may act as a hormone and passively enter cells or through organellar membranes (such as the nucleus or mitochondria), or that albumin transfers all-trans-retinoic acid to a plasma membrane receptor, acting as a channel, delivering all-trans-retinoic acid to CRABP (78).

Mechanism of Action of Retinoids: Nuclear Receptors

Retinoids have been shown to modulate fundamental physiologic processes, including embryonic development, cellular proliferation, differentiation, and apoptosis. These effects are mediated by changes in gene expression or function. A major breakthrough in the understanding of the mechanism by which retinoids exert their effects on gene expression was the discovery that certain members of the large steroid hormone receptor superfamily can bind retinoic acid (83, 84). Two families of nuclear retinoid receptors exist, termed retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both with three subtypes: α, β, γ (83, 84). RARs can bind all-trans-retinoic acid or 9-cis-retinoic acid with high affinity; RXRs selectively interact with the latter ligand (85). RARs function as heterodimers with RXRs and bind retinoic acid responsive elements (RARE) upstream from the promoters of target genes in the regulation of gene transcription (83, 84) (Figure 5). RARs form heterodimers with the three RXR subtypes, and RXRs form heterodimers with members of the nuclear receptor family, including peroxisome proliferator-activated receptor γ (PPARγ) (86).
Addition of retinol or all-
trans-
retinoic acid to different cell types such as the lung results in induction of CRBP I (87, 88). Smith et al. reported that a RARE is present in a CRBP promoter, suggesting that control of CRBP gene transcription by all-
trans-
retinoic acid or RAR may represent a positive feedback mechanism important in regulating cellular uptake of retinol and retinyl ester storage (89).

In order to overcome any issues with vitamin A delivery or absorption of vitamin A, we first evaluated the short-term effects of orally administering vitamin A (VA: retinyl palmitate), all-
trans-
retinoic acid (all-
trans-
RA), and a 10:1 molar combination of VA and all-
trans-
RA, (labeled “VARA”), in newborn C57BL/6 mice exposed to air (normoxia) or hyperoxia (95% O₂) for 4 days. Next, lung retinyl ester content was measured, and lung injury and development were evaluated. Additionally, mediators of inflammation and alveolar development were evaluated. The short-term study demonstrated that VARA was effective in increasing lung retinyl ester content and in reducing lung injury in newborn mice exposed to hyperoxia, possibly through modulation of pro-inflammatory mediators.

Next, we wanted to determine the maturational and functional benefits of an increase in lung VA. We exposed newborn C57BL/6 mice to air (normoxia) or hyperoxia (85% O₂) for 7 or 14 days while they were administered either vehicle or VARA. Next, lung retinol content was measured, and alveolar development and lung function were evaluated. Additionally, mediators of oxidative stress, inflammation and alveolar development were assessed. This longer-term study determined that an increase in lung VA improved alveolar development and lung function in hyperoxia-exposed newborn mice, possibly by modulation of oxidative stress and/or inflammation. Equally important
is that both the short- and longer-term exposures identified common potential pathways that may be affected by retinoids, many of which are known to be contributing factors to the development of BPD. The short-term hyperoxia exposure findings can be found in the second section of this dissertation, and the longer-term hyperoxia exposure findings can be found in the third section of this dissertation. The fourth and final section contains an integrated synthesis of results from the previous sections within the context of the current field of literature, and concludes with a discussion of a potential mechanism of action of retinoids that will be of benefit to future research of retinoids and hyperoxia-induced impaired alveolar development.
Vitamin A and Retinoic Acid Act Synergistically to Increase Lung Retinyl Esters During Normoxia and Reduce Hyperoxic Lung Injury in Newborn Mice

by

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Format adapted and errata corrected for dissertation
ABSTRACT

We have shown that vitamin A (VA) and all-trans-retinoic acid (RA) synergistically increase lung retinyl ester content in neonatal rats. To confirm if this biochemical synergism attenuates early neonatal hyperoxic lung injury in mice, we exposed newborn C57BL/6 mice to 95% O₂ or air from birth to 4 days. The agent (vehicle, VA, RA or the combination VARA) was given orally daily. Lung and liver retinyl ester content was measured, and lung injury and development were evaluated. We observed that lung, but not liver, retinyl ester levels were increased more by VARA than by VA or RA alone. Hyperoxic lung injury was reduced by VA and RA, and more so by VARA. VARA attenuated the hyperoxia-induced increases in macrophage inflammatory protein (MIP)-2α mRNA and protein expression, but did not alter hyperoxia-induced effects on peptide growth factors (Platelet-derived growth factor [PDGF], vascular endothelial growth factor [VEGF], and transforming growth factor [TGF]-β1). The 4 day exposure to hyperoxia or retinoids did not lead to observable differences in lung development. We conclude that the VARA combination has synergistic effects on lung retinyl ester concentrations and on the attenuation of hyperoxia-induced lung injury in newborn mice, possibly by modulation of inflammatory mediators.
INTRODUCTION

Very premature infants are at high risk for bronchopulmonary dysplasia (BPD) (1). Vitamin A (VA) deficiency often observed in premature infants is associated with a higher incidence of BPD (2). Randomized controlled trials and a recent systematic review indicate VA supplementation decreases BPD and/or death (3, 4). Despite supplementation, 25% of infants remain VA deficient (4). The persistence of biochemical VA deficiency may be due impaired VA transport as transthyretin, a major VA transport protein, is reduced by inflammation (5).

Retinoids are important for normal lung development and maturation (6, 7). Hyperoxia induces lung injury, and may contribute to BPD (1). Animal models have shown that administration of RA (the principal metabolite of VA) attenuates hyperoxic lung injury (8, 9). We have shown that a combination of VA (the nutrient) and RA (the metabolite) improved tissue retinoid stores much more than either VA or RA alone in infant rats (10, 11). It is possible that the functional consequence of this increase in tissue retinoid stores is a synergism in the attenuation of hyperoxic lung injury, which may be of therapeutic relevance in premature neonates as the administration of RA may circumvent the effects of a reduction in VA transport.

The objective of the present study was to determine whether the combination of VA and RA in the newborn mouse model would increase lung retinyl ester (RE) content and reduce early hyperoxic lung injury to a greater extent as compared to VA or RA administered alone.
METHODS

Experimental animals and animal care

The study was approved by the Institutional Animal Care and Use Committee (IACUC) and was consistent with the PHS policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare, 2002).

Neonatal mouse model of hyperoxic lung injury (*in vivo*):

Newborn C57BL/6 mice along with their dam were exposed to hyperoxia (95% O₂) or room air from birth to four days of age in a plexiglas chamber. The O₂ concentration (OM-100 oxygen analyzer, Newport Medical Instruments, Newport Beach, CA), humidity, temperature, and barometric pressure (Fisherbrand Digital Barometer, Fisher Scientific, Pittsburgh, PA) within the chamber were continuously monitored.

Experimental design

Newborn mouse pups while exposed to either air or hyperoxia were given one of four agents: vehicle (canola oil), VA, RA, or VARA daily for 4 days, and then sacrificed. At least four litters were used for each of these eight groups. Two litters in each group were used for evaluation of lung injury, and the other two litters were used for evaluation of lung and liver RE content. Additional litters (Air-vehicle, Air-VARA, Hyperoxia-vehicle, and Hyperoxia-VARA groups) were used either for bronchoalveolar lavage fluid (BALF) collection, or for calculation of the wet to dry weight ratio.

Administration of vehicle, VA, RA, or VARA

VA (all-trans-retinyl palmitate, Sigma-Aldrich, St. Louis, MO) was diluted directly in canola oil to a concentration of 0.1 mmol/g oil (2x concentrate). For RA (all-trans-RA,
Sigma-Aldrich), 200 µl ethanol was added to 3 mg RA, and 800 µl oil was then added to achieve a concentration of 0.01 mmol/g (2x concentrate). The 2x concentrates of VA and RA were mixed 1:1 (wt:wt) with oil to form the 1x doses for administration, or 1:1 with each other to form the VARA dose (0.05 mmol VA: 0.005 mmol RA per g dose). Retinoids were stored at 4°C in foil-wrapped vials. The doses were administered into the mouse pup’s mouth at 2 µL per day daily for 4 days. These doses were based upon our previous studies in rat pups (10). The dose selected was 20,000 IU (6 mg retinol)/kg for VA (100 IU for a 5 g mouse pup), based on Humphrey et al who provided 50,000 IU to 2.5 kg neonates (12). For RA, the dose was 500 µg/kg with an assumed 80% absorption (3.1 µg for a 5 g pup), based on Massaro and Massaro’s work (13).

**Evaluation of lung and liver retinol levels**

Lung and liver retinol and RE were measured by HPLC (10).

**Evaluation of lung injury**

**Histology**

Lung injury was evaluated in at least six animals per group from two litters. Lungs were fixed in inflation (25 cm H₂O pressure) using 10% formalin. Ten random high power (400x) fields from 5µm H&E stained sections of a mid-coronal section from apex to base of both lungs were evaluated, using a scoring system by an observer masked to treatment group (Table 1). Lung sections were stained with Pan Macrophage Marker F4/80 (Abcam, Cambridge, MA) and Myeloperoxidase (Thermo Scientific, Fremont, CA) for evaluation of macrophages and neutrophils, respectively. Primary antibodies were used at 1:100 dilution for 30 minutes followed by appropriate secondary antibodies conjugated with AlexaFluor 594 (Invitrogen, Eugene, OR). Slides were then evaluated
for macrophage and neutrophil abundance using a scoring system (Table 1) by an observer masked to slide identity. Quantitative morphometric analysis was carried out by light microscopy interfaced with a image analysis system (MetaMorph 6.2v4 software, Universal Imaging Corp., Downingtown, PA).

**BALF measurements**

BALF was collected by tracheostomy and cannulation with a 24G cannula, and two instillations of 0.3 ml of sterile PBS. BALF was assayed for total protein using the Bradford Assay (Bio-Rad) and for cytoplasmic lactate dehydrogenase (LDH, a marker of cell injury) (Sigma-Aldrich).

**Cytokine assays**

Quantitative real-time PCR for mRNA was performed as described previously (14, 15) on homogenized lungs for cytokines known to be involved in hyperoxic lung injury or with BPD/death: IL-1β (16, 17), IL-6 (16, 17, 18), TNF-α (17-19), IL-10 (16), monocyte chemoattractant protein (MCP)-1 (20,21), macrophage inflammatory protein (MIP)-2α (21, 22), and regulated upon activation, normal T-cell expressed, and secreted (RANTES) (16). The mouse primer sequences are listed in Table 2. Protein concentrations of these cytokines were evaluated in whole lung homogenates by ELISA (R&D Systems, Minneapolis, MN & SA Biosciences, Frederick, MD), and normalized to total protein measured using the Bradford Assay. In order to batch-process all the samples simultaneously for better validity, only the four main groups (21% O₂ Vehicle, 21% O₂ VARA, 95% O₂ Vehicle, and 95% O₂ VARA) were evaluated.
Peptide growth factor assays

Quantitative real-time PCR was performed for peptide growth factors known to be involved in hyperoxic lung injury: PDGF (23), VEGF (24, 25), and TGF-β1 (26). These peptide growth factors were also measured by ELISA (R&D Systems) in whole lung homogenates.

Evaluation of lung development

Alveolar development was evaluated by mean linear intercept (MLI) (27), radial alveolar counts (RAC) (28), and secondary septal crest density (15) as previously described (14, 15).

Statistics

Data are presented as the mean ± SEM. Lung and liver retinyl ester contents, lung developmental indices, and lung injury scores were analyzed by a three-way ANOVA (Factors: Oxygen, VA, RA), followed by multiple comparison testing by the Holm-Sidak method if significant differences (at p<0.05) were noted by the ANOVA. When the variance terms were unequal among groups, log₁₀ transformation was performed prior to statistical testing.

RESULTS

The mouse pups exposed to hyperoxia did not have obvious respiratory distress or impaired survival. Administration of retinoids was well tolerated. No differences in growth were noted with hyperoxia or with retinoid administration at 4 days.
Lung retinyl ester concentration

70 mouse pups were evaluated for lung RE (6-15/group). In air-exposed animals, VA and RA both increased lung RE content to a comparable extent, while the combination of VA and RA led to a marked synergistic increase in lung RE content, as the increase by VARA was more than the increase by the same amount of VA or RA alone (Figure 1A). Hyperoxia-exposed animals given vehicle had lower lung RE content, while VA and VARA both led to significant and comparable increases in lung RE content. The synergistic effect of the VARA combination on lung RE content that was seen in air-exposed animals was attenuated in hyperoxia. RA alone did not significantly increase lung RE in hyperoxia (Figure 1A). The relative proportions of lung REs were similar for all treatment groups, showing a major peak of retinyl palmitate with a shoulder of retinyl oleate, followed by a second peak of retinyl stearate (data not shown).

Liver retinyl ester concentration

53 mouse pups were evaluated for liver RE (4-7/group). In both air-exposed animals and hyperoxia-exposed animals, VA and VARA moderately increased liver RE content (Figure 1B). RA did not change liver RE in air-exposed animals, although a small but statistically significant decrease was noted in the hyperoxic mice (Figure 1B).

Lung injury

Histology

63 mouse pups were evaluated by histology (6-13/group). In air-exposed animals, VA, RA, and VARA did not change lung injury scores (Table 3). Hyperoxia increased lung injury scores in vehicle-exposed mice, although the magnitude of injury was mild (average score per field <2) (Table 3; Figure 2). In the hyperoxia-exposed animals,
administration of VA, RA, and VARA lowered injury scores compared to the vehicle group, and there was a further decrease in injury scores in the VARA group compared to the RA group (Table 3; Figure 2). There was no significant increase in alveolar macrophages in the hyperoxia-exposed mice (Table 3). Very few neutrophils were observed on the MPO staining, indicating a lack of neutrophilic infiltrate at this time point. No differences in wet-to-dry weight ratio or lung volumes were noted between the groups (data not shown).

**Cytokines**

Hyperoxia increased mRNA of IL-1β, IL-6, MCP-1, MIP-2α, and TNF-α, and decreased that of IL-10 (Figure 3). VARA supplementation prevented the hyperoxia-induced increases of IL-1β, IL-6, and TNF-α but did not affect the MCP-1 increase. The hyperoxia-induced reduction in IL-10 was prevented by VARA. VARA supplementation in air exposed newborn mice did not change the gene expression of any of the evaluated cytokines. RANTES gene expression did not change with either hyperoxia or VARA.

Changes in protein concentration of these cytokines in the lung homogenates however did not follow the changes in mRNA, with the exception of MIP-2α which was increased in the hyperoxia-vehicle group and was similar to air-vehicle and air-VARA in the hyperoxia-VARA group (Figure 3). IL-1β and RANTES protein were not significantly changed with either hyperoxia or VARA. IL-6 increased with hyperoxia, and VARA did not change IL-6 significantly in either air or hyperoxia compared to vehicle at the same oxygen concentration. Hyperoxia did not alter MCP-1 and TNF-α, and VARA reduced MCP-1 and TNF-α in both air and hyperoxia conditions compared to
vehicle. IL-10 protein was increased in the hyperoxia-vehicle group, and this increase was prevented by VARA.

**Peptide growth factors**

Neither hyperoxia nor VARA supplementation significantly changed PDGF mRNA or protein in lung homogenates (Figure 4). VEGF mRNA was increased only in the hyperoxia-VARA group, but VEGF protein was increased in both hyperoxia-vehicle and hyperoxia-VARA groups (Figure 4). A trend toward increased TGF-β1 mRNA expression was noted in hyperoxia-vehicle and hyperoxia-VARA groups (0.05<p<0.20 for effect of 95% O₂), but total (latent+active) TGF-β1 protein was similar in all groups, and active TGF-β1 protein was reduced in the hyperoxia-vehicle and hyperoxia-VARA groups (Figure 4).

**BALF analysis**

LDH was increased in the BALF of the hyperoxia-vehicle group (A₄₉₀⁻₆₇₀: 0.027±0.006 [mean+SEM], p<0.05 vs. other groups), while LDH concentrations were similar in the air-vehicle (0.016±0.002), air-VARA (0.013±0.002), and hyperoxia-VARA (0.013±0.002) groups. Protein concentrations were not significantly different (mean+SEM in µg/ml, air-vehicle: 6.4±1; air-VARA: 7.2±1; hyperoxia-vehicle: 11.8±4; hyperoxia-VARA: 7.2±4; p = 0.2 by 2-way ANOVA)

**Lung development**

Changes in MLI and secondary crest density over this brief time period were not statistically significant (p>0.05) (Table 4). RAC was lower in pups exposed to hyperoxia, but administration of VA, RA, and VARA did not change RAC significantly (Table 4).
DISCUSSION

We observed that an equimolar combination of VA and RA increased lung retinyl ester concentration in a newborn mouse model more than either VA or RA alone, confirming our previous studies in neonatal rats that the VARA combination synergistically increases lung RE content (10). A novel observation was that the synergistic effect of VARA on lung RE content seen in air-exposed newborn mice was blunted in hyperoxia-exposed mice, suggesting increased consumption or decreased uptake of lung RE during hyperoxia. In addition, VARA demonstrated synergism in attenuating early hyperoxic lung injury, accompanied by prevention of hyperoxia-induced increases in MIP-2α mRNA and protein. VARA also prevented hyperoxia-induced increases in gene expression of several pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and hyperoxia-induced reduction of IL-10, with less consistent changes in protein concentrations of these cytokines.

Lung development in the human from week 24 of gestation through the first 2 years of age parallels lung development of mice in the first two postnatal weeks (29, 30), and the newborn mouse lung is therefore a good model for lung injury in preterm infants. Exposure of neonatal mice to hyperoxia during this period of rapid lung development leads to pathophysiology similar to human BPD with decreased alveolarization (31). Retinoids are essential for normal lung morphogenesis and postnatal maturation (32). Veness-Meehan et al. (9) demonstrated that all-trans-RA treatment of newborn rats during hyperoxia improves survival and alveolarization. The exact mechanisms by which retinoids attenuate hyperoxia effects in the newborn lung are not certain, but modulation of cytochrome P4501A enzymes (8), growth factor expression (33,34), or CRABP-I (35)
may play a role. Regardless of the mechanism by which retinoids exert their effect, an increase in lung VA content is essential for this benefit. Neonates have very low VA reserves due to the limited transplacental VA passage (36), and lung RE content declines abruptly before birth (37). As all-trans-RA administration increases alveolar septation even in normally nourished rodents (13), physiologically low levels of RE at birth may be marginal and rate-limiting for lung development. We have previously shown that the VARA combination is several-fold more effective in increasing lung RE content than either VA or RA separately in newborn rats (10, 11). The present study determined that this increase in lung RE content may be of functional significance.

The hyperoxia-induced increases in pro-inflammatory cytokines and reduction in the anti-inflammatory IL-10 mRNA, and the attenuation of these changes with VARA indicates a possible mechanism of action of retinoids. However, except for MIP-2α, we observed that changes in protein concentrations of these cytokines did not correlate with mRNA changes. It is possible that post-transcriptional changes in protein synthesis or stability maybe involved and possibly regulated by retinoids. An alternative explanation is that techniques for measuring mRNA and protein differ in sensitivity. We have observed similar discordance between mRNA and protein estimations of various cytokines at later time points (7 days and 14 days) in the hyperoxic newborn mouse model (unpublished data). These results emphasize the importance of measurement of not just gene expression but also corresponding protein concentrations, as well as the complexity of inflammatory regulation in this model. MIP-2α, the murine equivalent of IL-8 in humans, is a potent neutrophil and macrophage chemoattractant, and blockade of its receptor CXCR2 reduces hyperoxic lung injury in adult animals (38). It is possible
that retinoid-induced attenuation of hyperoxic increases in MIP-2α mRNA and protein contribute to its mechanism of action by a reduction in subsequent lung inflammatory cell infiltrate.

We noted that hyperoxia increased VEGF protein and decreased active TGF-β1 in lung homogenates. Increased VEGF has been noted by other investigators from days 4-9 followed by a decrease from days 12-14 (25), indicating that temporal profiles of these mediators may be important. VEGF protein levels are also initially high followed by a decline in premature infants who subsequently develop BPD/death (24). Studies involving hyperoxia-exposed newborn animal models have demonstrated increased TGF-β signaling (26, 39), but these studies have not evaluated early time points. We speculate that differences in TGF-β signaling may be isoform-specific and time-dependent in the newborn lung, and the initial reduction in active TGF-β may possibly initiate impaired alveolarization, while later increased TGF-β signaling may contribute to a profibrogenic state.

The demonstration of VARA synergism is of clinical relevance. Increased VA dosing is not sufficient to alleviate VA deficiency in ELBW infants with lung inflammation, due to a lack of transport of VA to the lung (5). The current study overcomes this hurdle by co-administration of VA and RA. As RA increased lung RE, and RA is not converted to RE, increased lung RE content must be due to regulation of retinol metabolism or distribution by RA. While RA regulates retinol metabolism and increases RE content modestly, VA is rate-limiting for the storage of larger amounts of RE in the lungs (10). In the VARA preparation, the molar ratio of RA to VA is 1 to 10, suggesting that a small relative amount of RA influences the distribution of a larger
amount of VA. The synergistic effect of the VARA combination on lung RE content indicates that VA and RA have different but interacting roles in lung RE storage (10). Similar to our previous results in newborn rats, liver RE content increased only modestly and only to the VA component of VARA, possibly due to rapid oxidation of RA in the liver (40). The synergistic effect of VARA on lung RE was blunted with hyperoxic exposure, possibly due to increased RE consumption or decreased uptake, although this hypothesis was not specifically investigated in this study.

However, there are limitations to this study. First, while hyperoxia-exposed newborn mice are a common reproducible animal model, this model may not simulate all aspects of human BPD. Effects of VA and RA in newborn mice may not be very similar to their effects in preterm infants. As our study was designed to evaluate the effects of acute hyperoxia on lung retinyl esters and early lung injury, we did not observe effects on lung development and noted only mild lung injury and few inflammatory cells. Studies in newborn rats have shown that lung myeloperoxidase is not increased until day 6 of hyperoxia (22), and newborn mice exposed to 80% oxygen for 8 days did not have neutrophil infiltration (41). The rationale for evaluation at the early four day time point, even though lung injury is not severe, was to identify the early hyperoxia-induced changes that set into motion subsequent inflammation/injury. Once lung inflammation is established, it is difficult to determine the sequence and relevance of mediators as many cytokines and growth factors are released by inflammatory cells. Despite these limitations, this study has many strengths, including the analysis of not only biochemical evidence of RE storage, but also the functional impact on lung injury in a clinically relevant model.
In conclusion, this study demonstrates the efficacy of a combination of VA and RA in increasing not only lung RE concentrations, but also in reducing hyperoxic lung injury in a newborn mouse model. The combination of VA and RA has the therapeutic potential of reducing BPD to a greater extent than with VA supplementation alone. Further investigation is required to determine if the synergism of VARA combination attenuates other models of lung injury, and if this synergism is limited to the neonatal period.

ACKNOWLEDGEMENTS

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ABBREVIATIONS:

BALF: Bronchoalveolar lavage fluid
BPD: Bronchopulmonary dysplasia
MIP: Macrophage inflammatory protein
MCP: Monocyte chemoattractant protein
RA: all-trans-Retinoic acid
RANTES: Regulated upon Activation, Normal T-cell Expressed, and Secreted
RE: Retinyl esters
VA: vitamin A
VARA: vitamin A + all-trans-Retinoic acid
REFERENCES


Figure 1. Lung and liver retinyl ester concentrations in neonatal mice exposed to room air or hyperoxia, while being treated with vehicle (Veh), vitamin A (VA) alone, retinoic acid (RA) alone, or vitamin A + retinoic acid (VARA). Lung and liver RE (nmol/g) were analyzed on day 4. **Panel A: Lung retinyl ester concentrations.** [mean ± SEM; n per group shown at base of bar]. **Panel B: Liver retinyl ester concentrations.** [mean ± SEM of log_{10} transformed data; n per group shown at base of bar] (*p<0.05 vs. corresponding Vehicle, † p<0.05 vs. corresponding VA, ‡ p<0.05 vs. corresponding RA, § p<0.05 vs. corresponding Air)
Figure 2. Photomicrographs of lungs from neonatal mice exposed to room air or hyperoxia while being treated with vehicle (Veh), vitamin A (VA) alone, retinoic acid (RA) alone, or vitamin A + retinoic acid (VARA) (H&E stain; 400x; calibration bar = 50 µm; Br = Bronchus). Exposure to hyperoxia led to hemorrhage (erythrocytes within alveoli and in alveolar septae) and airway epithelial injury (denuded epithelial cells within Br) that were attenuated with VA, RA, and to a greater extent with VARA.
Figure 3. Cytokine gene expression and protein levels in lungs of neonatal mice treated with vehicle (Veh) or vitamin A + retinoic acid (VARA) while exposed to room air or hyperoxia. Gene expression and protein levels of cytokines in lung homogenates were analyzed on day 4 by competitive real-time PCR and by ELISA, respectively (Mean ±SEM, n= 6 animals per group; *p<0.05 vs. corresponding Vehicle, § p<0.05 vs. corresponding Air).
Figure 4. Peptide growth factor gene expression and protein concentration in lungs of neonatal mice treated with vehicle (Veh) or vitamin A + retinoic acid (VARA) while exposed to room air or hyperoxia. Gene expression and protein concentrations of PDGF, VEGF, and TGF-β1 in lung homogenates were analyzed on day 4 by competitive real-time PCR and by ELISA, respectively (Mean ±SEM, n= 6 animals per group; *p<0.05 vs. corresponding Vehicle, § p<0.05 vs. corresponding Air).
Table 1
Scoring system for lung injury and macrophage abundance

<table>
<thead>
<tr>
<th>Epithelial Injury</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Normal intact epithelium</td>
<td>1 Rare cells have separated from epithelium</td>
<td>2 10-25% of epithelial cells have separated from underlying basement membrane</td>
<td>3 25-50% of epithelium has separated</td>
<td>4 51-75% of epithelium has separated</td>
<td>5 &gt;75% of epithelial cells have separated</td>
<td></td>
</tr>
<tr>
<td>1 Few interstitial RBC</td>
<td>2 Few RBC in some alveoli</td>
<td>3 Moderate number of RBC in some alveoli</td>
<td>4 Many RBC in most alveoli</td>
<td>5 Large numbers of RBC in all alveoli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Moderate number of RBC in some alveoli</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Hemorrhage</th>
<th>0 No red blood cells (RBC) outside of blood vessels</th>
<th>1 Few interstitial RBC</th>
<th>2 Few RBC in some alveoli</th>
<th>3 Moderate number of RBC in some alveoli</th>
<th>4 Many RBC in most alveoli</th>
<th>5 Large numbers of RBC in all alveoli</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Many RBC in most alveoli</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Macrophage abundance                   | 0 None-Rare                                         | 1 1-10% of alveoli/saccules contain macrophages | 2 10-25%                        | 3 25-75%                       | 4 >75% |
### Table 2
Mouse primer sequences for real time quantitative PCR

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>18S Forward</td>
<td>GTC TGC CCT ATC AAC TTT CG</td>
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<tr>
<td>18S Reverse</td>
<td>ATG TGG TAG CCG TTT CTC A</td>
</tr>
<tr>
<td>PDGF Forward</td>
<td>TAA CAC CAG CAG GTT CAA GTG</td>
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<td>PDGF Reverse</td>
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<td>VEGF Forward</td>
<td>GAG CGG AGA AAG CAT TGG TTT G</td>
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<td>VEGF Reverse</td>
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</tr>
<tr>
<td>TGF-β1 Forward</td>
<td>GCC CTG GAT ACC AAC TAT TGC TT</td>
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<tr>
<td>TGF-β1 Reverse</td>
<td>AGT TGG CAT GGT AGC CCT TG</td>
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<td>IL-1β Forward</td>
<td>CCA TCC ACG CTG TTT TGA CC</td>
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<td>IL-1β Reverse</td>
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<td>MIP-2α Forward</td>
<td>CCACTCTCAAGGGCGGTCAAA</td>
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<td>MIP-2α Reverse</td>
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</tr>
<tr>
<td>MCP-1 Forward</td>
<td>AGG TCC CTG TCA TGC TTC TGG</td>
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<td>MCP-1 Reverse</td>
<td>GTG AAT GAG TAG CAG CAG GTG AG</td>
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<td>TNF-α Forward</td>
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<td>TNF-α Reverse</td>
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</tr>
<tr>
<td>IL-10 Reverse</td>
<td>GTG AAG ACT TTC TTT CAA ACA AAG</td>
</tr>
<tr>
<td>RANTES Forward</td>
<td>TGG CAG GAG TGC AAC AAG AA</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>RANTES Reverse</td>
<td>CTC AAG TTC GCT CAG CTT TCC T</td>
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### Table 3
Lung injury and macrophage abundance scores

<table>
<thead>
<tr>
<th></th>
<th>21% O₂ (n=6/gp)</th>
<th>95% O₂ (n=6/gp)</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>VA</td>
</tr>
<tr>
<td>Airway epithelial injury</td>
<td>0.35±0.1</td>
<td>0.32±0.15</td>
</tr>
<tr>
<td>Hemorrhage</td>
<td>0.17±0.1</td>
<td>0.25±0.14</td>
</tr>
<tr>
<td>Macrophage index</td>
<td>2.16±0.4</td>
<td>2.0±0.0</td>
</tr>
</tbody>
</table>

*Definitions of abbreviations: VA, Vitamin A; RA, retinoic acid; Group mean values (±SEM)*

* p<0.05 compared with corresponding vehicle

† p<0.05 compared with corresponding VA

‡ p<0.05 compared with corresponding RA

§ p<0.05 compared with corresponding Air (21% O₂)
Definitions of abbreviations: VA, Vitamin A; RA, retinoic acid; MLI, mean linear intercept; RAC, radial alveolar counts; SSC, secondary septal crests. MLI, RAC, and SSC showed no statistically significant differences between groups, except for a reduction in RAC in the 95% O₂-Vehicle group compared to the 21% O₂-Vehicle group.
VARA ATTENUATES HYPEROXIA-INDUCED IMPAIRED ALEVOLAR
DEVELOPMENT AND LUNG FUNCTION IN NEWBORN MICE

by

MASHEIKA L. JAMES, A. CATHERINE ROSS, TEODORA NICOLA, CHAD
STEELE, NAMASIVAYAM AMBALAVANAN

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Format adapted for dissertation
ABSTRACT

We have recently shown that a 10:1 molar combination of VA and all-trans-RA (VARA) synergistically increases lung retinoid content in newborn rodents. We hypothesized that the increase in lung retinoids would translate into functional benefits in the attenuation of alveolar simplification and abnormal lung function in newborn mice exposed to hyperoxia through reduction of oxidative stress markers and pro-inflammatory cytokines. Newborn C57BL/6 mice were exposed to 85% O₂ (hyperoxia) or air (normoxia) for 7 or 14 days from birth and given vehicle or VARA every other day. Lung retinol content was measured by HPLC, function was assessed by flexiVent, and development was evaluated by radial alveolar counts, mean linear intercept, and secondary septal crest density. Mediators of oxidative stress, inflammation, and alveolar development were evaluated in lung homogenates. We observed that VARA attenuated hyperoxia-induced alveolar simplification and improved lung function, with higher compliance and lower resistance, associated with increased lung retinol stores. VARA attenuated hyperoxia-induced increases in DNA damage and protein oxidation but did not alter malondialdehyde adducts, nitrotyrosine, or myeloperoxidase concentrations. IFN-γ and MIP-2α mRNA and protein increased with hyperoxia and this increase was attenuated by VARA. Supplementation and inhibition experiments indicated that MIP-2α and IFN-γ were sufficient but not necessary for hyperoxia-induced inhibition of alveolar development. Our study demonstrates that the VARA combination increases lung retinol and attenuates hyperoxia-induced inhibition of alveolar development and abnormal lung function, accompanied by a reduction in oxidative stress and pro-inflammatory cytokines.
KEYWORDS

Bronchopulmonary dysplasia; vitamin A; Retinoic acid; 8-OH deoxyguanosine; Protein carbonyls; Macrophage inflammatory protein-2α; Interferon-γ
INTRODUCTION

Bronchopulmonary dysplasia (BPD) in preterm infants, a common cause of morbidity and mortality, is characterized by alveolar simplification (fewer and larger alveoli with loss of septation) and reduced microvascular development (1-3). The etiology of BPD is multifactorial and includes genetic predisposition, barotrauma, and volutrauma from mechanical ventilation, reactive oxygen species production from prolonged oxygen use and high oxygen concentrations, and infections which lead to inflammation, damage, and attenuation of normal growth and repair of the developing neonatal lung (4).

Extremely low birth weight (ELBW; birth weight \( \leq \) 1000g) infants are susceptible to the development of BPD (5). Low plasma and tissue concentrations of vitamin A (VA; retinol) are often observed in ELBW infants and are associated with a higher incidence of BPD (6). Randomized controlled trials and a recent systematic review indicate vitamin A supplementation decreases BPD and/or death (7) and trends for improvement have also been noted in longer-term outcomes at 18-22 months of age (8). Despite supplementation, 25% of infants remain vitamin A deficient (9) and the mechanisms by which VA improves outcomes have not been determined. We have shown that a 10:1 molar combination of vitamin A (VA; the nutrient) and all-trans-retinoic acid (RA; the metabolite) (labeled “VARA”) improved tissue retinoid stores much more than either VA or RA alone in neonatal rats and mice (10, 11). However, it is important to determine whether such an increase in retinoid stores translates into an improvement in alveolar development and lung function during hyperoxia exposure. We hypothesized that VARA
supplementation would attenuate hyperoxia-induced alveolar simplification and abnormal lung function by reducing oxidative stress and pro-inflammatory cytokines.

METHODS

All protocols were approved by the Institutional Animal Care and Use Committee of UAB. All experiments were done with a minimum of six mice from at least two litters for each condition.

Study Groups

C57BL/6 dams and litters were exposed to either 85% O₂ (hyperoxia) or air (normoxia) (11). Dams were switched every 24 hours to prevent oxygen toxicity. Newborn mice were orally administered vehicle (canola oil) or VARA (0.05 mmol VA: 0.005 mmol RA per g dose) on alternate days (10, 11). Additional sets of mice received intranasal recombinant interferon- gamma (IFN-γ) and macrophage inflammatory protein-2 alpha (MIP-2α) (200ng; R&D systems) on postnatal days (P) 4, 7, 10, or intraperitoneal IFN-γ and MIP-2α neutralizing antibodies (4µg; R&D systems) on P4, P7, and P10.

On P7 or P14, pups were anesthetized with ketamine/xylazine and pulmonary function was evaluated on a flexiVent (12, 13). Mice were then euthanized and lungs were either inflation-fixed for histology, or lung homogenates prepared for RNA (11-14), protein analysis (11-14), and for markers of oxidative stress. Lung retinol was measured by HPLC (10, 11).
Analysis of alveolar morphometry

Lung alveolar morphometry was performed as previously described (11-14). Alveolar development was evaluated by radial alveolar counts (RAC) (15), mean linear intercept (MLI) (16), and secondary septal crest density (SSC) (11, 12).

Analysis of markers of oxidative stress

Lung homogenates were analyzed for protein carbonyls, 3-nitrotyrosine, and malondialdehyde (MDA) adducts by ELISA (Cell Biolabs, Inc, San Diego, CA). Myeloperoxidase (MPO) was measured by colorimetric activity assay (BioVision, Mountain View, CA). DNA was isolated from whole lung using a Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) and analyzed for 8-OH deoxyguanosine (8-OHdG) by ELISA (Cell Biolabs).

Analysis of mRNA

Total RNA was isolated using a Qiagen total RNA isolation kit and then quantified and reverse transcribed with SYBR Green/Fluorescein Mastermix Reagents (Qiagen). Mouse Angiogenesis RT² Profiler PCR Arrays (PAMM-024, Qiagen) were used for the analysis of 84 angiogenic and pro-inflammatory genes on a Bio-Rad iCycler System (Table 1). Normalization of gene expression values were to the average geometric mean of hypoxanthine guanine phosphoribosyl transferase 1, heat shock protein 90 alpha class B member 1, and beta-actin expression. Quantitative real-time PCR (qPCR) was performed with specific primers (Table 2) as previously described (11-14).
**Analysis of protein**

Lungs were homogenized in 1 ml of a tissue protein extraction reagent (TPER, Pierce Biotechnology) containing protease inhibitor (Roche Diagnostics), centrifuged at 7,000 x g for 5 min, and the supernatant analyzed for 32 key mouse cytokines and chemokines using the Milliplex MAP mouse cytokine/chemokine Premixed 32 Plex (Millipore, Billerica, MA) on a Luminex 200 platform (Luminex Corporation, Austin, TX) (**Table 3**). Samples were further analyzed for MIP-2α and INF-γ, by ELISA (R&D Systems; Qiagen).

**Statistical analysis**

Data were expressed as means ± SE. Data were analyzed by two-way ANOVA to test for separate and combined effects of VARA and hyperoxia. Multiple comparison testing (Student-Newman-Keuls) was performed if statistical significance (p<0.05) was noted by ANOVA.

**RESULTS**

**Group characteristics**

The mouse pups exposed to hyperoxia did not have obvious respiratory distress or impaired survival, and administration of VARA was well tolerated, with survival >90% in all groups. No significant differences in growth were noted with hyperoxia or with retinoid administration at 7 or 14 days. In both air-exposed and hyperoxia-exposed animals, administration of recombinant MIP-2α, MIP-2α neutralizing antibodies, recombinant IFN-γ, and IFN-γ neutralizing antibodies were well tolerated at all time points.
VARA increases lung retinol

VARA increased lung retinol in air-exposed mice (Figure 1). VARA also improved lung retinol in hyperoxia-exposed mice, although the magnitude of increase was less compared to that in air-exposed mice (Figure 1).

VARA attenuates hyperoxia-induced alveolar simplification

Hyperoxia-exposed mice receiving vehicle had alveolar simplification (reduced RAC, increased MLI, and decreased SSC) compared with the air-vehicle groups (Figure 2). VARA attenuated hyperoxia-induced alveolar simplification as evident by an increase in RAC, reduction in MLI, and an increase in SSC (Figure 2). Alveolar development in air-VARA mice was similar to that of air-vehicle mice.

VARA attenuates hyperoxia-induced alterations in lung function

Hyperoxia-vehicle mice had reduced lung compliance and increased lung resistance compared to air-vehicle mice (Figure 3). Hyperoxia-VARA mice had lung compliance and resistance similar to that of air-vehicle and air-VARA mice, indicating that VARA supplementation prevented the hyperoxia-induced reduction in lung compliance and the increase in resistance (Figure 3).

VARA attenuates hyperoxia-induced increases in mediators of oxidative stress

Hyperoxia increased 8-OHdG (Figure 4A), protein carbonyls (Figure 4B), and myeloperoxidase activity (Figure 4E). VARA supplementation attenuated hyperoxia-induced increases in 8-OHdG (Figure 4A) and protein carbonyls (Figure 4B). No changes in MDA-adducts (Figure 4C) and nitrotyrosine levels (Figure 4D) were observed between groups.
VARA attenuates hyperoxia-induced increases in the mRNA expression of key pro-inflammatory mediators

Hyperoxia-vehicle mice had increased mRNA expression of many pro-inflammatory and angiogenic genes (Table 1) such as neutrophil activating protein-3 (NAP-3; Cxcl1), MIP-2α (Cxcl2; IL-8), epithelial derived neutrophil-activating peptide 78 (Cxcl5; ENA-78), epieregulin (Ereg), frizzled homolog 5 (Fzd5), hypoxia inducible factor, 1 alpha subunit (HIF-1α), IFN-γ, interleukin-1beta (IL-1β), matrix metalloproteinase-9 (MMP-9), and tumor necrosis factor-alpha (TNF-α), and the increased expression of these genes was prevented by VARA (Table 1). Hyperoxia-vehicle mice had decreased mRNA expression of angiopoietin-2, fibroblast growth factor-1 (FGF-1), and matrix metalloproteinase-2 (MMP-2), and these effects were prevented by VARA (Table 1). During normoxia, VARA modulated the expression of many pro-inflammatory and angiogenic genes (Table 1) with MIP-2α being the only gene whose expression was decreased 4-fold (Table 1).

VARA attenuates hyperoxia-induced increases in the protein expression of key pro-inflammatory mediators

Hyperoxia-vehicle mice had increased protein expression of many pro-inflammatory markers (Table 3). The protein concentrations of MIP-2α and IFN-γ were increased by hyperoxia and reduced by VARA (Table 3). During normoxia, VARA reduced protein levels of eotaxin, interferon γ -induced protein-10 (IP-10), and monokine induced by interferon γ (MIG) (Table 3).
Inhibition of MIP-2α and IFN-γ does not attenuate hyperoxia-induced alveolar simplification

The administration of MIP-2α and IFN-γ neutralizing antibodies to hyperoxia-vehicle mice reduced MIP-2α by 90% and IFN-γ protein by about 50%, respectively (data not shown). However, these neutralizing antibodies to MIP-2α or IFN-γ did not improve hyperoxia-induced alveolar simplification, either when given alone or in combination (data not shown). Air-exposed mouse pups that were administered either recombinant MIP-2α or IFN-γ had alveolar simplification and inflammatory cell infiltrates similar to that seen in hyperoxia-exposed mouse pups (data not shown).

Hydroxia effects on growth factors known to be involved in lung development

Fibroblast growth factor-10 (FGF-10) mRNA was increased in hyperoxia-VARA mice, but not in hyperoxia-vehicle or air-vehicle mice, indicating an interaction between hyperoxia exposure and VARA in FGF-10 expression (Figure 5B). Hyperoxia exposure increased the mRNA of fibroblast growth factor-7 (FGF-7), fibroblast growth factor-18 (FGF-18), midkine, platelet derived growth factor-A (PDGF-A), sonic hedgehog (Shh), tenascin-C, and lysyl oxidase (LOX) regardless of the presence of VARA, as compared to air-vehicle mice (Figures 5 and 6).

DISCUSSION

The present study is the first to determine the functional benefits of increased lung retinoids on alveolar development and lung function in a newborn mouse model. We have previously shown that the VARA combination improved lung retinoid concentration more than either VA or RA alone in a 4 day acute lung injury mouse model (11). In the
present study, we have shown that the increase in lung retinoids translates into an improvement in alveolar development and lung function during hyperoxia exposure. In addition, we have identified potential pathways that may be modulated by retinoids and lead to improvement of hyperoxia-induced impaired alveolar development and lung function.

There are multiple strengths to our study. We evaluated markers of oxidative stress, mRNA and protein expression of angiogenic factors, the protein expression of key cytokines and chemokines, and the mRNA expression of important growth factors and extracellular matrix proteins during a critical phase of alveolar septation during postnatal lung development. Lung structure as well as function was evaluated following exposure to normoxia as well as hyperoxia, in combination with VARA, recombinant MIP-2α, recombinant IFN-γ, or neutralizing antibodies to either MIP-2α or IFN-γ. However, our study has some limitations. We evaluated important mechanisms associated with abnormal alveolar development (oxidative stress, angiogenesis, cytokines, chemokines, growth factors, and extracellular matrix), but retinoids may affect multiple pathways simultaneously, including those which were not evaluated in this study (17-20). Experiments using whole lung homogenates do not permit the identification of changes in gene expression or protein synthesis in selected cell populations in vivo, such as airway epithelia, endothelial cells, or interstitial cells. Mouse models may not closely resemble human disease due to inter-species differences. While hyperoxia-exposed newborn mice are a common reproducible animal model, this model does not simulate all aspects of human BPD.
Lung development in the human from week 24 of gestation through the first 2 years of age parallels lung development of mice in the first two postnatal weeks (21, 22) and the newborn mouse lung is therefore a good model for impaired alveolar development in preterm infants. Vitamin A (retinol) is essential for normal lung morphogenesis and postnatal maturation (23). Retinol is thought to be stored in lung lipid interstitial cells (LICS) within the lung alveolar wall which synthesize and secrete retinoic acid (24). All-trans-retinoic acid can then bind its receptors, RAR (α, β, or γ) and heterodimerize with RXR at retinoic response elements to regulate the expression of many genes (25, 26). Transgenic animal models have shown that the deletion of α and γ receptors decreases alveolar development (16) but the deletion of the β receptor leads to premature septation indicating its role in inhibiting the premature onset of alveolarization (27).

Chronic postnatal hyperoxia inhibits alveolar development in animals with postnatal alveolar septation (e.g. rats, mice), but not in animals with prenatal septation (e.g. sheep) (28). Newborn mice exposed to chronic hyperoxia have enlarged air spaces and reduced alveolar development similar to the histopathology of BPD (29). Veness-Meehan et al. (30) demonstrated that all-trans-retinoic acid treatment of newborn rats improves survival and alveolarization during hyperoxia exposure. The exact mechanisms by which retinoids attenuate hyperoxia effects in the newborn lung are uncertain, but modulation of oxidative stress mediators (20, 31), pro-inflammatory mediators (11), or growth factor expression (19) may play a role. Regardless of the mechanism by which retinoids exert their effect, an increase in lung retinoids is essential for this benefit. We have shown that a 10:1 molar combination of VARA, more so than VA or RA
administered alone, increased lung vitamin A in an acute exposure of newborn mice to hyperoxia (11). The present study determined that the VARA combination also improves impaired alveolar septation and lung function in hyperoxia-exposed newborn mice.

Prolonged hyperoxia exposure elicits the production of reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, and hydrogen peroxide which may be important in mediating hyperoxia-induced impaired alveolar development and lung function (32, 33). Hyperoxia-mediated ROS production has been directly linked to the oxidation of DNA, proteins, and lipids which may cause impaired alveolar development directly through multiple pathways and induce a variety of cellular responses through the generation of secondary metabolic reactive species (31, 34). DNA and protein oxidation have been found to be correlated with the development of BPD in preterm infants (35). The hyperoxia-induced increases in DNA and protein oxidation and the attenuation of these changes with VARA indicate a possible mechanism of the action of retinoids. It is possible that retinoids act as antioxidants, scavenging ROS via electron transfer, and preventing DNA and protein oxidation, thereby attenuating alveolar simplification in hyperoxia-exposed newborn mice (31). It is also possible that retinoic acid acts as a cofactor to modify increased oxygen consumption (and reduce ROS production) via regulation of protein kinase Cδ (PKCδ), thereby regulating mitochondrial energy homoeostasis and subsequent tissue injury (20).

Increased levels of ROS via hyperoxia exposure have also been implicated in initiating lung inflammation through the activation of transcription factors, signal transduction, and gene expression of pro-inflammatory mediators (36). Inflammation has been strongly correlated with the development of BPD in preterm infants through
increased production of pro-inflammatory cytokines such as IFN-γ, TNF-α, IL-6, IL-8 (analogous to mouse MIP-2α; Cxcl2), IL-10, and IL-1β (37). Animal studies have also indicated that blocking inflammation improves alveolar development and DNA oxidation (38, 39). Bry et al (40) found that inflammation prevents expression of retinoic acid binding proteins (e.g. CRABP-I) and receptors (e.g. RAR-γ2), and thereby reduces alveolar septation. Our data suggests that hyperoxia increases many pro-inflammatory mediators in newborn mice, which are attenuated by VARA. However, while MIP-2α and IFN-γ had concordant changes in mRNA and protein, we observed that changes in IL-1β and TNF-α protein did not correlate with mRNA changes. It is possible that post-transcriptional changes in protein synthesis or stability maybe involved with, and possibly regulated by retinoids (41). Our study also indicated that MIP-2α and IFN-γ are both sufficient but not necessary for hyperoxia induced impaired alveolar development in newborn mice, perhaps because other cytokines and mediators, both measured and unmeasured, also affect lung injury and development during hyperoxia.

Alveolar development is coordinated by multiple paracrine interactions between the fibroblastic, epithelial, and microvascular lung components, all of which also interact with the extracellular matrix (42). Our study indicates that hyperoxia modulates many growth factors that are important in alveolar development and that VARA influences many of these factors such as angiopoietin-2, epiregulin, FGF-1, frizzled homolog 5, HIF-1α, MMP-2, and, MMP-9. Some of the alterations induced by hyperoxia such as increases in MMP-9, Shh, and LOX and decreases in MMP-2 may contribute to impaired alveolar development (43, 44). Other genes changed with hyperoxia exposure such as FGF-7, FGF-18, midkine, PDGF-A, tenascin-C, tropoelastin, and vascular endothelial
growth factor (VEGF) might be attempts at compensation for impaired alveolar development (45, 46). Tenascin-C is also known to be involved in an autocrine loop in inflammation (47). Interestingly, FGF-10 was increased by VARA in hyperoxia exposed newborn mice indicating an interaction between hyperoxia exposure and VARA. Benjamin et al (48) showed that FGF-10 is important in the positioning of myofibroblasts around the saccular airways which is critical in alveolar development. Benjamin et al (49) also showed that pro-inflammatory mediators inhibited FGF-10 expression in fetal lung mesenchymal cells through NFκB activation. FGF-10 has also been shown to attenuate oxidative stress mediated alveolar epithelial cell DNA damage (50). Additional studies are required to determine the role of FGF-10 as well as other primary growth factors involved in VARA-mediated attenuation of hyperoxia-induced impaired alveolar development.

In summary, our study shows that the VARA combination improves alveolar development and lung function in newborn mice exposed to hyperoxia during the critical period of maximal alveolar formation. This improvement in alveolar development and lung function are associated with reductions in DNA damage and protein oxidation and beneficial changes in multiple pathways, including inflammation and growth factors.

ACKNOWLEDGEMENTS

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REFERENCES


Table 1: Real-time PCR microarray analysis of 84 key mouse angiogenic mediators from lung homogenates of mice administered vehicle or VARA during 7 days of exposure to air or hyperoxia. (n=3 mice per group; Gene expression was normalized to the average geometric mean of the following housekeeping genes: hypoxanthine guanine phosphoribosyl transferase 1, heat shock protein 90 alpha (cytosolic) class B member 1, and beta actin; Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate a negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change.*p<0.05 vs. 21% O2 vehicle and #p<0.05 vs. corresponding vehicle. Numbers in bold indicate fold changes in expression that are >4 with a p<0.05.)

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</tr>
<tr>
<td>Transmembrane serine protease 6</td>
<td>Tmprss6</td>
</tr>
<tr>
<td>Tumor necrosis factor-α</td>
<td>Tnfa</td>
</tr>
<tr>
<td>Tumor necrosis factor, alpha-induced protein 2</td>
<td>Tnfaip2</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Gene Symbol</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
</tr>
<tr>
<td>Tumor necrosis factor (ligand) superfamily, member 12</td>
<td>Tnfsf12</td>
</tr>
<tr>
<td>Vascular endothelial growth factor A</td>
<td>Vegfa</td>
</tr>
<tr>
<td>Vascular endothelial growth factor B</td>
<td>Vegfb</td>
</tr>
<tr>
<td>Vascular endothelial growth factor C</td>
<td>Vegfc</td>
</tr>
<tr>
<td>Glucuronidase, beta</td>
<td>Gusb</td>
</tr>
<tr>
<td>Hypoxanthine guanine phosphoribosyl transferase 1 (Housekeeping gene)</td>
<td>Hprt</td>
</tr>
<tr>
<td>Heat shock protein 90 alpha (cytosolic), class B member 1 (Housekeeping gene)</td>
<td>Hsp90ab1</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Gapdh</td>
</tr>
<tr>
<td>Actin, beta (Housekeeping gene)</td>
<td>Actb</td>
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Table 2. Mouse primer sequences (5’-3’) for qPCR.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>18S- Forward</td>
<td>GCT TTC AAC TAT CCC GTC TG</td>
</tr>
<tr>
<td>18S- Reverse</td>
<td>ACT CTT TGC CGA TGG TGT A</td>
</tr>
<tr>
<td>PDGF-A-Forward</td>
<td>TAA CAC CAG CAG CGT CAA GTG</td>
</tr>
<tr>
<td>PDGF-A-Reverse</td>
<td>CTG GAC CTC TTT CAA TTT TGG C</td>
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<tr>
<td>Midkine-Forward</td>
<td>AAA CCG AAC TCC AGG ACC AGA GAC</td>
</tr>
<tr>
<td>Midkine-Reverse</td>
<td>AAC ACT CGC TGC CCT TCT TCA C</td>
</tr>
<tr>
<td>Shh-Forward</td>
<td>CCA ATT ACA ACC CGA CAT C</td>
</tr>
<tr>
<td>Shh-Reverse</td>
<td>GCA TTT AAC TTG TCT TTG CAC CT</td>
</tr>
<tr>
<td>FGF7-Forward</td>
<td>ACG AGG CAA AGT GAA AGG GA</td>
</tr>
<tr>
<td>FGF7-Reverse</td>
<td>TGC CAC AAT TCC AAC TGC CA</td>
</tr>
<tr>
<td>FGF10-Forward</td>
<td>GTC ACA ATG GCA AAT GAT GC</td>
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<td>FGF10-Reverse</td>
<td>GGA TTC TGT GGG CCT TAC AA</td>
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<tr>
<td>FGF18-Forward</td>
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<tr>
<td>FGF18-Reverse</td>
<td>ACT GCT GTG CTT CCA GGT TC</td>
</tr>
<tr>
<td>Tropoelastin-Forward</td>
<td>TGG TAT TGG TGG CAT CGG</td>
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<tr>
<td>Tropoelastin-Reverse</td>
<td>CCT TGG CTT TGA CTC CTG TG</td>
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<tr>
<td>Tenascin-C-Forward</td>
<td>GTT TGG AGA CCG CAG AGA AAG AA</td>
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<tr>
<td>Tenascin-C-Reverse</td>
<td>TGT CCC CAT ATC TGA TGC CCA TCA</td>
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<tr>
<td>LOX-Forward</td>
<td>GTG ACA TTC GCT ACA CAG GAC ATC</td>
</tr>
<tr>
<td>LOX-Reverse</td>
<td>CCA AAC ACC AGG TAC GGC TTT ATC</td>
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</table>
Table 3: Analysis of protein amounts of 32 key mouse cytokines and chemokines from lung homogenates of mice administered vehicle or VARA for 7 days while being exposed to air or hyperoxia. (mean±SE; n=4-6 mice per group; *p<0.05 vs. corresponding air (21%O\textsubscript{2}); #p<0.05 vs. corresponding vehicle).

<table>
<thead>
<tr>
<th>Analyte (in pg/mL)</th>
<th>21% O\textsubscript{2}</th>
<th>21% O\textsubscript{2} VARA</th>
<th>85% O\textsubscript{2} Vehicle</th>
<th>85% O\textsubscript{2} VARA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
<td>1755±102</td>
<td>1447±39*</td>
<td>925±63*</td>
<td>912±49</td>
</tr>
<tr>
<td>Granulocyte colony stimulating factor (G-CSF)</td>
<td>9±0.8</td>
<td>7.2±1</td>
<td>39±12*</td>
<td>45±10</td>
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<tr>
<td>Interferon –gamma (IFN-γ)</td>
<td>73±14</td>
<td>69±15</td>
<td>105±12*</td>
<td>75±18*</td>
</tr>
<tr>
<td>Interleukin (IL)-1α</td>
<td>79±8</td>
<td>113±15</td>
<td>150±11*</td>
<td>133±18</td>
</tr>
<tr>
<td>IL-1β</td>
<td>42±6</td>
<td>40±4</td>
<td>42±2</td>
<td>52±4</td>
</tr>
<tr>
<td>IL-2</td>
<td>8±0.6</td>
<td>9±0.6</td>
<td>10±0.5</td>
<td>20±3*</td>
</tr>
<tr>
<td>IL-3</td>
<td>2±0.2</td>
<td>1±0.1</td>
<td>0.9±0.1</td>
<td>1±0.1</td>
</tr>
<tr>
<td>IL-4</td>
<td>3±0.4</td>
<td>4±0.9</td>
<td>2±0.1*</td>
<td>2±0.2</td>
</tr>
<tr>
<td>IL-5</td>
<td>60±9</td>
<td>35±13</td>
<td>6±1*</td>
<td>12±3</td>
</tr>
<tr>
<td>IL-6</td>
<td>12±4</td>
<td>20±0.8</td>
<td>33±9*</td>
<td>39±6</td>
</tr>
<tr>
<td>IL-7</td>
<td>5±0.7</td>
<td>4±0.8</td>
<td>5±0.6</td>
<td>7±1</td>
</tr>
<tr>
<td>IL-9</td>
<td>247±28</td>
<td>258±22</td>
<td>221±14</td>
<td>312±36</td>
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<tr>
<td>IL-10</td>
<td>14±2</td>
<td>11±1</td>
<td>13±1</td>
<td>15±2</td>
</tr>
<tr>
<td>IL-12 (p40)</td>
<td>2±0.7</td>
<td>0.9±0.3</td>
<td>0.7±0.4</td>
<td>2±0.7</td>
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<tr>
<td>IL-13</td>
<td>975±70</td>
<td>814±36</td>
<td>587±27*</td>
<td>570±28</td>
</tr>
<tr>
<td>IL-17</td>
<td>14±1</td>
<td>15±0.5</td>
<td>19±0.5*</td>
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<tr>
<td>Protein</td>
<td>Peak</td>
<td>Area 2</td>
<td>Area 3</td>
<td>Area 4</td>
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<tr>
<td>-----------------------------------------------------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>Interferon gamma-induced protein (IP)-10</td>
<td>405±40</td>
<td>177±13&quot;</td>
<td>314±26*</td>
<td>290±25</td>
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<tr>
<td>Keratinocyte chemoattractant (KC)</td>
<td>136±16</td>
<td>111±9</td>
<td>251±46*</td>
<td>292±24</td>
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<tr>
<td>Leukemia Inhibitory Factor (LIF)</td>
<td>8±1</td>
<td>5±0.73</td>
<td>31±2*</td>
<td>46±3.0&quot;</td>
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<tr>
<td>Monocyte chemotactic protein (MCP)-1 (Ccl2)</td>
<td>32±5</td>
<td>35±1</td>
<td>44±3*</td>
<td>46±4</td>
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<tr>
<td>Macrophage colony stimulating factor (M-CSF)</td>
<td>15±2</td>
<td>16±2</td>
<td>14±1</td>
<td>18±2</td>
</tr>
<tr>
<td>Monokine induced by interferon-gamma (MIG)</td>
<td>704±74</td>
<td>488±52&quot;</td>
<td>149±14*</td>
<td>282±42</td>
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<tr>
<td>Macrophage inflammatory protein (MIP)-1α</td>
<td>39±16</td>
<td>------</td>
<td>32±11</td>
<td>24±14</td>
</tr>
<tr>
<td>Macrophage inflammatory protein (MIP)-2α</td>
<td>156±25</td>
<td>153±20</td>
<td>210±30*</td>
<td>164±30&quot;</td>
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<tr>
<td>Regulated upon activation normal T-cell expressed, and presumably secreted (RANTES)</td>
<td>23±2</td>
<td>30±4</td>
<td>24±1</td>
<td>33±4</td>
</tr>
<tr>
<td>Tumor necrosis factor (TNF)-α</td>
<td>6±1</td>
<td>3±0.3</td>
<td>8±0.82*</td>
<td>6±0.5</td>
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<tr>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>360±29</td>
<td>451±22</td>
<td>656±40*</td>
<td>714±32</td>
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</tbody>
</table>
Figure 1: Lung retinol concentration in newborn mice at 14 days of age. Lung retinol (nmol/g) was analyzed on day 14 (mean±SE; n=6 mice/group; #p<0.05 vs. corresponding vehicle).
Figure 2: Alveolar development in newborn mice at 14 days of age. (A-D)
Representative photomicrographs of H&E stained sections of lungs from newborn mice administered either vehicle (A, B) or VARA (C, D) during 14 days of air (A, C) or hyperoxia (B, D) exposure (Calibration bars = 250 µm). In mouse pups given vehicle, alveolar size is larger in hyperoxia-exposed (B) compared to air-exposed mice (A), indicating delay in septation. Administration of VARA moderately attenuated the hyperoxia-induced increase in alveolar size (D), and did not change alveoli of air-exposed animals (C). (E) MLI, (F) RAC (G) SSC at 14 days of age in mouse pups given either vehicle or VARA, while being exposed to air or hyperoxia  (mean±SE; n=6 mice/group; *p<0.05 vs. corresponding air; #p<0.05 vs. corresponding vehicle).
Figure 3: VARA attenuates hyperoxia-induced alterations in lung function. Lung compliance (A) and total lung resistance (B) in 14 day mouse pups exposed from birth to air or hyperoxia, in combination with either vehicle or VARA. (mean±SE, n =6 mice/group; *p< 0.05 corresponding air, # p < 0.05 vs. corresponding vehicle).
Figure 4: VARA attenuates DNA and protein oxidation. 8-OHdG (A), protein carbonyls (B), MDA-adducts (C), and nitrotyrosine (D) were analyzed by ELISA, and MPO (E) was analyzed by a colorimetric activity assay in lung homogenates of 14 day mouse pups exposed from birth to air or hyperoxia, in combination with either vehicle or VARA (n=6 mice/group; mean±SE, *p<0.05 vs. corresponding air; #p<0.05 vs. corresponding vehicle).
Figure 5: Analysis of mRNA of growth factors important in alveolar development. (A-F) mRNA levels of FGF-7 (A), FGF-10 (B), FGF-18 (C), Midkine (D), PDGF-A (E), Shh (F) measured by qPCR in homogenized lungs from mouse pups exposed to air or hyperoxia, in combination with either vehicle or VARA from birth to 7 days (n=6 mice/group; mean±SE, *p<0.05 vs. corresponding air at same time point; #p<0.05 vs. corresponding vehicle at same time point).
Figure 6: Analysis of mRNA of mediators of the extracellular matrix. (A-C) mRNA levels of tenascin-C (A), tropoelastin (B), LOX (C) were measured by qPCR in homogenized lungs from mouse pups exposed to air or hyperoxia, in combination with either vehicle or VARA from birth to 7 days (n=6 mice/group; mean±SE, *p<0.05 vs. corresponding air at same time point; #p<0.05 vs. corresponding vehicle at same time point).
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Vitamin A (VA), commonly referred to as retinol, is important in the development and maturation of the lung and other organs such as the eye. VA accumulation in the fetal lung occurs during the third trimester of pregnancy; therefore, preterm infants with limited transplacental transfer of VA have reduced VA stores in the lung (30, 31). Extremely low birth weight preterm infants who are VA deficient are at higher risk for BPD (31). BPD, primarily an impairment of alveolar septation, is a multifactorial disorder to which hyperoxia exposure is a major contributor. Clinically, VA supplementation decreases the incidence of BPD and/or death (90). However, 25% of preterm infants remain VA deficient despite supplementation (9). Higher dosing regimens did not improve retinol levels or outcomes (91). Ambalavanan et al. suggested that inflammation, due to prolonged hyperoxia exposure, may further lower VA levels (due to impaired VA transport) in which in turn may accentuate impaired alveolar development and injury and predispose the developing lung to further inflammation, leading to a vicious cycle of additional lowering of VA transport proteins and available VA leading to greater inflammation (92).

All-trans-retinoic acid (all-trans-RA), a metabolite of vitamin A, has been the focus of much basic lung research involving animal models of BPD over the past 20 years. In humans, alveolar septation occurs from approximately 36 weeks of gestation.
until 1 to 2 years of age (17). Alveolarization in rodents occurs from postnatal day 5 until day 30 (17-19). Newborn rodents exposed to 14 days of hyperoxia have impaired alveolarization and this model is often used as an experimental model of BPD (34, 93). Lung VA content declines abruptly before birth in newborn rodents which further makes newborn rodents an ideal animal model for BPD (reduced VA stores and hyperoxia-induced alveolar simplification) (94). Veness-Meehan et al. showed in a newborn rat hyperoxia model that all-trans-RA did not initially improve septal formation or decrease airspace size in animals exposed to hyperoxia alone or to hyperoxia plus dexamethasone. However, a late improvement in alveolar septal formation was noted (34). The exact mechanisms by which all-trans-RA attenuates the effects of hyperoxia-induced impaired alveolar development in the newborn lung are uncertain, but modulation of the expression of antioxidants (35-40), pro-inflammatory mediators (41), or growth factors (42, 43) may play a role. Regardless of the mechanism by which retinoids exert their effect, an increase in lung retinoids is essential for this benefit.

Our group previously investigated the effects of orally administering VA, all-trans-RA, and a 10:1 molar combination of VA and all-trans-RA (VARA) in newborn rats on lung retinyl ester stores (95). We found that the VARA combination is several-fold more effective in increasing lung retinyl ester content than either VA or all-trans-RA administered alone. In a 6 hour metabolic study, [3H] retinol with VARA compared with VA or vehicle, increased the uptake of newly absorbed [3H] retinol by 3-fold, indicating that VARA simulated the uptake of [3H] retinol and its retention as [3H] retinyl esters in newborn rat lungs (95). After cessation of VARA, lung retinyl esters remained increased throughout the period of alveolar development. Ross et al. found that VARA
administration induced STRA6 (retinol binding protein receptor) expression in newborn rats accompanied by a synergistic increase in lung retinyl esters (81), indicating that all-
trans-RA modulates the expression of STRA6 which allows for increased mobilization and storage of vitamin A in the lung. Consequently, we investigated the effects of VARA on lung VA in a newborn mouse model to ensure the effects seen with VARA administration in newborn rats are not species-dependent and to determine if improved lung VA may be of maturational and functional significance.

For the first time, we show that oral administration of a 10:1 molar combination of VA and all-trans-RA (VARA) synergistically increased lung VA stores much more than administering VA or all-trans-RA alone in newborn mice in both our short-term (4 day) and longer-term (14 day) hyperoxia exposures, confirming our previous study in neonatal rats (95). The demonstration of VARA synergism is of clinical relevance. Increased VA dosing is not sufficient to alleviate VA deficiency in extremely low birth weight infants with lung inflammation, due to a lack of VA transport and uptake in the lung (10). Our studies overcome this barrier by co-administration of VA and all-trans-RA. As all-trans-RA increased lung retinyl esters, and all-trans-RA is not converted to retinyl esters, increased lung retinyl ester content must be due to regulation of retinol metabolism or distribution (e.g. upregulation of retinol binding protein (RBP; retinol transport protein) and STRA6 (RBP receptor)) by all-trans-RA. While all-trans-RA alone regulates retinol metabolism and increases retinyl ester content modestly, VA is rate-limiting for the storage of larger amounts of retinyl ester in the lungs (10). In the VARA preparation, the molar ratio of all-trans-RA to VA is 1 to 10, suggesting that a small relative amount of all-trans-RA influences the distribution of a larger amount of VA. The
synergistic effect of the VARA combination on lung retinyl ester content indicates that VA and all-trans-RA have different but interacting roles in lung retinyl ester storage (e.g. all-trans-RA increases STRA6 expression for the uptake of VA into the lung which undergoes reversible oxidation into retinyl esters) (10, 81). Our longer-term study further demonstrated that an increase in lung VA significantly improves alveolar development and lung function in newborn mice exposed to hyperoxia. Experiments undertaken to provide insight into the potential pathways that may be regulated by VARA in the improvement of alveolar development in hyperoxia-exposed newborn mice revealed that regulation of inflammation may be of critical importance. Our 4 day hyperoxia exposure experiment determined that monocyte inflammatory protein-2α (MIP-2α) mRNA and protein expression to be increased by hyperoxia and attenuated with VARA. MIP-2α, the murine equivalent of IL-8 in humans, is a potent neutrophil and macrophage chemoattractant and is increased in the lungs and blood of preterm infants who subsequently develop BPD (96, 97). Blockade of the MIP-2α receptor CXCR2 reduces hyperoxic lung injury in adult animals (98). These observations are consistent with our 14 day hyperoxia exposure experiment which demonstrated increased MIP-2α mRNA and protein expression as well as increases in mRNA and protein expression of interferon-γ (IFN-γ; macrophage activating factor). Our 14 day study also indicated that MIP-2α and IFN-γ are both sufficient but not necessary for hyperoxia-induced impaired alveolar development in newborn mice, perhaps because other cytokines and mediators, both measured and unmeasured, also affect lung injury and development during hyperoxia.
Our 14 day study also revealed that retinoids may reduce oxidative stress in the newborn lung. Prolonged hyperoxia exposure elicits the excess production of reactive oxygen species (ROS), such as superoxide anion (O2•-), hydrogen peroxide (H2O2), and hydroxyl radical (OH•) (99, 100). Excess production of ROS overwhelms the available antioxidant capacity and perturbs the reduction-oxidation (redox) equilibrium (99, 100). Excess ROS leads to oxidative stress in cells and tissues which may be important in mediating hyperoxia-induced impaired alveolar development (99, 100). Hyperoxia-mediated ROS production has been directly linked to the oxidation of DNA, proteins, and lipids in lung type I and II epithelial cells and fibroblasts which are principal cell types involved in alveolar development (101, 102). DNA and protein oxidation have been found to be correlated with the development of BPD in preterm infants (103). The hyperoxia-induced increases in DNA and protein oxidation and the attenuation of these changes with VARA indicate a possible mechanism of the action of retinoids. It is possible that this VARA-induced attenuation of oxidant injury is mediated via modulation of nuclear factor-erythroid 2 (NF-E2) related factor 1 and 2 (Nrf1 and Nrf2) which belong to the Cap’n’collar (CNC)-bZIP transcription factor family that mediate antioxidant response elements (ARE) (104). Nrf1 and Nrf2 have been found to be expressed in the murine lung (105). During the absence of oxidative stress, Nrf2 is sequestered in the cytoplasm by kelch-like ECH-associated protein 1 (Keap1) as a Keap1/Nrf2 complex, which prevents nuclear accumulation of Nrf2 (106). Keap1 regulates rapid proteosomal degradation of Nrf2 (107). On exposure to oxidative stress, Nrf2 is activated by phosphorylational modification of Nrf2 and/or Keap 1 via several protein kinase pathways, which leads to the disassociation of Nrf2 from Keap1 and
nuclear Nrf2 translocation (106). Cho et al. found that Nrf2 attenuates hyperoxia-induced lung injury in adult mice (108). Furthermore, McGrath-Morrow et al. determined Nrf2 attenuates hyperoxia-induced impaired alveolar growth and lung injury in newborn mice (109). It is possible that there is a retinoic acid response element (RARE) within the Nrf2 promoter, which enables lung type I and II epithelial cells or endothelial cells (both of which are key cell types in alveolar development) to attenuate oxidant stress in the lungs of hyperoxia-exposed newborn mice. All-trans-RA may increase the expression and activation of Nrf2 (e.g. through upregulation of NADPH oxidase or ERK which in turns activates Nrf2), thereby preventing oxidative stress through Nrf2 modulation of ARE-mediated antioxidant (e.g. superoxide dismutases; SOD1) and detoxifying enzyme (e.g. NADP(H); quinone oxidoreductase (NQO1) or heme oxygenase (HO)-1) expression.

Another novel finding from our 14 day study was the increased expression of FGF-10 by VARA indicating an interaction between hyperoxia exposure and VARA. Benjamin et al (110) showed that FGF-10 is important in the positioning of myofibroblasts around the saccular airways which is critical in alveolar development. Benjamin et al (111) also showed that pro-inflammatory mediators inhibited FGF-10 expression in fetal lung mesenchymal cells through NFκB activation. FGF-10 has also been shown to attenuate oxidative stress mediated alveolar epithelial cell DNA damage (112). Additional studies are required to determine the role of FGF-10 as well as other primary growth factors involved in VARA-mediated attenuation of hyperoxia-induced impaired alveolar development.

Overall, these findings support the hypothesis that a combination of VA and all-trans-RA is effective in increasing lung retinoids in newborn mice in both settings of
short-term and longer-term hyperoxia exposure, as well as improving alveolar
development and lung function in the longer-term hyperoxia model. Importantly, because
oxidative stress has been associated with BPD, these findings provide initial evidence
that retinoids may influence oxidative stress which may result in beneficial changes in
multiple pathways, including inflammation and alveolar maturation in a newborn mouse
model of BPD.

Future Directions
Based on these findings and from other studies utilizing retinoids, it is increasingly
evident that retinoids may reduce hyperoxia-induced oxidative stress in a newborn mouse
model of BPD (40, 113, 114) which in turn attenuates impaired alveolar growth. Joung et
al. suggested that DNA damage may be the crucial mechanism in the pathogenesis of
BPD (103). Our findings indicate that retinoids attenuate hyperoxia-induced increases in
DNA and protein oxidation in newborn mice. Taken together, these findings present the
question: How do retinoids reduce oxidative stress and lower DNA and protein oxidation
in a newborn mouse model of BPD?

We hypothesize that all-trans-RA may modulate Nrf2 gene expression and
activation in neonatal mouse lung epithelial cells which consequently increases
antioxidant production (e.g. SOD1) which will preserve their cellular integrity and permit
alveolar septation despite hyperoxia exposure (Figure 1). We also hypothesize that all-
trans-RA may act as a cofactor to modify increased oxygen consumption (and reduce
ROS production) within the mitochondria of lung epithelial cells, or as a transcription
factor in the influence of genes involved in apoptosis (Figure 1). Acin-Perez et al.
determined that vitamin A is essential in the metabolic fitness of mitochondria (115). They found that when mouse embryonic fibroblast cells were deprived of vitamin A, respiration and ATP synthesis were decreased (115). They also determined that vitamin A is a cofactor of pyruvate kinase Cδ (PKCδ) and that the complex signals the pyruvate dehydrogenase complex for enhanced flux of pyruvate into the Krebs cycle, indicating that vitamin A is of fundamental importance for energy homeostasis (115). Similarly, Chiu et al. found that vitamin A deficiency in mouse embryonic fibroblasts leads to ROS generation, drastic reductions in ATP and NAD+ levels, and activation of poly-(ADP-ribose) polymerase (PARP)-1 (113). PARP-1 is a sensor of DNA damage (116) which is involved in regulation of transcription, cell cycle, and cell death (117). Overexpression of PARP-1 during oxidative stress induces cell death via energy failure, due to rapid utilization of NAD+ and ATP, transcriptional derangement, and PARP-1 mediated signaling to the mitochondria (118-122). Jameel et al. determined that vitamin A deficient hepatic stellate cells, principal storage cells for vitamin A, were susceptible to superoxide-induced apoptosis, indicating that vitamin A may be important in modulating antioxidant production and/or apoptosis (114). This data indicates that vitamin A may regulate PARP-1 expression and thereby preserve energy metabolism and prevent cell death. It is also important to note that both these hypotheses may be inter-dependent as increased oxidative stress, accompanied by decreased antioxidant production may lead to overall cellular stress resulting in mitochondrial dysfunction and apoptosis. To test hypothesis 1, we can first evaluate the expression and localization of Nrf2 in our newborn mouse model of hyperoxia-induced impaired alveolar development with or without VARA. If VARA increases Nrf2 expression and activation more so than in the hyperoxia
Figure 1: A schematic of potential mechanisms of action of retinoids in hyperoxia-induced impaired alveolar development in newborn mice. Prolonged exposure to hyperoxia induces oxidative stress in the lungs of newborn mice. Oxidative stress is a source of reactive oxygen species (ROS) that can directly damage lung epithelial and/or endothelial cells (key cell types in alveolar development) and/or indirectly through ROS production from inflammatory cells (e.g. macrophages and neutrophils). ROS damages lung epithelial and/or endothelial cells through DNA and protein oxidation which elicits mitochondrial dysfunction and apoptosis. VARA may inhibit the oxidation of DNA and protein by the modulation of Nrf2; thereby, increasing antioxidant production. VARA may prevent apoptosis of these key cell types by the modulation of key pro-apoptotic (e.g. Bax and Caspase 3) and anti-apoptotic mediators (e.g. Bcl-2). VARA may modulate energy homeostasis through regulation of poly-(ADP-ribose) polymerase (PARP)-1 and by acting as a cofactor to pyruvate kinase Cδ (PKCδ). VARA may also prevent inflammation through modulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).
vehicle group (allowing for a balance between antioxidants and oxidants, despite prolonged hyperoxia exposure), we can then determine if all-trans-RA increases the expression and activation of Nrf2 in neonatal mouse alveolar epithelial cells as this cell type is vital to alveolar development and has been shown to undergo DNA damage and protein oxidation in response to hyperoxia exposure (123). We can expose neonatal mouse lung epithelial cells to air or hyperoxia in combination with either vehicle or all-trans-RA. If Nrf2 expression is increased in hyperoxia-exposed newborn mouse lung epithelial cells administered all-trans-RA, accompanied by increased SOD1 expression and reduced apoptosis, these data will demonstrate that retinoids are important in mediating the Nrf2-antioxidant response. Additional studies will be necessary to determine if there is a RARE in the promoter region of Nrf2 or one of its activators such as NADPH oxidase (NOX). To test hypothesis 2, we can determine the expression of PARP-1 in our newborn mouse model of hyperoxia-induced impaired alveolar development administered with or without VARA. If PARP-1 expression is reduced in hyperoxia-exposed newborn mice and VARA attenuates this effect, this will indicate that retinoids regulate PARP-1, and thereby energy homeostasis. We can further evaluate the regulation of energy homeostasis by retinoids by first exposing neonatal mouse lung epithelial cells to air or hyperoxia in combination with either all-trans-RA or vehicle, and measure mitochondrial bioenergetics. In the setting of hyperoxia, if PARP-1 expression is reduced and energy homeostasis is preserved with retinoid exposure, this will signify the importance of retinoids on energy homeostasis in a key cell type that is involved in alveolar development. Next, we can evaluate the effects of all-trans-RA on the
expression of key mediators involved in apoptosis, such as Bax, activated Caspase-3, and anti-apoptotic mediators such as Bcl-2.

It is important to make the distinction that these hypotheses do not preclude the presence of other contributing factors or mechanisms that may be involved in hyperoxia-induced impaired alveolar development or may be regulated by vitamin A in the attenuation of this effect. It is possible that retinoids may also regulate the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is activated in response to various stimuli such as oxidative stress and cytokines and leads to the expression of genes mediating inflammation. NF-κB exists in the cytoplasm in an inactive form associated with regulatory proteins, called inhibitors of κB (IκB) (124). NF-κB activation is achieved through the signal-induced proteolytic degradation of IκB in the cytoplasm which frees NF-κB to interact with the nuclear transport machinery and translocates to the nucleus, where it binds to the promoter of its target genes to initiate transcription (125, 126). Data suggest that upregulation of cellular antioxidant systems prevents NF-κB activation by cytokines (127, 128). NF-κB activation has been implicated in mediating hyperoxia-induced IL-8 (analog of mouse MIP-2α in mouse) inflammatory response (129, 130). Charoensit et al. determined that all-trans-RA inhibits liver injury-induced NF-κB activation in mice (131). Kim et al. determined that all-trans-RA suppressed cytokine production from mouse peritoneal macrophages (132). Based on these findings, we hypothesize that all-trans-RA inhibits hyperoxia-induced increased expression and activation of NF-κB in mouse lung macrophages (Figure 1). In order to test this hypothesis, we can evaluate the expression of NF-κB and IκB in hyperoxia-exposed mouse lung macrophages with or without administration of all-trans-RA. If all-
trans-RA reduces NF-κB expression accompanied by increased protein levels of IκB in hyperoxia-exposed mouse alveolar macrophages, this will indicate that retinoids may be important in modulating hyperoxia-induced inflammation through regulation of NF-κB.

In closing, when one considers that these hypotheses presented above (Figure 1) are not mutually exclusive and that each may very well contribute independently in the improvement of hyperoxia-induced impaired alveolar development by retinoids, one can begin to appreciate how the findings in this dissertation and those from past studies potentially begin to create a complex, yet plausibly unified model. We sincerely hope that our initial work, as presented herein, will provide a foundation for later studies by others, such that in time a combination of vitamin A and all-trans-RA is evaluated for therapeutic efficacy in the clinical setting or that molecular targets of retinoids are identified for therapeutic intervention in the prevention of BPD.
GENERAL REFERENCES


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APPENDIX

IACUC APPROVAL FORMS
NOTICE OF RENEWAL

DATE: January 11, 2012

TO: NAMASIVAYAM AMBALAVANAN, M.D.

176E-9380 7335
FAX: (205) 934-3100

FROM: [Signature]
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Retinoids and Hypoxic Lung Injury
Sponsor: NIH
Animal Project Number: 120309058

As of March 15, 2012, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>A</td>
<td>330</td>
</tr>
</tbody>
</table>

Animal use must be renewed by March 14, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 120309058 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.
MEMORANDUM

DATE: January 11, 2012

TO: NAMASIVAYAM AMBALAVANAN, M.D.
176F-9380 7335
FAX: (205) 934-3100

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on January 11, 2012.

Title of Application: Retinoids and Hyperoxic Lung Injury
Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)