

The Pennsylvania State University
The Graduate School
College of Health and Human Development

**INVESTIGATION ON THE MECHANISM OF VITAMIN A UPTAKE,
ACCUMULATION AND METABOLISM IN THE LUNGS OF
THE NEONATAL RAT**

A Thesis in
Nutrition
by
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ABSTRACT

The lungs of humans, rodents and other mammals are immature at birth and undergo rapid development during the postnatal periods. Vitamin A (VA) is an essential nutrient that is required by the lung tissue in the postnatal period to help complete lung septation and maturation. Therefore, it is important to keep sufficient lung vitamin A in an early time of life. Previous studies have shown that retinol combined with its metabolite, retinoic acid (RA), is able to increase lung RE contents synergistically. A metabolic study has shown that RA redirects more of the VA given as a supplement into the neonatal lung. And this synergistic effect of combination of VA and RA (VARA) is only specific for the lung tissue. However, the mechanism of the synergy is unknown.

To better understand vitamin A metabolism in the lungs, we investigated how RA and its synthetic, metabolism-resistant analog, Am580, affect the distribution and metabolism of newly absorbed retinol in the neonatal rat. We also studied the transcript level of some genes involved in vitamin A metabolism when neonatal rats were treated with RA or Am580. Our results had shown that treatment of RA and Am580 not only increased neonatal lung RE significantly but also drive more oral-taken retinol to the lung. At the gene level, both RA and Am580 can significantly up-regulate the expression of some retinoids metabolism genes, like lecithin: retinol acyltransferase (LRAT), a member of cytochromes P450 (CYP26B1) and stimulated by retinoic acid gene 6 (STRA6) in neonatal rat lung. Compared to a transient up-regulation with RA, Am580 shows

prolonged effect in increasing the expression of LRAT and CYP26B1. In addition, a 6-h and a 12-h study showed that although both LRAT and CYP26B1 expression response to treatment of RA greatly, LRAT reaches to its peak level much earlier than CYP26B1. In conclusion, the administration of acidic retinoids redirects the flow of supplemented VA in the neonate body. Some important retinoids metabolism genes like LRAT, CYP26B1 and STRA6 may contribute to the redirection of VA flow and play a key role in VA metabolism in neonatal lung.

From these data, a model is proposed that treatment of RA greatly up-regulates expression of LRAT in the neonatal rat lung (at about 6 hours). Although CYP26B1 expression is highly induced by RA too, the change is not as big as LRAT. The change in gene pattern results in more retinol uptake into lung tissue and more RE formation. Meanwhile, the increased expression of STRA6, which codes a membrane receptor for retinol binding protein (RBP), also contributes to VA uptake into the lung. Thus the synergistic effect of VARA in RE formation can be explained.

Each year, more than 300,000 premature infants are born in the United States. This population is born with inadequate body stores of vitamin A and is prone to respiratory diseases. Considering the beneficial effects of VA in promoting lung development and maturation, it is desirable to develop an efficient and safe way to increase neonatal lung RE content in premature infants. Our study in VA metabolism in neonatal lung provides promising therapeutic option in clinical medicine.

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LIST OF ABBREVIATIONS

ATRA: all-trans-retinoic acid

9cRA: 9-cis-retinoic acid

BPD: bronchopulmonary dysplasia

CRBP: cellular retinol-binding protein

CRABP: cellular RA-binding protein

CYP: cytochrome P450

h: hour(s)

HPLC: high performance liquid chromatography

LRAT: lecithin:retinol acyltransferase

RAR: retinoic acid receptor

RXR: retinoic X receptor

RARE: retinoic acid response element

RXRE: retinoid X-response elements

RBP: retinol binding protein

RDS: respiratory distress syndrome

RE: retinyl ester

RT: reverse transcription

ROH: retinol

RXR: retinoid X receptor

TTR: transthyretin

VA: vitamin A

VARA: vitamin A combined with retinoic acid

CHAPTER 1

INTRODUCTION TO THESIS

The lungs of humans, rodents and other mammals are immature at birth and undergo rapid development during the postnatal periods (1). Vitamin A (VA) is an essential nutrient that is required by the lung tissue in this period to help complete lung septation and maturation (2) (3). In full term infants, a process of significant vitamin A accumulation in the lungs has begun from the third trimester until birth. But preterm infants usually are born with a very low level of VA in the lungs. As a result, these infants are more prone to developing respiratory diseases (4). Therefore, it is important to increase lung vitamin A in this population in an early time of life. Previous studies have shown that retinol combined with its metabolite, retinoic acid (RA), is able to increase lung RE contents synergistically (5). A metabolic study has shown that RA redirects more of the VA given as a supplement into the neonatal lung (6). However, the mechanism is unknown. The purpose of this thesis is to investigate how RA and its synthetic metabolism resistant analog, Am580, affect the distribution and metabolism of newly absorbed retinol in the neonatal rat. At the same time, we also want to study the transcript level of some vitamin A metabolism genes when neonatal rats are treated with RA or Am580.

General Introduction to Vitamin A

In 1913, at the University of Wisconsin, E.V. McCollum and M. Davis found that certain “lipins” extracted from eggs, butter and cod liver oil were necessary for the normal growth of young rats (7). Later on, this fat soluble compound was named vitamin A and reported as an essential micronutrient in the diet. Since then, further research has revealed many intriguing characteristics associated with vitamin A. Up to now, the importance of vitamin A in human health has gone far beyond what people had expected.

Keeping an adequate but not excessive amount of vitamin A in the body has profound consequences in maintaining human health. The major functional activities of vitamin A include 1) promoting vision (8), 2) participating in protein synthesis and cell differentiation in epithelial tissues and skins (9, 10) ,3) supporting reproduction and growth (11), 4) maintaining the integrity of immunity (12, 13). Persons with vitamin A deficiency are often afflicted with such conditions as night blindness, xerophthalmia, metaplasia, epithelial keratinization, poor growth, decreased resistance to infection, etc (14). On the other hand, just as a deficiency of vitamin A affects all body systems, so does an overabundance. The manifestations of vitamin A toxicity include headache, vomiting, diplopia, alopecia, dryness of the mucous membranes, bone and joint pain, liver damage and coma (15). High intake of vitamin A by pregnant women may increase the incidence of teratogenic effects in fetuses (16).

The term “vitamin A” refers to the retinoids, which are defined as a class of chemical compounds structurally related to vitamin A. The compounds in natural form

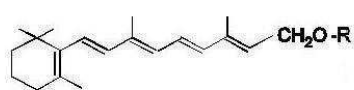
include retinol and its metabolites: retinyl ester (RE), retinal, retinoic acid (RA) and some water-soluble metabolites. The basic structure of a retinoid is composed of a substituted cyclohexenyl ring, a tetraene side chain and a functional group at the end of the side chain. Retinol (also named vitamin A) has a hydroxyl group at its terminal end. When this group is esterified with a fatty acid, retinol becomes RE, the form in which vitamin A is stored. The hydroxyl group also undergoes oxidation to produce an aldehyde (retinal), which may be further oxidized to a carboxylic acid (retinoic acid). In addition to these naturally occurring retinoids, a large number of synthetic analogs, for example, Am580, an aromatic amide, belong to this large family as well (Fig. 1A). Am580 has a similar chemical structure to at-RA, but the two methyl-groups at site C4 prevent the access of RA metabolizing enzyme, CYP26 to Am580. This feature makes Am580 resistant to oxidative metabolism by CYP26.

Although vitamin A is widespread in many diets and is easily taken up by the body, the transport of vitamin A in the body is a very complicated process. In an individual, the dietary RE is first hydrolyzed to retinol prior to intestinal absorption. Following the hydrolysis of dietary REs, the free retinol taken up by the intestinal mucosal cell is re-esterified to RE. The newly formed RE is incorporated into chylomicrons for delivery to the liver through lymphatic and blood circulation. As the center of vitamin A metabolism, the liver has four major functions: 1) the uptake and processing of chylomicron RE (17); 2) the storage of vitamin A as RE (18); 3) the synthesis of retinol-binding protein (RBP) (19); and 4) the metabolism of retinoids (20). When vitamin A is required, liver-stored RE is mobilized to form retinol again and delivered

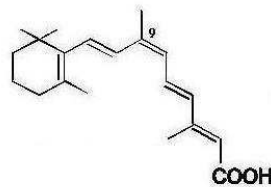
from the liver to target organs bound to RBP (20). The binding of retinol with RBP makes the transport of this fat-soluble micronutrient through plasma possible (21). After plasma retinol is taken up into the cells of the target tissue, part of it may be esterified to RE again, while the other part will be metabolized to retinal, an aldehyde form of vitamin A, then further oxidized to RA, a biological active form of vitamin A, and ultimately metabolized to its polar metabolites, 4-oxo-RA, 4-hydroxy RA and 18-hydroxy RA, etc (22, 23) (Fig 1B, 2).

A

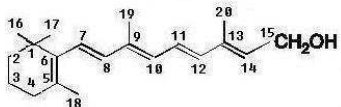
Naturally Occurring and Synthetic Retinoids



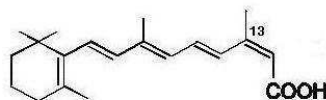
retinyl ester



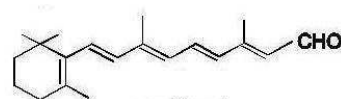
9-cis-retinoic acid



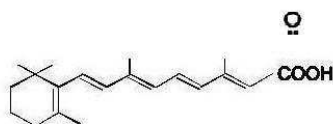
retinol (vitamin A)



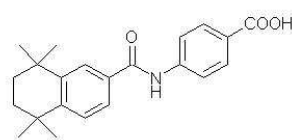
13-cis-retinoic acid



retinal

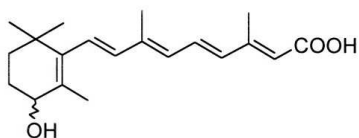


all trans-retinoic acid

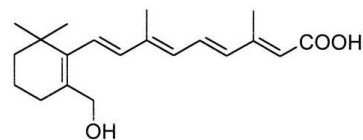


Am580 (Synthetic)

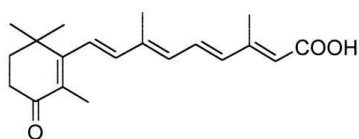
B



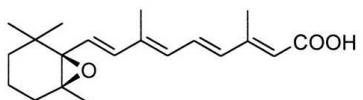
4-hydroxy-retinoic acid (4-OH-RA)



18-hydroxy-retinoic acid (18-OH-RA)



4-oxo-retinoic acid (4-oxo-RA)



5,6-epoxy-retinoic acid (5,6-epoxy-RA)

Figure 1. Naturally occurring and synthetic retinoids. (A) Chemical structures of some main members of natural and synthetic retinoids (from internet resources); (B) At-RA polar metabolites (24).

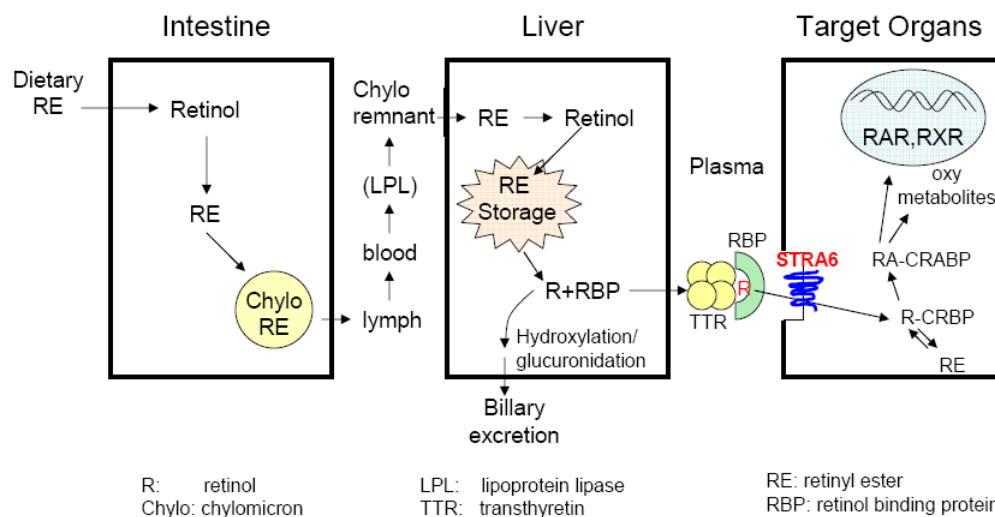


Figure 2. Vitamin A transport and metabolism in the body. Schematic overview of vitamin A transport and cellular metabolism (25).

Within the cytoplasm of tissue cells, retinol and its oxidized form RA bind with cellular retinol-binding proteins (CRBP) and cellular retinoic acid-binding proteins (CRABP), respectively (26). By binding with these retinoid-binding proteins, the concentration of free cellular retinoid is limited, and the bound retinoid is directed to specific enzymes for metabolic processing (27). CRBP-retinol directed to the enzyme lecithin: retinol acyltransferase (LRAT) undergoes an esterification reaction and turns into the storage form of RE (28). CRBP-bound retinol is also a substrate for alcohol dehydrogenase (ADH), which catalyzes oxidization of retinol to retinal. Retinal is further oxidized to retinoic acid by aldehyde dehydrogenase (ALDH) (29). CRABP has the similar function of mediating intracellular RA concentration and directing RA to the enzyme CYP26, which metabolizes RA into polar inactive metabolites, such as 4-oxo-RA, 4-OH-RA, 18-OH-RA and 5,18-epoxy-RA, etc. Therefore, the biological effects of excess RA are limited (30, 31) (Fig. 3). Along the metabolic pathway, retinol and retinal are interconvertible through oxidation and reduction reactions, whereas RA is not able to be converted back to retinal (32). For that reason, the oxidative reaction from retinal to RA is considered the rate-limiting step of the retinol metabolic pathway (33) (Fig. 4).

Within the family of retinoids, RA is believed to be the most important physiologically active metabolite. Although most of RA is formed within cells through oxidative metabolism, a small fraction of dietary retinoids is converted to RA in the intestine or is contained in the diet, absorbed via blood circulation bound to albumin (34, 35). And plasma RA contributes to pools of RA in different tissues in various extents (36). Orally administrated all-trans RA turns over rapidly in plasma, the average half-life for

at-RA is 19 minutes in adult rhesus monkey (37). In fasting condition, plasma at-RA is 4-14 nmol/dl in human (~0.2-0.7% of plasma retinol level) (38, 39).

RA impacts many aspects of human health by affecting the transcription level of numerous genes. However, RA can not do this alone; its important function is accomplished by cooperation with retinoid receptors in the nucleus. There are two main types of retinoid receptors, the retinoic acid receptor (RAR) and retinoid X receptor (RXR) in the nuclear. Both of them are functionally related to the steroid/thyroid hormone receptor family (40). All-trans-RA can bind to RAR with high affinity, whereas 9-cis-RA binds to RXR with high affinity (41).

Once at-RA and 9-cis-RA enter the nucleus, they bind and activate RAR or RXR, which in turn form the RAR/RXR heterodimer (bound with at-RA) or the RXR/RXR homodimer (bound with 9-cis-RA). The RA/receptor complexes then interact with specific regions of DNA termed retinoid responsive elements (RARE or RXRE). The simultaneous binding of RA with the receptors triggers a conformational change in the RAR/RXR complex that reduces its affinity for the transcriptional corepressors while enhancing its interaction with other transcriptional coactivators. The replacement of corepressors by coactivators initiates gene transcriptional activity and subsequently induces the expression of genes which contain one or more RARE or RXRE (40) (Fig. 5). RA is able to induce genes like RAR β and CYP26A1 rapidly and greatly via RAREs in their promoter regions (42). Gene LRAT and CYP26B1 also respond to RA's regulation rapidly and greatly. However, the promoter regions of these two genes lack the sequence

of RARE. How are these two genes regulated by RA is still unknown. Some other molecular mechanism maybe involved in these genes' regulation by RA. For example, RA might perform its regulating function through the interaction between RA/receptor complex and some transcription factors which bind to specific binding site on DNA and subsequently initiate transcription of target genes.

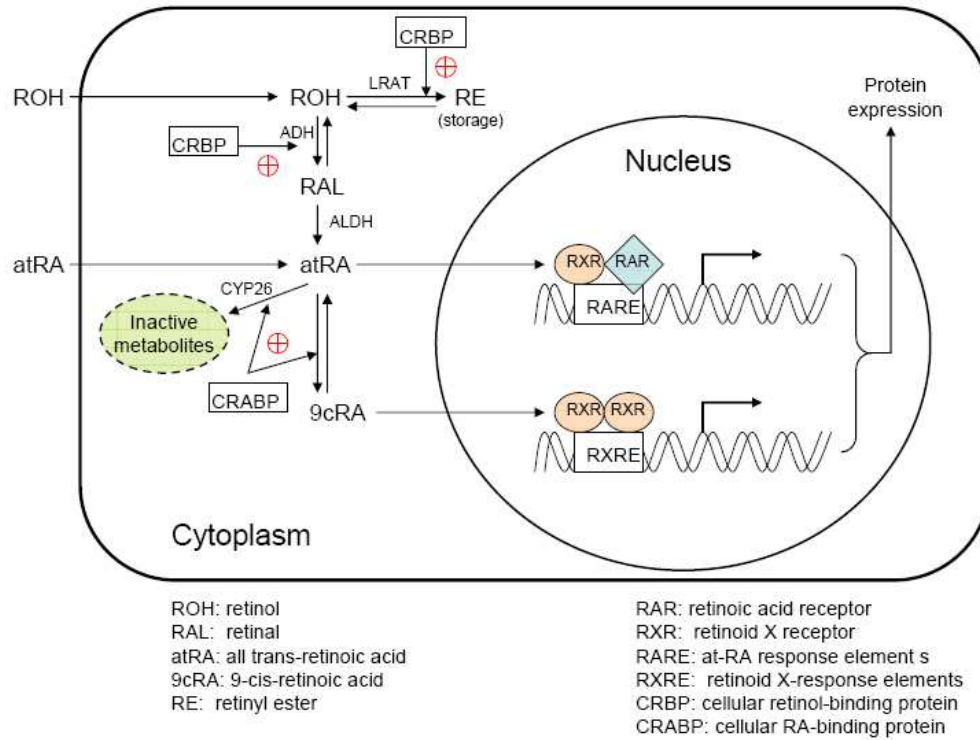


Figure 3. Cellular retinoid metabolism and signaling pathway. A model of events in retinoid metabolism (43).

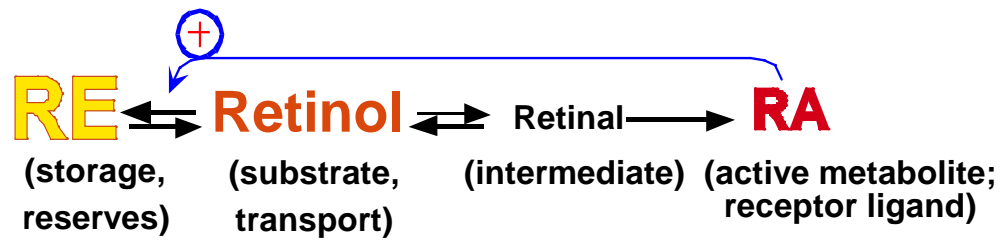


Figure 4. A model of retinoid metabolic pathway.

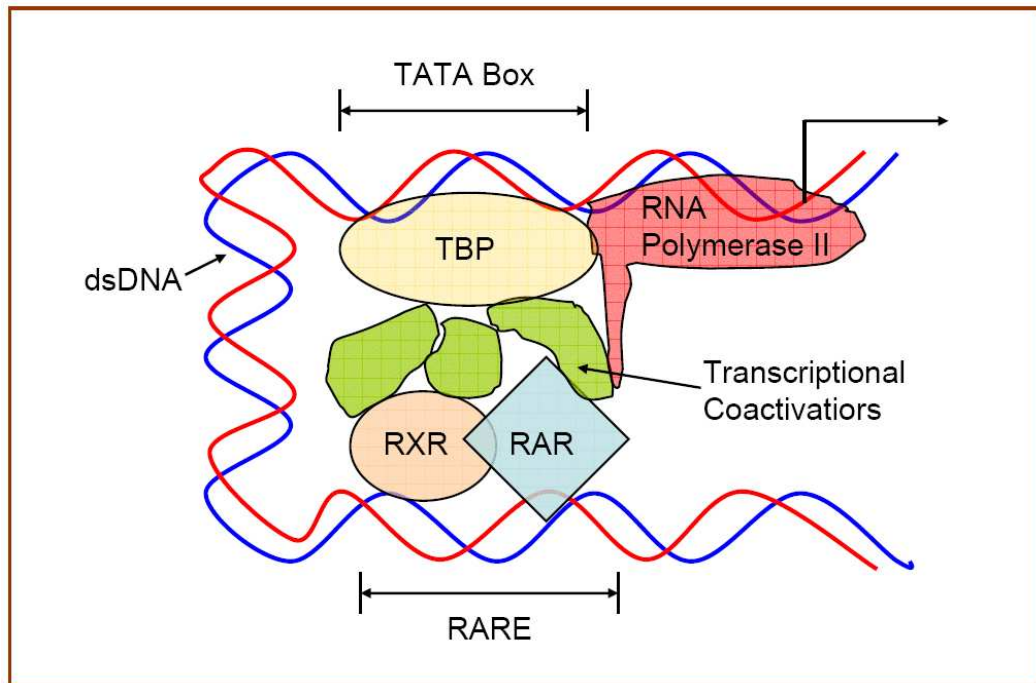


Figure 5. A model of transcriptional mechanism of RAR/RXR (from NUTR446 notes)

Among many genes which are under RA's regulation, two important genes, LRAT and CYP26, have demonstrated great importance in vitamin A metabolism (44). As mentioned before, LRAT catalyzes the esterification of retinol to its storage form of RE, whereas CYP26 mediates the oxidation of RA to inactive products (Fig. 6). Physiologically, the role of RA in regulating the expression of LRAT and CYP26 enables RA to control vitamin A metabolism under different levels of vitamin A status (44). During vitamin A sufficiency, the continued presence of RA is a signal of high vitamin A levels in the body and up-regulates the expression of LRAT and CYP26, which subsequently limit the availability of RA and prevent excess retinol. On the contrary, vitamin A deficiency generates a condition of very low RA concentration, which down-regulates the expression of LRAT and CYP26. Therefore, to a certain extent, the self-regulatory mechanism of RA renders the body able to avoid both vitamin A deficiency and toxicity.

Recently, A multitransmembrane domain protein in bovine retinal pigment epithelium cells, STRA6, was identified as a receptor for retinol binding protein (RBP) and can mediate cellular uptake of vitamin A (45). STRA6 is a RA-responsive gene and is expressed in a variety of embryonic and adult cells or organs (46). Although it is highly expressed in eyes, STRA6 expression level is still considerable in adult lung organ (47). But, whether STRA6 gene is expressed in pup lung and involved in vitamin A uptake process during postnatal period is still unclear.

Certain synthetic retinoic acid analogs have aroused researchers' interests due to

their potent RA activities and property of resistance to metabolism by CYP26A1. Among these compounds, Am580 is an aromatic amide (Fig. 1A) and exhibits high selectivity for the nuclear RA receptors RAR alpha (48). Previous studies have showed enhanced effects of Am580 on induction of CYP26 gene expression and all-trans-retinoic acid metabolism in the intestinal cells (49). Investigation on this synthetic compound might contribute more in the research of vitamin A metabolism and clinical therapeutic application.

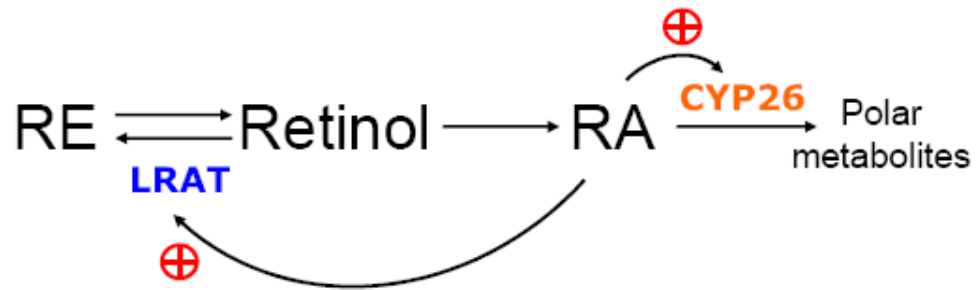


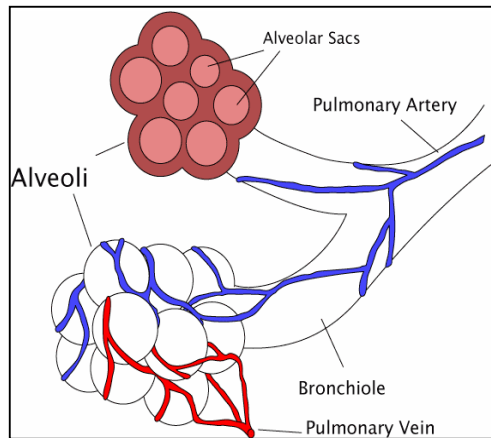
Figure 6. Regulatory role of RA in retinoid metabolism. Simplified model of roles of lecithin:retinol acyltransferase (LRAT), and cytochrome P450 (CYP26) in retinoid metabolism. (44)

Vitamin A and the neonatal lung

The lung is the essential respiration organ in air-breathing vertebrates. Its principal function is to transport oxygen from the atmosphere into the bloodstream and to excrete carbon dioxide from the bloodstream into the atmosphere (50). This exchange is accomplished in the alveoli, the basic structure units in the lung. Alveoli are lung air sacs made of simple squamous epithelial cells for diffusion of gases. Millions of these tiny, exceptionally thin-walled air sacs form the particular structure of the lung (50) (Fig. 7).

In the postnatal period, the lung undergoes rapid development and significant morphologic alterations (51). The large, thick-walled gas exchange saccules divide into numerous small, thin-walled alveoli. The formation of alveoli during lung development is termed alveolization and is essentially completed in the neonatal period (52). It is well known that impairment of the alveolar wall is the root of pulmonary diseases such as bronchopulmonary dysplasia (BPD) in the early life of preterm infants and emphysema in adulthood (53). The impaired alveolization or damage to alveolar sacs results in loss of function in gas exchange. Therefore, therapies to promote alveolization are of great interest in clinical applications.

A



B

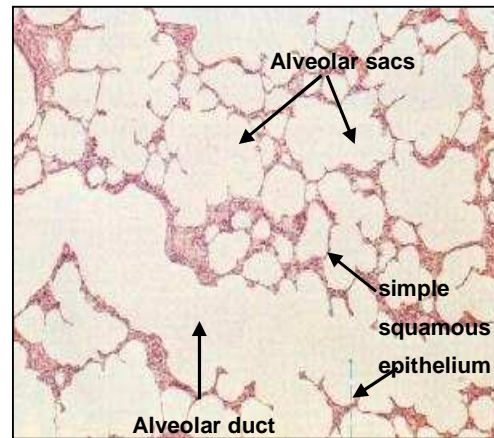


Figure 7. Diagram and histology of alveoli. (A) Gas exchange happens between the atmosphere and pulmonary blood; (B) Alveoli are chambers of simple squamous epithelium, they connect to each other and to the alveolar ducts to form sponge-like structure (from internet resources).

In the later 20th century, vitamin A was found to be required for the development of the neonate lung (54). Animal studies on vitamin A storage in the prenatal and postnatal lung indicated that the fetal lung begins to dramatically increase its vitamin A storage in the latter 1/3 of prenatal life and then quickly depletes this storage during late pregnancy and early postnatal life, which is an important period for lung maturation (3). The consistency of lung vitamin A consumption and lung maturation implies a strong relationship between vitamin A and lung development.

In the population of preterm infants [particularly those infants with very low birth weights (VLBW)] who are born with inadequate vitamin A storage (55-57), a progressive sequence of histopathologic changes in the epithelial lining of pulmonary system is observed. The normal columnar or cubical mucous-secreting epithelial cells become flattened, dry and keratinized, and lost the functions (58, 59). In this population, BPD is the most prevalent form of chronic lung disease observed.

Considering the observed changes in lung vitamin A storage during the perinatal period of rats and the histopathologic changes in preterm infants with low vitamin A levels, it is believed that vitamin A is an essential micronutrient which promotes the lung development and may prevent BPD. In several clinical trials, intramuscular administration of vitamin A in a small group of VLBW infants showed a reduced biochemical evidence of vitamin A deficiency and slightly decreased risk of chronic lung disease (60, 61). Other animal model studies also have shown that vitamin A deficiency during the postnatal period led to an increased size of rat lung air-exchange spaces and

reduced elastin in the parenchyma, and the administration of RA to the rats in this period resulted in the recovery of the septation and formation of alveoli (62, 63).

Infant respiratory distress syndrome ("RDS"), a syndrome caused in premature infants by developmental insufficiency of surfactant production and structural immaturity in the lungs, is the leading cause of death in preterm infants (64). To reduce incidence of RDS and infant mortality from RDS, premature infants are frequently exposed to oxygen therapy or treated with dexamethasone, a glucocorticoid, which can increase production of surfactant and accelerate lung maturation (65, 66). However, increasing evidence has shown that oxidative stress and side effects caused by the therapies of hyperoxia and dexamethasone are implicated in the failure of septation and development of BPD (67, 68). Several animal studies reported that RA treatments may partially rescue the failed septation and reduce the lung injuries in rats or mice which were treated with glucocorticoid or hyperoxia exposure (63, 69, 70). All these reports proved the physiological action of RA in promoting lung septation and maturation.

CHAPTER 2

HYPOTHESES AND AIMS

Aim 1. To determine how the storage of retinyl esters (RE) in the neonatal rat lung is affected by the administration of RA. We also will study how a non-metabolizable analog of RA, Am580, affects the RE contents in the same organs. The combination of vitamin A and its bioactive metabolite retinoic acid (VARA) has a synergistic effect in increasing RE contents in postnatal lung tissue (5). We want to know how the RE status in neonatal pup lung changes kinetically after VA supplementation with or without RA. We also want to know whether the combination of VA and RA's non-metabolizable analog, Am580 is capable to further increase the synergistic effect of VARA. Postnatal rats will be treated orally with one single dose of oil, VA, RA, a combination of VA and RA (VARA), Am580, and a combination of VA and Am580 (VAAm). The lung tissue will be collected 6 hours and 12 hours after dose administration. Lipid extracts from each sample will be analyzed to quantify the total lung and liver retinol contents (RE plus retinol) by HPLC. We anticipated that the combined dose of VA and RA or VA and Am580 will increase the total retinol level in the neonatal lung compared to VA alone.

Aim 2. To determine the effects of RA on the distribution of newly absorbed vitamin A (^3H -labeled) and formation of retinyl ester (^3H -labeled) in the

neonatal rat lung. RA might play an important role in the delivery and storage of VA in the lungs during the postnatal period (71). We will use the same animals from the 12 hour study for a metabolic study. All the treatment doses will be labeled with ^3H -retinol. The newly formed ^3H -retinol, ^3H -RE and ^3H -labeled polar metabolites of retinol in the lung and liver will be counted by liquid scintillation spectrometry and the percentage of the oral dose in each tissue fraction will then be calculated. We hypothesized that the treatment of RA or Am580 will redirect the flow of the new absorbed VA into the lung tissue in neonatal rats. These treatments might also change the metabolic pattern of VA in the neonatal lung.

Aim 3. To determine the response of certain genes related to VA uptake and metabolism in the neonatal lungs after treatment with VA, RA and the combination of VA and RA, as well as Am580. Several important genes, like lecithin: retinol acyltransferase (LRAT) and a member of the cytochrome P450 family, CYP26, and newly identified vitamin A transporter gene, STRA6, play important roles in VA uptake and metabolism. By analyzing the mRNA levels of these genes, we may be able to better understand how RA affects the mechanisms of VA uptake, storage and oxidation in the neonatal lung. In this study, specific genes like LRAT, CYP26A1, CYP26B1 and STRA6 will be analyzed for their transcript levels using real-time polymerase chain reaction (RT-PCR). Microarray analysis may also be conducted to screen for some

other genes which are regulated by RA or Am580 in these tissues. We expect that these genes will respond to treatment with RA or Am580 rapidly. We also hypothesized that the expression of the genes in the samples treated with Am580 will induce a higher level of gene's expression than RA.

CHAPTER 3

RESEARCH DESIGN AND METHODS

Experimental Design

We conducted two studies with treatments of 6 hours and 12 hours respectively (considering the peak expression time of genes and completed process of VA absorption from GI tract). In each study, 7-8 days old neonatal Sprague-Dawley rats were divided into 6 groups. Since the pups were from several litters, sexes were evenly distributed to each group. Average body weight of each group was close to each other. (Table 1)

Treatment Strategy

Doses:

- Vehicle: (oil)
- VA (RP): This dose is based on a dose of 50,000 IU/2.5 kg in young children (72, 73). By converting international unit to mass unit with the factor of 0.548 μg retinyl palmitate (RP)/IU, we get the VA doses of 10.96 μg RP (or 6 μg retinol) /g BW.
- RA: Based on prior usage (63) in neonatal rats: 500 μg RA ip/kg body weight, we adjusted for oral delivery: estimate about 80% of dose will be absorbed.
- VARA: the same amount of VA and RA will be combined at a molar ratio of 10:1.
- Am580: molar equivalent to RA dose.
- VAAM580: Same amount of VA and Am580 will be combined at a molar ratio of 10:1.

	Vehicle	VA	RA	VARA	Am580	VAAm580
Study I (6-h study)	N=4 (2M; 2F)	N=4 (2M; 2F)	N=4 (2M; 2F)	N=4 (2M; 2F)	N=4 (2M; 2F)	N=4 (2M; 2F)
Study II (12-h study)	N=5 (2M; 3F)	N=5 (2M; 3F)	N=5 (2M; 3F)	N=5 (2M; 3F)	N=5 (2M; 3F)	N=5 (2M; 3F)

Table 1. Animal experiment design. One dose of treatment was given orally 6 hours or 12 hours before tissues were collected for analysis. An appropriate amount of ^3H -retinol will be provided with the doses as a tracer for a retinol metabolic study.

Experimental Methods

Doses were prepared in the concentration of 0.05 mmol VA/L for VA dose, 0.005 mmol VA/L for RA and Am580 doses. For the combinations of VARA and VAAm580, the prepared VA and RA or Am580 doses were mixed with a ratio of 10:1 in volume. The volume of each dose provided to pup is 0.4 μ l/g body weight and the exact volume of dose was dependent on the pup's body weight. After 6 hours or 12 hours, pups were killed with carbon dioxide. The lung and liver tissues were removed, trimmed and weighed. All samples were frozen in liquid nitrogen immediately. Then, we stored the lung and liver tissues at -80 oC for later analysis.

Retinoid Analysis

Portions of the lung tissue were cut, weighed and extracted in chloroform:methanol, 2:1 v:v, mixed solvent overnight. Then, samples were processed by Folch wash procedure (74). After the final wash, the extract was dried down under argon; the samples were redissolved in 2 ml hexanes. Portion of the hexanes volume was dried again and underwent a hydrolysis reaction by a saponification procedure. A known amount of an inner standard, trimethylmethoxyphenyl-retinol (TMMP) was added to each sample and the samples were dried under argon and reconstituted in 100 μ l of methanol for HPLC analysis. Portions of each sample (usually 18-22 μ l) were injected into a C-18 HPLC column and eluted with a gradient of 92.5:7.5 methanol:water at a flow rate of 1.5 ml/min for 5 min. The eluate was monitored by a Waters 960 photodiode array detector and the areas of the peaks for TMMP and retinol were analyzed by Millennium-32 (Waters)

software.

³H-retinol metabolic study

We used ³H-retinol as a tracer to study the distribution of newly given retinol in the neonatal rats. ~2 μCi ³H-retinol contained in each dose described above were delivered to each neonatal rat along with dose. After 12 hours, pup lung and liver tissues were cut, weighed and extracted in chloroform:methanol, 2:1 v:v, mixed solvent overnight. After Folch wash procedure, a portion of hexanes containing organic extracts was counted for unmetabolized ³H-retinol by a liquid scintillation spectrometry. The other portion of hexanes was dried, redissolved in hexanes, subjected to alumina column chromatography and counted for ³H-RE. The upper aqueous phases from the Folch wash were pooled as aqueous extracts and a small portion was counted to assess the formation of aqueous ³H-labeled polar metabolites of retinol (75).

Gene mRNA level determination

Total RNA from individual pup lung was extracted using a guanidine extraction method and reverse transcribed into its complementary DNA (cDNA). The diluted reaction product was used for real-time PCR analysis. Primers designed to detect mRNA expression were: 5'-ATA GGA TCC TGA CCA ACA CTA CAT CCT CTC-3' (forward) and 5'-ATT CTC GAG TCT AAG TTT ATT GAA ACC CCA GA-3' (reverse) for rat LRAT; 5'-TTG AGG GCT TGG AGT TGG T-3' (forward) and 5'-AAC GTT GCC ATA CTT CTC GC-3' (reverse) for rat CYP26B1; 5'-GTG CCA GTG ATT GCT GAA GA-3'

(forward) and 5'-GGA GGT GTC CTC TGG ATG AA-3' (reverse) for rat CYP26A1; 5'-CCG ATC CTG GAC AGT TCC TA -3' (forward) and 5'-CCA CCT GGT AAG TGG CTG TT -3' (reverse) for rat STRA6. The mRNA expression level of each sample was corrected by calculating mRNA-to-ribosomal 18S RNA ratio. Two liver mRNA samples from normal adult rat (C1) and vitamin A sufficient rat (C2) were included in each PCR run as references. Data were normalized to the average value for the control group, set at 1.00, prior to statistic analysis.

Statistic Analysis

Data were presented as group means \pm SEM (standard error of the mean). In lung total retinoids analysis and ^3H -retinol metabolic study, group differences were tested by one-factor ANOVA followed by Fisher's protected least significant difference test. To test the differences in gene expression level between each group and different time point, we conducted two-factor ANOVA followed by Fisher's protected least significant difference test. The software used for statistic analysis is SuperANOVA (software; Abacus, Berkeley, CA). For comparison, we converted the mean mRNA value of the lung control group to a value of 1, and the mean values of the other groups were converted accordingly. To reduce variance of each group mean, value was transformed to log₁₀ form before statistic analysis. Differences with $p \leq 0.05$ were considered significant.

CHAPTER 4

RESULT I

VA combined with RA or Am580 increases lung RE significantly at 6-h and 12-h

In the group of VA, lung total retinol was increased ~3-fold at 6-h and 12-h compared to control group (Fig. 8). The treatment of RA increased lung total retinol ~2-fold at 6-h and 12-h as well. VARA also can increase lung total retinol to the average levels of ~21 nmol/g at 6-h and ~24 nmol/g at 12-h. Treatment of Am580 increased lung total retinol contents nearly to the same extent as the treatment of RA in both the 6-h and 12-h studies. VAAm580 also promoted lung total retinol contents to the same extent of VARA at 6 hours, but this increase declines to ~13 nmol/g at 12 hours. These data suggested that the acidic retinoids RA and Am580 promote the uptake and metabolism of retinol by the neonatal lung, leading to a synergistic increase in RE concentration compared to treatment with VA alone. However, compared with RA, the non-metabolized Am580 combined with VA has the effect of quickening retinol metabolism rate in the pup lung.

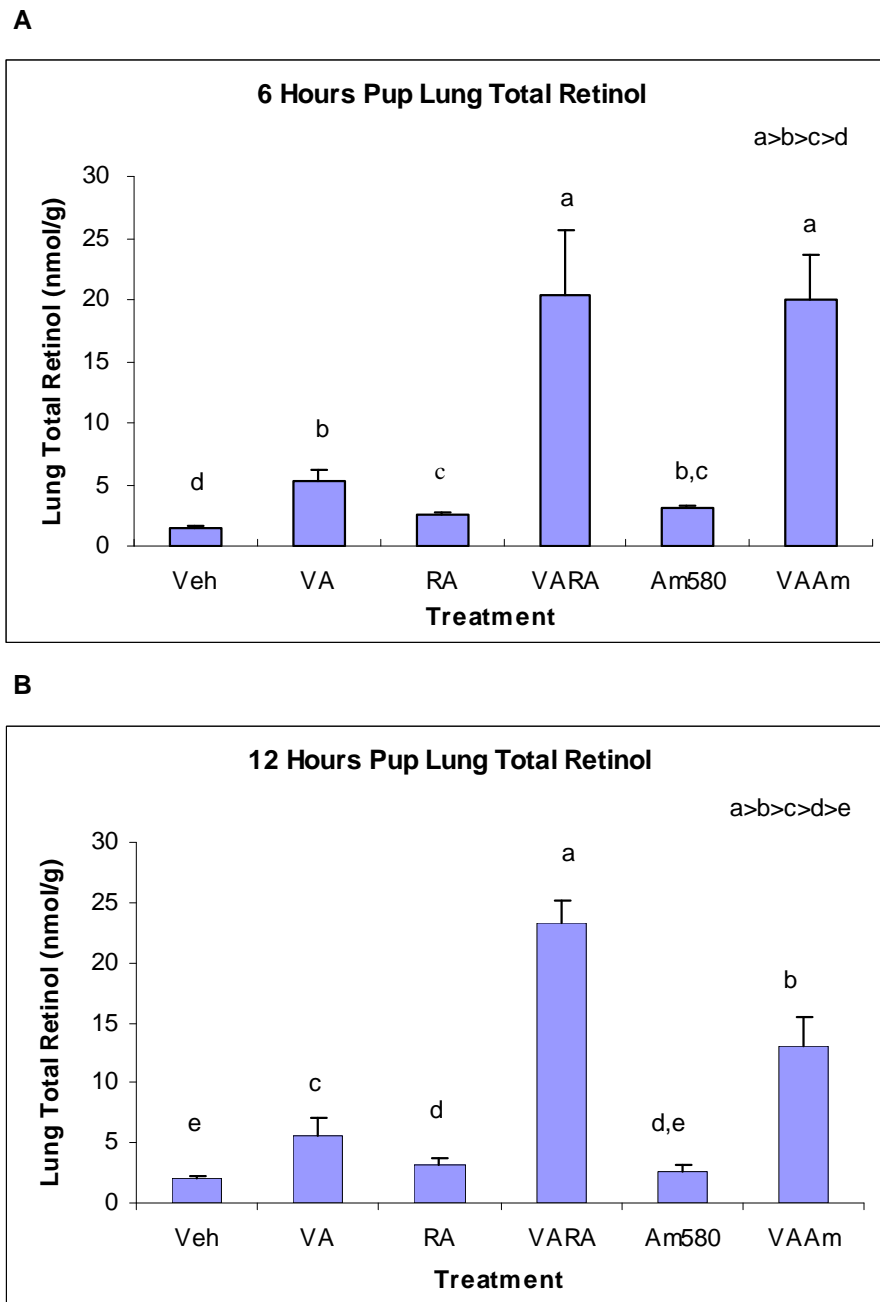


Figure 8. Pup Lung total retinol concentrations analysis. Neonatal rats were given a single dose of oil (vehicle), vitamin A (VA) alone, retinoic acid (RA) alone, combination of VA and RA (VARA), Am580 and combination of VA and Am580 (VAAm) (n=4-6) on postnatal day 7. Tissues were collected after 6 hours and 12 hours respectively. Results are presented as group means \pm SEM; Groups were compared by one-factor ANOVA. Fisher's protected LSD. Data were transformed by log₁₀ prior to ANOVA. ($a>b>c>d>e$, $p\leq 0.05$) (A) Concentration of total retinol in the neonatal rat lung of 6-h study. (B) Concentration of total retinol in the neonatal rat lung of 12-h study.

RA or Am580 promote higher lung uptake of newly absorbed ^3H -retinol

In the 12-h study we conducted a metabolic study to investigate how the newly absorbed ^3H -retinol was distributed into the neonatal rat lung and the liver and whether the treatment of VARA or VAAM580 can promote the uptake of newly absorbed retinol into the lung which in turn increased lung RE formation. Tissues were analyzed 12 h after oral dosing, a time chosen so that the absorption of orally given ^3H -retinol would be totally completed. The results showed that VA has the same percentage (~1% of oral dose) of lung ^3H -retinol uptake as the control group (Fig. 9A). The uptake of ^3H -retinol was higher in the lungs of neonates treated with RA, VARA, Am580, and VAAM580 as compared to control or VA alone. However, VARA was more effective than VAAM580 in promoting lung ^3H -Retinol uptake and ^3H -RE formation at 12 h. This result might be due to the different retinol metabolism rate affected by RA and Am580. In contrast to the pup lung, the similar effects are not observed in pup liver. RE formation in the liver was increased only by VA and VARA (Fig. 9C). In the pup lung, the results of ^3H -RE formation did not show significant differences within groups of VA, RA and VARA, but the Am580 and VAAM580 groups had a lower percentage of ^3H -RE (Fig. 9B). We infer that the lower ^3H -retinol percentage and ^3H -RE formation in the lung treated with VAAM580 is due to the higher rate of metabolism affected by Am580 as well. In the liver, no significant differences in ^3H -RE level were observed within groups (Fig. 9D). These results indicated that both RA and Am580 can promote lung uptake of newly absorbed ^3H -retinol, but do not affect ^3H -RE accumulation in the liver.

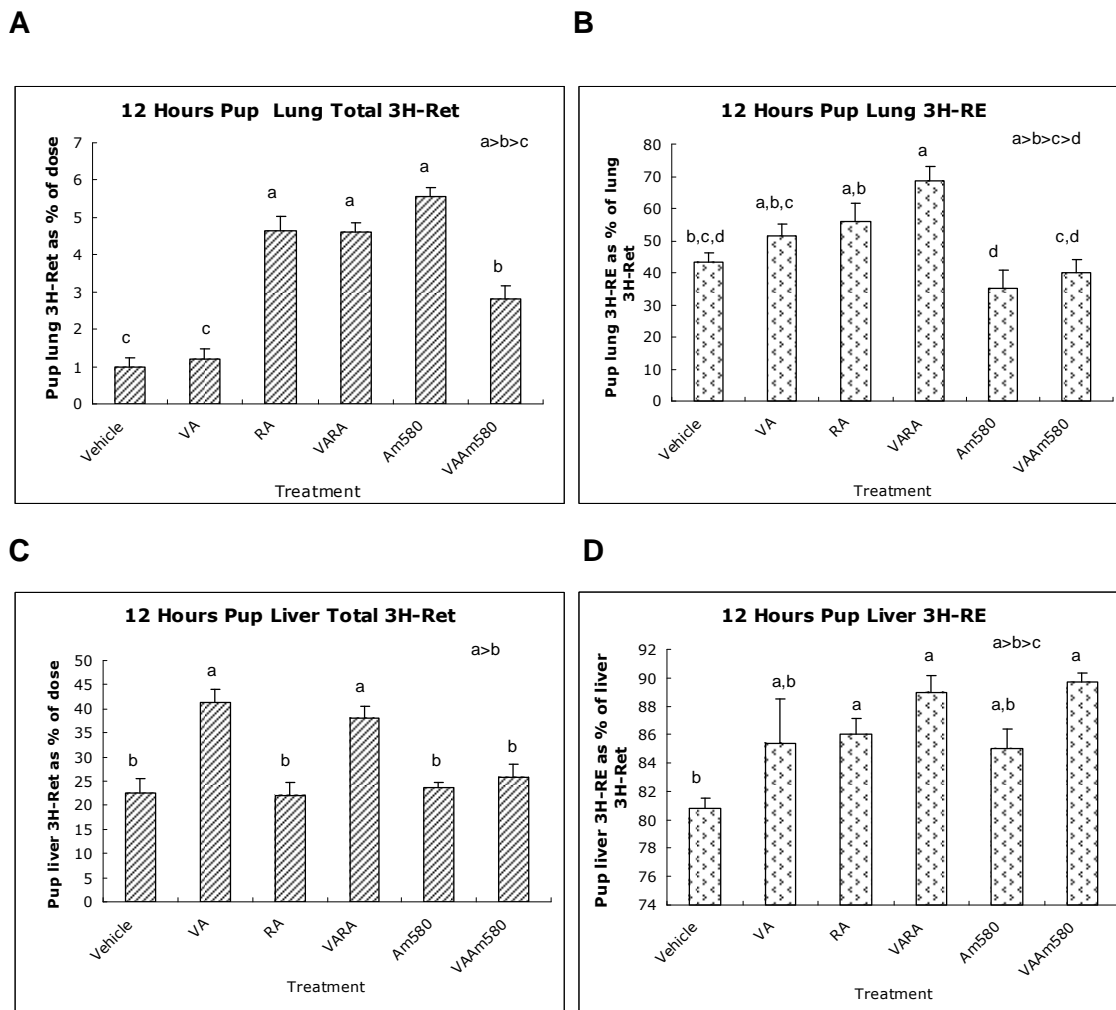


Figure 9. Uptake and metabolism of newly absorbed ^3H -retinol in the lung and liver. Neonatal rats were given a single dose of oil (vehicle), vitamin A (VA) alone, retinoic acid (RA) alone, combination of VA and RA (VARA), Am580 and combination of VA and Am580 (VAAm) ($n=4-6$) on postnatal day 7. ^3H labeled dose was given to each pup orally 12 hours prior to sacrifice. Tissues were collected after 6 h and 12 h respectively. Treatments are the same as in the retinoid analysis study. Results are presented as group means \pm SEM; Groups were compared by one-factor ANOVA. Data were transformed by \log_{10} prior to ANOVA. Fisher's protected LSD. ($a>b>c>d$, $p\leq 0.05$) (A) Lung total ^3H -retinol as percent of dose; (B) Lung total ^3H -RE as percent of lung total ^3H -retinol; (C) Liver total ^3H -retinol as percent of dose; (D) Liver total ^3H -RE as percent of liver total ^3H -retinol. ROH, retinol.

CHAPTER 5

RESULT II

Expression of LRAT, CYP26B1, CYP26A1 and STRA6 genes in pup lung at 6-h and 12-h after treatment with VARA or VAAm580

Because LRAT and CYP26B1, CYP26A1 and STRA6 are important genes involved in the vitamin A metabolism and are regulated by RA, we next consider that those genes might play a role in regulating vitamin A metabolism and result in the synergistic effect of increasing lung RE content in the neonatal lung by administration of VARA or VAAm580. Genes level were analyzed in both the 6-h and 12-h studies.

In the 6-h study, treatment of RA can induce a ~7-fold elevation in pup lung LRAT expression, but this elevation is transient and returned to basal levels at 12 h. By comparison with RA, Am580 not only dramatically increase LRAT expression (~15-fold increase), but also maintain high expression level of LRAT even at 12 h (Fig. 10).

The expression of lung CYP26B1 was increased to about ~3.5-fold by both RA and Am580 at 6 h, but the increases induced by RA returned to basal levels at 12 h. However, in comparison to RA, Am580 further and dramatically increased CYP26B1 level at 12 h (Fig. 11).

Lung CYP26A1 did not increase in response to RA, but it still can be up-regulated by Am580 at 6 h and 12 h (Fig. 12). The increases of CYP26A1 at 12 h are not as

significant as CYP26B1. Since pup lung CYP26B1 level is much higher than CYP26A1 (data not shown), CYP26B1 might play the major role in retinoid metabolism in neonatal rat lung.

We also determined pup lung STRA6 level at 6 h. Results showed that both RA and Am580 induced STRA6 expression to 3~4 times high. The elevated STRA6 level suggested an increase in retinol uptake from extracellular RBP-retinol complex.

This study showed that LRAT, CYP26B1 and STRA6 are concomitantly up-regulated in the neonatal lungs after treatment with RA (\pm VA), but the increases were transient, compared to a higher magnitude, prolonged increase with Am580 (\pm VA). Overall, acidic retinoids given concomitantly with VA can dramatically increase the levels of some important retinoid homeostatic genes, like LRAT, CYP26B1 and STRA6 which may stimulate retinol uptake and increase its metabolism in the lungs of neonates.

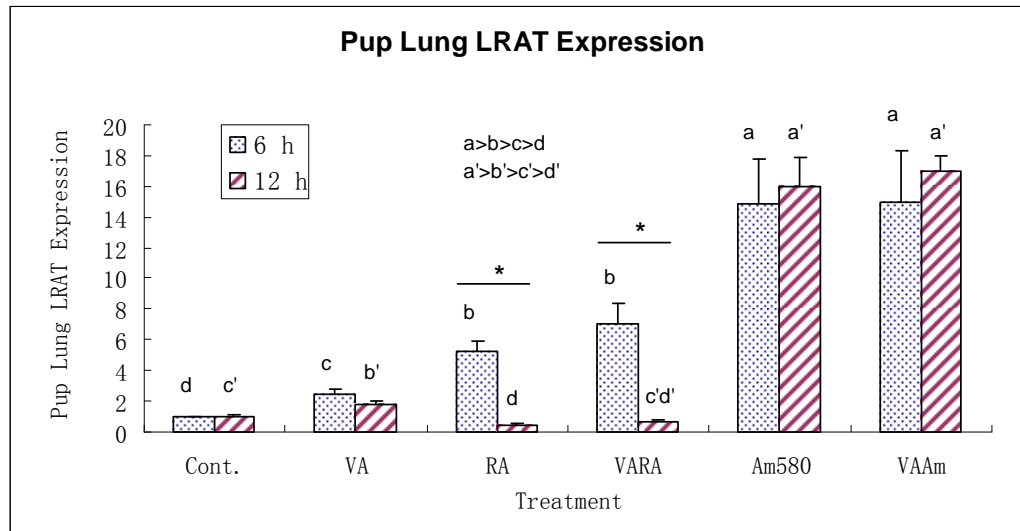


Figure 10. Pup lung LRAT expression level at 6h and 12h. 7-8 days old neonatal rats treated with oil, VA, RA VARA, Am580 and VAAM580 were sacrificed 6h and 12h after the treatment respectively. Lung tissue was collected and processed for total RNA isolation. Lung LRAT expression levels were compared between each group and different time points. Results are presented as group means \pm SEM; Groups were compared by two-factor ANOVA. Data were log₁₀ transformed prior to ANOVA. Fisher's protected LSD. (n=5, p \leq 0.05). Oil (Cont.), vitamin A (VA), retinoic acid (RA), Am580, combination of vitamin A and retinoic acid (VARA), combination of vitamin A and Am580 (VAAM580).

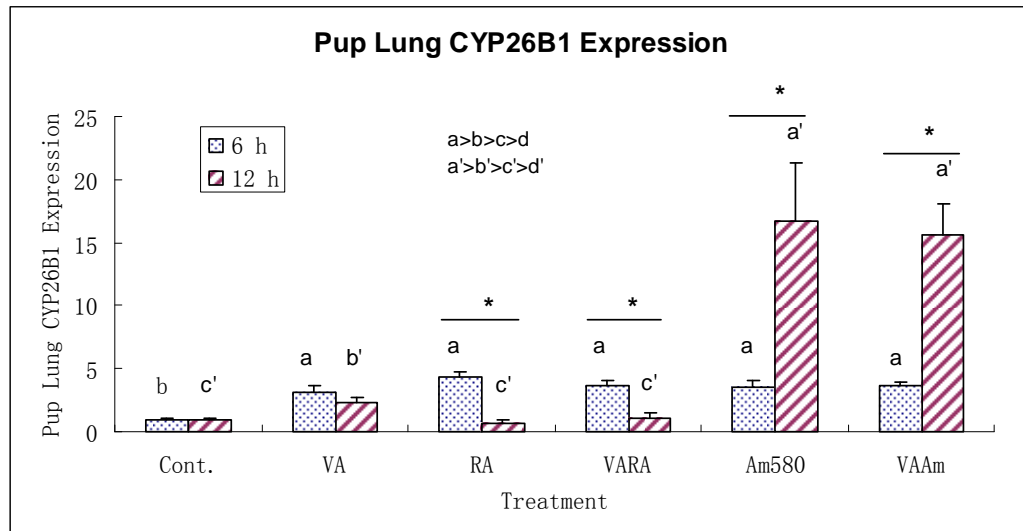


Figure 11. Pup lung CYP26B1 expression level at 6h and 12h. 7-8 days old neonatal rats treated with oil, VA, RA VARA, Am580 and VAAM580 were sacrificed 6h and 12h after the treatment respectively. Lung tissue was collected and processed for total RNA isolation. Lung CYP26B1 expression levels were compared between each group and different time points. Results are presented as group means \pm SEM; Groups were compared by two-factor ANOVA. Data were log₁₀ transformed prior to ANOVA. Fisher's protected LSD. (n=5, p \leq 0.05). Oil (Cont.), vitamin A (VA), retinoic acid (RA), Am580, combination of vitamin A and retinoic acid (VARA), combination of vitamin A and Am580 (VAAM580).

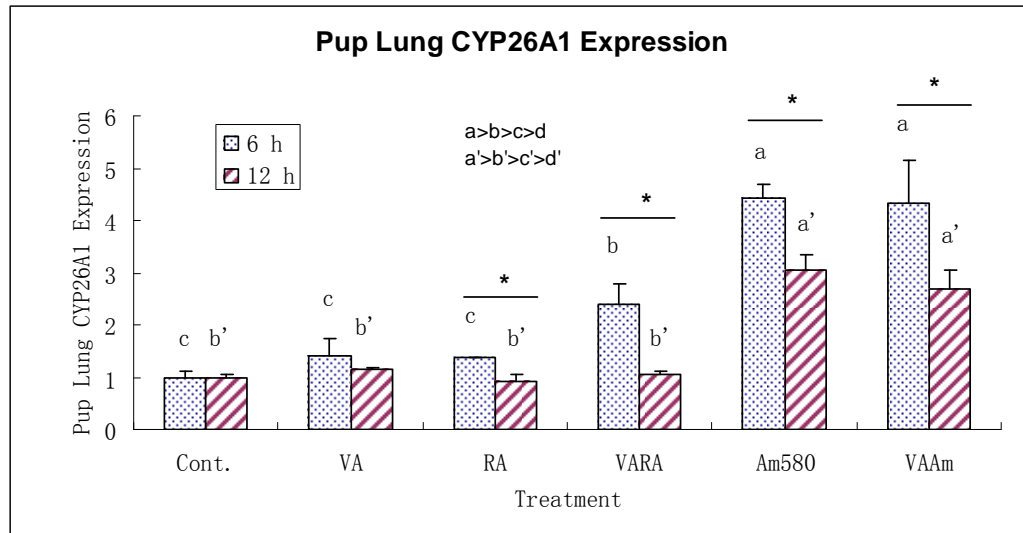


Figure 12. Pup lung CYP26A1 expression level at 6h and 12h. 7-8 days old neonatal rats treated with oil, VA, RA VARA, Am580 and VAAm580 were sacrificed 6h and 12h after the treatment respectively. Lung tissue was collected and processed for total RNA isolation. Lung CYP26A1 expression levels were compared between each group and different time points. Results are presented as group means \pm SEM; Groups were compared by two-factor ANOVA. Data were log10 transformed prior to ANOVA. Fisher's protected LSD. (n=5, $p \leq 0.05$). Oil (Cont.), vitamin A (VA), retinoic acid (RA), Am580, combination of vitamin A and retinoic acid (VARA) , combination of vitamin A and Am580 (VAAm580).

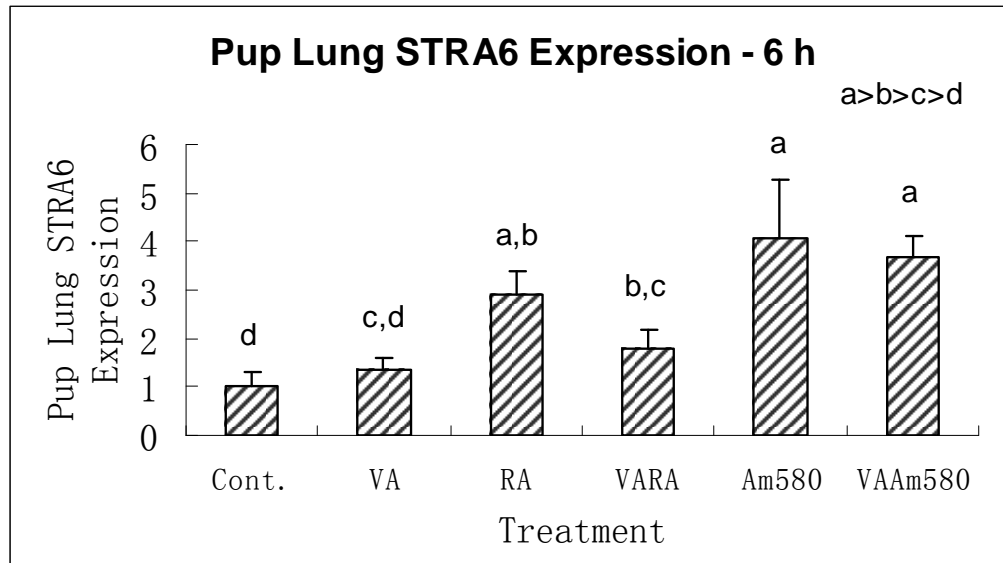


Figure 13. Pup lung STRA6 expression level at 6h. 7-8 days old neonatal rats treated with oil, VA, RA VARA, Am580 and VAAM580 were sacrificed 6h after the treatment. Lung tissue was collected and processed for total RNA isolation. Lung STRA6 expression levels were compared between each group. Results are presented as group means \pm SEM; Groups were compared by one-factor ANOVA. Data were log₁₀ transformed prior to ANOVA. Fisher's protected LSD. (n=4~6, $p \leq 0.05$). Oil (Cont.), vitamin A (VA), retinoic acid (RA), Am580, combination of vitamin A and retinoic acid (VARA), combination of vitamin A and Am580 (VAAM580).

CHAPTER 6

DISCUSSION

Human lung development goes through a long journey from as early as the fourth week of embryonic stage until birth to 8 years of age. During the late fetal and postnatal period, the lung undergoes a complex change in morphogenesis and differentiation of the respiratory epithelium and eventually forms the mature and functional lung organ. In this stage, Vitamin A (VA) has a major role in promoting lung development and maturation. In the rat, significant storage of vitamin A in the lungs starts in late gestation just before the onset of alveolization and surfactant synthesis. These stores are rapidly depleted during late pregnancy and postnatal life as the lungs still develop (3, 76, 77). Usually, the septation of lung in rat and mouse begin from about postnatal day 4 to 14 but mainly from day 4 to 7 (1, 78), that's why we used 7 days old neonatal rat as our animal model. Weaning rats fed with vitamin A deficient diet shows the characteristics of keratinized metaplasia in the trachea and the bronchopulmonary tree (2). Supplement of retinol or RA can repair epithelial lesions and increase surfactant phospholipid synthesis in fetal rat lung (79). In the population of preterm infants, premature delivery accompanied with vitamin A deficiency usually has been associated with an increased susceptibility to lung injury, for example, neonatal RDS and subsequent BPD, due to deficit in pulmonary surfactant. However, neonatal supplementation with retinol reduces the risk of BPD (60, 61);

Since vitamin A has shown its essential activity in neonatal lung development and promising effects on reducing lung injuries and dysfunction, there could be a biological advantage in increasing or maintaining adequate vitamin A storage in the neonatal lung. Supplementing VA to the offspring directly may be efficient in increasing lung vitamin A level. However, VA dosage is still a concern for the clinical use due to the toxicity of high amounts of VA. Our previous study has indicated that VA given orally in combination with RA (VARA, 10:1 ratio, 10% RA) increased retinol uptake and lung RE formation more effective compared with VA or RA given individually. This result indicates that VA and RA act in a synergistic manner in promoting neonatal lung RE concentration (5). In a dose-dilution study, VARA had about 4 times the effect as compared with a standard dose of VA alone(6). In contrast to lung tissue, VA and VARA increased RE equally in the liver (5), and therefore it appears that the synergistic effect of VARA is only specific for the lung tissue. So we concluded that the administration of RA redirects the flow of supplemented VA in the neonate body.

Then we investigated the mechanism of the Vitamin A metabolism in the neonatal rat lung at mRNA level. It is well known that retinoids exert their influence in the development, cell differentiation mainly through the binding of RA with two main types of retinoid receptors, the retinoic acid receptor (RAR) and retinoid X receptor (RXR) in the nuclear. Typically, RAR and RXR form the heterodimer and targets the retinoic acid-responsive element (RARE) to regulate the downstream genes (40). This RA signaling pathway modulates a big number of RA-responsive genes which play important roles in lung development, cellular differentiation and vitamin A it selves metabolism.

Among these genes, LRAT and CYP26 are the most important genes involved in vitamin A metabolism (44). By converting retinol to its storage form RE or oxidizing retinoic acid to the inactive polar metabolites (Fig. 6), LRAT and CYP26 control the balance of the metabolism pathway and regulate the homeostasis of retinoids in the lung tissue. Therefore, to develop a better and efficient method for increasing lung retinol store in VA deficient population like very low birth weights (VLBW) infants, we should have a better understanding of gene expression pattern affected by RA.

The previous neonatal lung vitamin A metabolism study only selected the time point of 6 h, the uptake and esterification of newly absorbed ³H-retinol probably had not reached a steady state at this time point. Thus, in our recent studies, we added a 12-h study to observe the prolonged effect of VARA and make comparison between these two time points. Since RA could be metabolized rapidly by CYP26, we also used a metabolizing-resistant analog of RA, Am580 in our study. Our results suggested that effects of VARA at 6 h and 12 h did not differ from each other and Am580 also can synergistically increase the uptake of newly absorbed retinol and lung RE content to the same extent of that caused by VARA at 6 h. However, the administration of VAAM580 resulted in a lower lung RE concentration at 12 h compared with VARA at 12 h or VAAM580 at 6 h (Fig. 8A & 8B). To explain why there is a decline in lung RE with treatment of VAAM580 at 12 h, we checked gene expression level at 6 h and 12 h respectively. By investigating several gene including CYP26A1, CYP26B1, LRAT and STRA6, we found that the expression of LRAT, CYP26B1 and STRA6 were highly induced by RA at 6 h. However, gene levels of LRAT and CYP26B1 returned to the basal

level at 12 h. By contrast, Am580 not only be able to maintain the high expression level of LRAT, it also further up-regulate the expression of CYP26B1 at 12 h. Highly induced genes, especially dramatically increased CYP26B1, results in accelerated oxidative metabolism rate and change the balance of the metabolic pathway toward the direction of oxidation. In the meantime, due to the completed absorption of retinol at 12 h, the uptake of retinol into lung tissue is limited. Although CYP26A1 in lung tissue was up-regulated by Am580 as well, but it did not increase in response to RA and there is no significant difference between 6 h and 12 h. Our new data have shown that CYP26B1 is expressed in the lung about 10-fold higher than CYP26A1 and responds to RA's regulation stronger than CYP26A1 in the lung (data are not shown). Since CYP26A1 is not a predominant isomer of CYP26 in the lung, it has limited effects in regulating VA metabolism in this tissue. However, CYP 26A1 expression level significantly responses to RA and Am580 in the pup liver (data are not shown), which suggests its important role in VA metabolism in the liver. Based on these findings, we concluded that LRAT and CYP26B1 are the critical genes in regulating neonatal lung vitamin A homeostasis (Fig. 14).

We also compared the metabolism of newly absorbed ^3H -retinol in the 12-h metabolic study. This experiment revealed that more ^3H was presented in the lung of RA- or Am580-treated groups compared with the control or the VA group. In addition, the newly formed ^3H -RE was proportional to the ^3H -retinol obtained by lung tissue which implies an accordingly increased esterification of retinol in neonatal rat lung. These results indicated that more newly supplemented ^3H -retinol could be directed into the lung tissue and converted to RE when neonates were treated concomitantly with RA or Am580.

It appears most likely that the induced LRAT and STRA6 expression by RA or Am580 contributes to the increased lung ^3H -retinol uptake and ^3H -RE formation.

In summary, our studies further confirmed the effect of VARA in promoting the uptake of newly absorbed retinol by the neonatal lung and increasing lung RE level synergistically. The induced expression of several important genes, LRAT, CYP26B1 and STRA6 by RA or Am580 reveals a possible molecular mechanism for the synergistic effect of VARA, although the inductions caused by RA were transient. The higher magnitude and prolonged increase in gene expression induced by Am580 provided us another evidence for a better understanding in vitamin A metabolism in neonatal rat lung.

Each year, more than 300,000 premature infants are born in the United States, and ~60,000 are VLBW infants who are usually born with inadequate body stores of vitamin A and are prone to respiratory diseases. As a promising therapeutic method in clinical use, VARA treatment might rapidly correct retinoid deficiency in neonatal lung tissue in these young infants during the early time of life and thus reduce infant mortality and prevent respiratory infection. Our studies not only present a model of the molecular mechanism for the synergistic effect by VARA, but also provide clues in developing a better therapeutic option in clinical medicine.

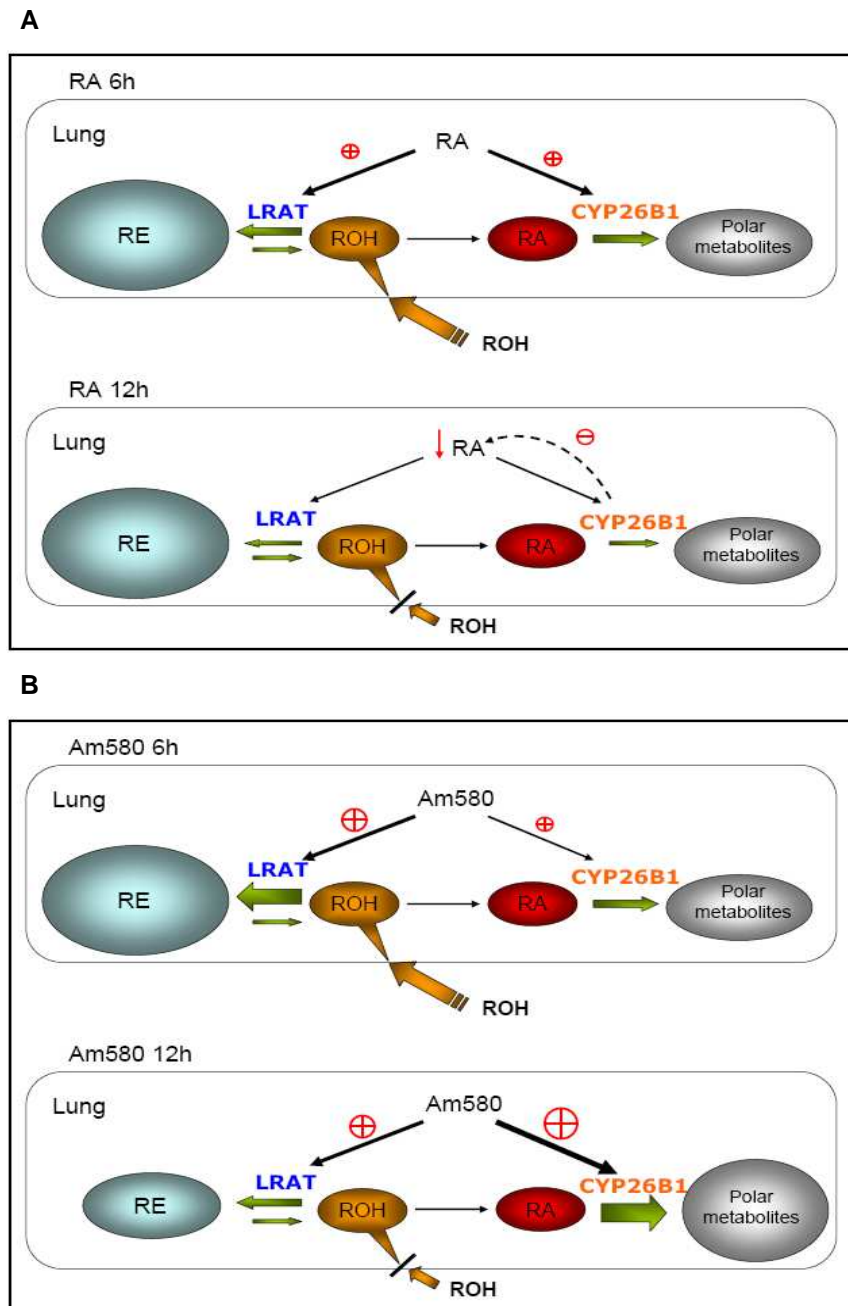


Figure 14. Model of vitamin A metabolism in neonatal rat lung. (A) When RA is administrated with retinol to neonatal rats, RA up-regulates expression of LRAT and CYP26B1 to the same extent at 6 h, the pathway flow of retinoids to RE formation or to polar metabolites is kept in balance. More dietary retinol is taken up by lung tissue. At 12 h, RA itself is metabolized by CYP26B1. Although the activity of RA has declined, the pathway balance is still kept. (B) When Am580 is administrated with retinol to neonatal rats, Am580 promotes LRAT expression higher than CYP26B1 at 6 h. Due to its resistance to CYP26B1, Am580 continues its regulatory activity, and dramatically up-regulates CYP26B1 to a higher magnitude than LRAT at 12 h. Thus, the balance of the pathway is broken and more retinoids moves in the oxidative direction.

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