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Retinoic acid reverses the airway hyperresponsiveness but not the parenchymal defect that is associated with vitamin A deficiency

Stephen E. McGowan, Amey Jo Holmes, and Jennifer Smith

Research Service, Veterans Affairs Medical Center and the Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, Iowa 52242

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McGowan, Stephen E., Amey Jo Holmes, and Jennifer Smith. Retinoic acid reverses the airway hyperresponsiveness but not the parenchymal defect that is associated with vitamin A deficiency. *Am J Physiol Lung Cell Mol Physiol* 286: L437–L444, 2004; 10.1152/ajplung.00158.2003.—Airway hyperresponsiveness (AHR) is influenced by structural components of the bronchial wall, including the smooth muscle and connective tissue elements and the neuromuscular function. AHR is also influenced by parenchymally derived tethering forces on the bronchial wall, which maintain airway caliber by producing outward radial traction. Our previous work has shown that vitamin A-deficient (VAD) rats exhibit cholinergic hyperresponsiveness and a decrease in the expression and function of the muscarinic-2 receptors (M2R). We hypothesized that if decreases in radial traction from airway or parenchymal structures contributed to the VAD-related increase in AHR, then the radial traction would normalize more slowly than VAD-related alterations in neurotransmitter signaling. Rats remained vitamin A sufficient (VAS) or were rendered VAD and then maintained on the VAD diet in the presence or absence of supplementation with all-*trans* retinoic acid (RA). VAD was associated with an approximately twofold increase in respiratory resistance and elastance compared with VAS rats. Exposure to RA for 12 days but not 4 days restored resistance and elastance to control (VAS) levels. In VAD rats, AHR was accompanied by decreases in bronchial M2R gene expression and function, which were restored after 12 days of RA supplementation. Subepithelial bronchial elastic fibers were decreased by ~50% in VAD rats and were significantly restored by RA. The increase in AHR that is associated with VAD is accompanied by decreases in M2R expression and function that can be restored by RA and a reduction in airway elastic fibers that can be partially restored by RA.

asthma; retinoids; retinol; cholinergic; muscarinic; elastin

VITAMIN A DEFICIENCY (VAD) is an important public health problem for children in developing countries, and the World Health Organization has estimated that up to 190 million children may have subclinical VAD, worldwide (40). Unlike clinical VAD, which usually manifests with ocular symptoms, subclinical VAD is asymptomatic. However, children with subclinical VAD have an increased mortality from measles, diarrheal illnesses, and possibly from acute respiratory tract infections (17, 32). Because respiratory tract infections in children may be complicated by airway hyperresponsiveness (AHR), it is possible that some of the excess mortality from respiratory infections in children with VAD relates to AHR. Vitamin A (retinol) is acquired from dietary retinol or carotenoids but undergoes oxidative conversion to

more active metabolites, retinoic acids (RA), before exerting its biological effects (10). Two isomers of RA (all *trans* and 9-*cis*) exert their biological effects by binding to retinoic acid receptors and retinoid-X receptors, respectively (23). These two receptors are members of the steroid hormone receptor superfamily and form heterodimers, which bind to RA responsive elements in numerous genes, including those that are expressed in the pulmonary epithelium and the airway wall. We have previously shown that VAD increases airway reactivity in rats and that this is associated with a decrease in airway muscarinic receptor-2 (M2R) expression and function (28). In prejunctional nerves, M2R serve as autoinhibitory receptors to limit the release of ACh from prejunctional neurons in bronchial smooth muscle (18). This results in an attenuation of vagally induced bronchoconstriction, which has been shown to be an important regulator of airway resistance during viral infections and in asthma (1, 13). M2R are also located in bronchial smooth muscle cells, where they participate in bronchoconstriction, but appear to be less influential than the muscarinic receptors-3 (5, 37, 38).

The mechanisms that are responsible for the VAD-related increased reactivity to cholinergic agents and the alteration in M2R function remain incompletely understood. They could involve a decrease in M2R gene expression, an influence of VAD on the development and maintenance of junctional cholinergic ganglia, or a combination of both. Alternatively, VAD could promote structural changes in the lung parenchyma or airway walls that may promote responsiveness to a cholinergic stimulus. Lung parenchymal or airway-wall tethering forces, referred to as airway-parenchymal interactions, may apply outward retractive forces and thereby tether the noncartilaginous airways and prevent luminal narrowing during bronchoconstriction (19, 22). Others have shown that alterations in the mechanical properties of the pulmonary parenchyma and airway wall influence airway responsiveness to cholinergic agents (2, 30). Therefore, we desired to learn whether VAD-related alterations in parenchymal and airway architecture contribute to AHR. We hypothesized that RA would more rapidly reverse the AHR if the VAD-mediated AHR resulted from a decrease in M2R gene expression than if it resulted from a more generalized lung parenchymal defect. To address this hypothesis, we have studied the effects of VAD on airway and lung parenchymal architecture and how RA supplementation reverses the structural, functional, and biochemical alterations that are observed in VAD rats.

Address for reprint requests and other correspondence: S. McGowan, C33B G. H., Dept. of Internal Medicine, Univ. of Iowa Hospitals and Clinics, 200 Hawkins Dr., Iowa City, IA 52242 (E-mail: stephen-mcgowan@uiowa.edu).

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METHODS

Preparation of VAD rats. Specific pathogen-free female Lewis rats were obtained from Harlan-Sprague Dawley (Madison, WI). All animals were maintained in HEPA-filtered cages, and sentinel animals were used to establish that the colony remained specific-pathogen free. The protocol was approved by the animal use committees at the Veterans Affairs Medical Center and the University of Iowa. The rats were weaned at *postnatal day 19* and were placed on a modified VAD diet (catalog number 96022; ICN, Aurora, OH) for 7–10 wk to achieve VAD (34). Vitamin A-sufficient (VAS) rats were littermates of the VAD animals or age-matched females purchased from Harlan-Sprague Dawley. The general health of the VAD rats was monitored, and the VAD animals were used before the onset of weight loss or keratitis. VAD was confirmed by analyzing hepatic stores of retinyl esters and plasma retinol at the time of death. Retinoids were extracted from the plasma or liver tissue as previously described (26). RA (25 μg) in safflower oil was administered orally daily for 4, 8, or 12 days to some rats to determine whether this reversed the effects of VAD on airway reactivity. Supplementation of VAD rats for 12 days is sufficient to completely restore the expression of retinaldehyde dehydrogenase, a retinoid-responsive gene product that is present in the tracheal epithelium (9).

Analysis of respiratory resistance and elastance. Lung resistance and elastance were analyzed in anesthetized, ventilated rats. Rats were anesthetized with ketamine and xylazine, a tracheostomy was performed, and a 16-gauge Teflon tracheal catheter was inserted. A femoral venous cannula was inserted to administer succinylcholine to abrogate spontaneous respiratory movements. The rats were ventilated with a volume-cycled rodent respirator (Inspra; Harvard Apparatus, Holliston, MA). Transpulmonary pressure (P_L) was measured as the difference between intrapleural (esophageal) pressure, measured using an esophageal catheter, and airway pressure, measured at the external end of the endotracheal tube. Flow was measured by a pneumotachograph attached between the mechanical ventilator and the endotracheal tube, and volume was calculated by integrating the flow. Data were acquired and sampled at 50z using an RSS 100HR Research Pneumotach system (Hans Rudolph, Kansas City, MO). This provides primary data for volume, flow, and pressure. Lung resistance (R_L) was calculated from the pressure and flow data. Lung elastance (E) was calculated from the equation $P_L = R_L Q + EV + K$, where K is a parameter reflecting the end-expiratory pressure, Q is flow, and V is volume (7). In this analysis, R and E are dependent on both airway and tissue factors. A series of measurements was made before and after delivering methacholine as an aerosol using a DeVilbiss AeroSonic ultrasonic nebulizer for 30 s. A series of aerosolizations with increasing concentrations of methacholine were delivered at 5-min intervals, and the lungs were inflated by delivering a sigh that comprised three successive tidal breaths without intervening exhalation. This counteracted the effects of atelectasis that were induced by the previous dose of methacholine (20).

Histological analyses of airway wall thickness, airway elastic fibers, and gas-exchange surface area. At the time of death, the left lung was lavaged and inflated to 20 cmH_2O pressure with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.0, and maintained at 4°C for 24 h. The fixed lungs were sliced sagittally near the hilum into 3-mm sections, dehydrated, and embedded in paraffin. The paraffin blocks were sectioned at 3.5- μm intervals and stained with hematoxylin and eosin. The sections were initially photographed using an Olympus BX40 microscope, 35-mm slides were prepared, and these were scanned using a Nikon 4000ED scanner (Melville, NY). The digitized images of the airways were analyzed using the Image-Pro Plus program [Media Cybernetics (Silver Spring, MD), kindly made available by Dr. Jeanne Snyder, Department of Anatomy and Cell Biology, University of Iowa]. The bronchi that had been transected perpendicularly to the long axis of the airway and presented a closed circle or oval profile were selected to quantify bronchial wall

thickness (4). The perimeters defined by the subepithelial basement membrane and the peripheral border of the outer wall of adventitia were traced electronically. The total bronchial wall area was calculated as the difference between the area defined by the adventitial perimeter and the area defined by the luminal perimeter (31). This analytical tool corrects for the variability in the wall thickness within a particular section. The wall area was divided by the circumference of the subepithelial basement membrane to normalize for variation in the sizes of the airways (11).

Airway elastic tissue was analyzed by staining paraffin-embedded sections with a modified Van Gieson stain (elastic stain; Sigma Chemical, St. Louis, MO). Three medium-sized, noncartilaginous, muscularized airways were analyzed in three VAS rats, three VAD rats, three VAD rats that received RA for 4 days, and three VAD rats that received RA for 12 days. Airways with an oval or circular profile (3 from each rat) were selected at random and photographed. The digitized photographic images were enlarged using Adobe Photoshop 6.0, and the brightness and contrast were adjusted to optimize visualization of the elastic fibers. The number of fibers that traversed from the epithelial basement membrane to the smooth muscle layer was enumerated for each airway independently by two observers. To adjust for airway size, the number of elastic fibers was normalized to the circumference of the subepithelial basement membrane of that particular airway.

To analyze the architecture of the gas-exchange portion of the lung, portions of the parenchyma were randomly selected and photographed, and 35-mm slides were prepared. Six photographic fields containing alveolar structures were analyzed for the right caudal lobe of each rat, and four rats were analyzed for each treatment group. The slides were scanned, digitized, uniformly enlarged, and printed. The prints were overlaid with transparent grids and used for morphometric analysis (16, 27). The volume densities of air space and tissue were determined using point counting using a 10-by-10 grid with 100 evenly spaced points, $\sim 42 \mu\text{m}$ apart. Mean cord lengths were determined by counting intersections with an array of 70 lines, each $\sim 33 \mu\text{m}$ long. The two observers were unaware of the vitamin A status and experimental treatment of the animals. The mean cord lengths and alveolar surface areas were calculated as described previously (27, 42).

Physiological assessment of M2R function. Studies were conducted to compare the sensitivity of the prejunctional M2R to pilocarpine in VAS and VAD rats following a procedure that we described previously (28). In the doses that were used (0.3–50 μg), pilocarpine acts as an M2R agonist and enhances the suppression of vagally induced bronchoconstriction (8, 36). Rats were anesthetized and placed on constant-volume mechanical ventilation, an intrapleural catheter was inserted, and the animals were then paralyzed with intravenous succinylcholine (28). Both vagal nerves were isolated, sectioned, draped over bipolar silver wire electrodes, and insulated. The sympathetic nerve activity was blocked with propranolol. Transpulmonary pressure and flow were measured, and transpulmonary resistance was calculated from the peak inspiratory pressure and flow. Both vagus nerves were stimulated with square-wave stimulation at 17 Hz (8). The optimal voltage was determined for each animal at the beginning of the experiment to give an increase in transpulmonary resistance of 0.3–0.4 $\text{cmH}_2\text{O}\cdot\text{ml}^{-1}\cdot\text{s}^{-1}$ at 17 Hz and a 0.4-ms pulse duration (35). The nerves were allowed to recover for 3 min after each stimulation. Once the optimal voltage was ascertained, two control stimulations (in the absence of pilocarpine) were performed. Next, incremental doses (ranging from 0.25 to 10 $\mu\text{g}/\text{kg}$) of pilocarpine were administered, and the resistance was determined before and after stimulation for each dose of pilocarpine. A cumulative dose-response curve was generated to identify the dose at which the maximal attenuation of the vagal response was achieved.

Immunoblotting for M2R protein. Western immunoblotting was used to analyze M2R protein in bronchial tissues from VAS and VAD rats that either had or had not received RA (28). The immunoblots

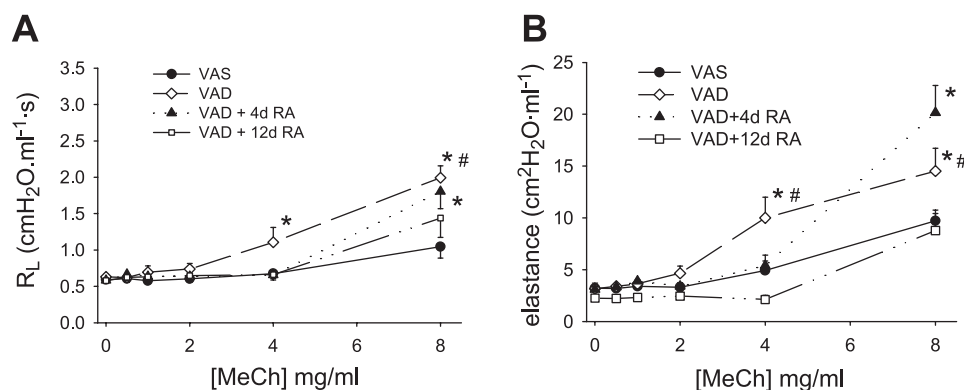


Fig. 1. Vitamin A deficiency (VAD) alters the magnitude of the methacholine-induced change in lung resistance and elastance. *A*: increase in lung resistance (R_L) produced by various concentrations of aerosolized methacholine (MeCh) in anesthetized, ventilated rats. Experimental groups containing 5 animals each were vitamin A-sufficient (VAS), untreated VAD, or VAD rats that were treated with retinoic acid (RA) for either 4 or 12 days (d). *B*: methacholine-induced increase in lung elastance in the same animals used for *A*. In *A* and *B*, data are expressed as means \pm SE. * $P < 0.05$, VAS vs. VAD or VAD + 4d RA rats at the same concentration of methacholine using a 2-way ANOVA. # $P < 0.05$, VAD vs. VAD + 12d RA.

were developed using rabbit-anti-human M2R (Research and Diagnostic Antibodies, Benecia, CA), anti-rabbit IgG-peroxidase (Roche Biochemicals, Indianapolis, IN), and enhanced chemiluminescence (Amersham-Pharmacia, Piscataway, NJ). The fluorograms were imaged using an image-processing system (Alpha Innotech, San Leandro CA), and the densities of the bands comprising the M2R protein were determined. All lanes contained the same quantity of protein, and the densities were normalized to those for a sample from one VAS rat that was included in all of the analyses.

Competitive PCR assay of M2R mRNA. An RT-based assay of M2R mRNA was developed to assess the steady-state level of mRNA in bronchial tissue from VAS and VAD rats and VAD rats that had been exposed to RA. RT was performed using the Superscript Kit (GIBCO-BRL, Grand Island, NY) and an oligo(dT) primer. Oligonucleotide primers specific for the rat M2R (forward, 5'-TAAAGTCAACCGC-CACCTTC-3' and reverse, 5'-GTCGTCCGCTTAACTGGGTA-3') were used to amplify a 266-bp cDNA. To account for differences in primer binding and amplification efficiency among samples, an M2R internal control was included. The internal control was a 174-bp segment of cDNA that contains a deletion within the region that is flanked by the primer-binding sites in the native M2R mRNA. This truncated control was obtained by RT-PCR-based cloning using the method described by Celi and associates (12). To account for minor differences in the quantities of total RNA used for the RT reactions, rat β -actin cDNA, generated by the RT reaction, was also quantified. To amplify the β -actin RT product, the forward and reverse primers were 5'-CCTTCTACAATCAGC-3' and 5'-ACGTCACACTTCA-TG-3', respectively, and yielded a 550-bp amplicon. A β -actin internal control was used, which was a 374-bp segment of cDNA that contains a deletion within the region that is flanked by the primer-binding sites in the native β -actin mRNA. PCR was conducted using a standard buffer (Boehringer Mannheim, Indianapolis, IN) containing 1.5 mM $MgCl_2$ and primers at final concentrations of 50 pM. Preliminary experiments were performed using PCR to determine the

number of cycles required to ensure that the template DNA was the limiting component of the reaction. The optimal PCR conditions for M2R amplification were as follows: initial denaturation at 94°C for 1 min, addition of *Taq* polymerase using the "hot start method," denaturation for 1 min at 93°C, annealing at 52°C for M2R or 40°C for β -actin for 1 min, extension at 72°C for 1 min, and a final extension for 10 min at 72°C. Amplifications of M2R RT product proceeded for 27 cycles, and 25 cycles were used to amplify the β -actin RT product. The PCR products were resolved on a 2% agarose gel that contained ethidium bromide and imaged using the Alpha Innotech system.

Statistical analyses. The data are expressed as means \pm SE. The resistance or elastance at each concentration of methacholine, or the change in airway resistance at each dose of pilocarpine, was compared in VAS, VAD, and VAD rats that received RA using a two-way ANOVA. The abundance of M2R proteins in the bronchial tissue or retinoids in plasma and livers of VAS and VAD rats and VAD rats that had been treated with RA were compared using a one-way ANOVA. The linear density of elastic fibers and the three parameters of gas-exchange morphology (mean chord length, volume density, and surface area) were also analyzed using a one-way ANOVA. Differences were considered significant at $P < 0.05$ (33).

RESULTS

Documentation of VAD. Vitamin A stores were evaluated by assessing the quantities of vitamin A esters in the livers of VAS and VAD rats. The liver is the major storage organ, and nearly all of the retinoids in the liver are retinyl esters. VAS and VAD rats had 605.4 ± 75.4 and 1.8 ± 0.5 (SE) nmol retinyl esters/g liver, respectively ($P < 0.01$, for VAS $n = 5$, for VAD $n = 9$). Supplementation with RA did not increase retinyl ester stores (data not shown), so data from RA-supplemented VAD rats

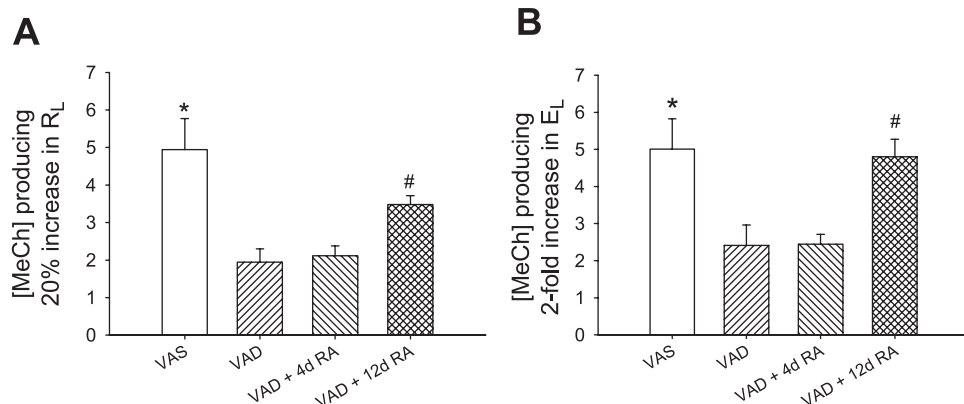


Fig. 2. VAD alters the airway sensitivity to methacholine, which is reflected in the lung resistance and lung elastance (E_L). The mean concentrations (mg/ml) of aerosolized methacholine that produced a 20% increase in resistance (*A*) or a 2-fold increase in elastance (*B*) relative to aerosolized PBS were calculated using linear regression analysis of the data obtained from the studies shown in Fig. 1. Error bars are 1 SE. * $P < 0.05$, VAS vs. VAD and VAD + 4d RA using 2-way ANOVA. # $P < 0.05$, VAD + 12d RA vs. VAD using 2-way ANOVA.

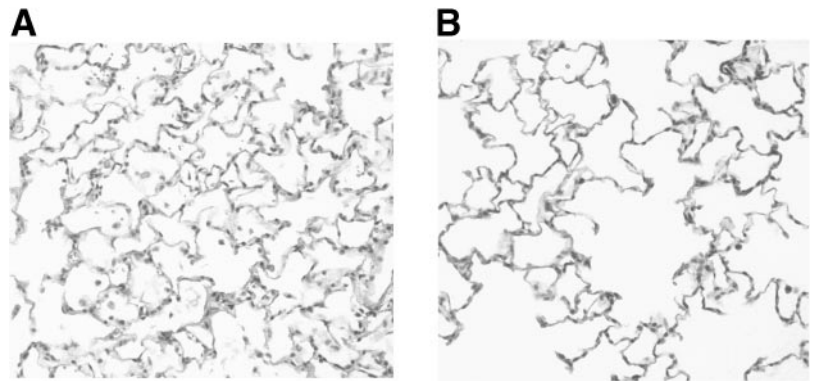


Fig. 3. Representative photomicrographs of lung parenchymal tissues from VAS (A) and VAD (B) rats at the same magnification of $\times 50$.

were combined with those from rats that were not supplemented with RA.

RA reverses the VAD-induced AHR to methacholine. When RA was administered to VAD rats, this returned the responsiveness to aerosolized methacholine to a concentration-response pattern that was similar to that for VAS rats. Rats with VAD developed a higher level of airway resistance when exposed to 4 or 8 mg/ml methacholine compared with VAS rats (Fig. 1A). Treatment with RA for 12 days restored the methacholine responsiveness to a pattern that was more similar to that of VAS rats. Treatment for 4 days did not significantly restore the resistance in VAD rats after exposure to 8 mg/ml methacholine, whereas the resistance at 4 mg/ml was similar to VAS. Lung elastance was also analyzed and was significantly increased in VAD rats in response to 4 and 8 mg/ml methacholine (Fig. 1B). Treatment of VAD rats with RA for 12 days restored the effect of methacholine on elastance to a level that was similar to that observed in VAS rats. However, after treatment for 4 days, elastance in VAD rats remained higher than in VAS rats after exposure to 8 mg/ml methacholine but was similar to VAS at 4 mg/ml. The data in Fig. 1 illustrate how RA exposure altered the magnitude of the methacholine-induced elevations in resistance and elastance. Figure 2 shows that the sensitivity to methacholine was increased by VAD and significantly restored by administration of RA for 12 days. The concentrations of methacholine that were required to produce a 20% increase in resistance and a twofold increase in elastance are shown in Fig. 2, A and B, respectively.

RA alters elastic fiber number but does not alter alveolar surface area or airway wall thickness. Because alterations in alveolar architecture can impact airway mechanics, in particular the elastance, we assessed the effects of RA supplementa-

tion on alveolar structure in VAD rats (7, 15). Representative portions of VAS and VAD lungs are shown in Fig. 3. VAD rats demonstrated a decrease in alveolar wall density, an increase in mean chord length, and a decrease in gas-exchange (includes both alveolar ducts and alveoli) surface area compared with VAS rats. The results of the analyses for four rats from each group are shown in Table 1. These VAD-related changes were not reversed by the administration of RA for up to 12 days, indicating that a restoration of alveolar structure did not account for the restoration of lung resistance and elastance that occurred after RA administration.

Elastance is influenced by both alveolar and airway parameters. We evaluated airway smooth muscle and epithelial thickness in VAS, VAD, and RA-supplemented VAD rats. The results are shown in Table 1 and demonstrate that epithelial and smooth muscle area did not vary with vitamin A nutritional status. Therefore, it is unlikely that the improvement in elastance after RA administration resulted from a change in the thickness of the airway wall. We did not rigorously evaluate the thickness of the epithelial basement membrane, but we did perform Gomori trichrome staining. This did not reveal any obvious alteration in the amount of collagen or smooth muscle. Because the area of the smooth muscle layer (which would include collagen and smooth muscle, as determined from hematoxylin and eosin staining) was not altered by vitamin A, we did not perform a morphometric analysis of the trichrome-stained sections.

We also compared the density of airway elastic fibers in VAD and VAS rats. Representative photomicrographs of muscularized airways are shown in Fig. 4. Compared with VAS rats, the muscularized airways of VAD rats contain significantly fewer elastic fibers that extend from the epithelial

Table 1. Morphometric analysis of airways and alveoli of VAS and VAD rats

Parameter	Vitamin A Status				
	VAS	VAD	VAD + 4d RA	VAD + 8d RA	VAD + 12d RA
Epithelial area/circumference	7.23 \pm 0.46(3)	6.33 \pm 0.23(3)	6.79 \pm 0.33(2)	5.96 \pm 0.30(2)	
Smooth muscle area/circumference	1.12 \pm 0.43(3)	1.11 \pm 0.43(3)			
Elastic fibers/mm subepithelial basement membrane	206 \pm 21(3)	106 \pm 9†(3)	118 \pm 15†(3)		170 \pm 10‡(3)
L_m (4)	36.3 \pm 0.8*	50.9 \pm 2.4	50.2 \pm 2.1	44.7 \pm 1.9	47.4 \pm 1.1
V_d alveolar wall (4)	0.35 \pm 0.01*	0.23 \pm 0.01	0.24 \pm 0.01	0.24 \pm 0.02	0.25 \pm 0.02
Alveolar surface area (4)	378.2 \pm 20.7*	194.5 \pm 16.1	198.1 \pm 16.4	227.5 \pm 27.2	218.2 \pm 16.6

Data are means \pm SE; no. of animals in parentheses. VAS, vitamin A sufficient; VAD, vitamin A deficient; d, day; RA, retinoic acid; L_m , mean chord length; V_d , volume density. * $P < 0.01$, VAS vs. VAD, VAD + 4d RA, or VAD + 12d RA. † $P < 0.01$, VAD or VAD + 4d RA vs. VAS. ‡ $P < 0.05$, VAD + 12d RA vs. VAD or VAD + 4d RA.

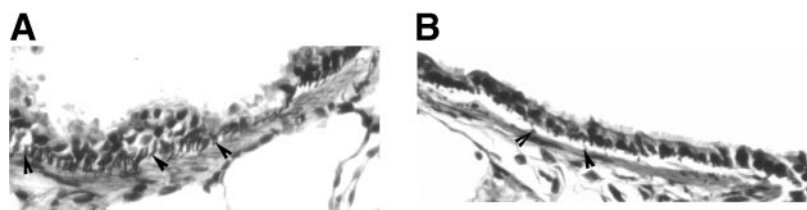


Fig. 4. Representative photomicrographs of airways from VAS (A) and VAD (B) rats. A modified Van Gieson stain was used to allow visualization of elastic fibers that connect the basal epithelial layer to the smooth muscle. Magnification is $\times 100$.

basement membrane to the smooth muscle (Table 1). There was no significant restoration of elastic fibers after 4 days of exposure to RA. However, exposure for 12 days resulted in a significant, although partial, restoration in the number of elastic fibers. Therefore, a decrease in subepithelial elastic fiber density is a potential explanation for the alteration in AHR in VAD rats. Because our previous studies showed that VAD is associated with an alteration in the function of airway M2R, we also analyzed how RA altered the expression of the M2R (28).

RA reverses the VAD-induced decrease in M2R protein and mRNA. VAD resulted in a decrease in M2R expression that was evident as a decrease in both protein and mRNA. A representative Western immunoblot is shown in Fig. 5. Treatment with RA for 12 days, but not 4 days, completely restored the quantity of M2R protein in VAD airway to a level that is comparable to that found in VAS rats. Figure 6 shows the combined results from the Western analyses of three samples in each treatment group. Three separate gels were analyzed, each of which contained one sample from each treatment group. These data indicate that exposure to RA for 4 days was insufficient to completely restore the M2R protein to a level that is comparable to that observed in VAS rats. VAD was also associated with a decrease in M2R mRNA, which was restored by treating VAD rats with RA for 12 days. A representative competitive PCR analysis for M2R and β -actin mRNA, from the bronchus of a VAD rat, is shown in Fig. 7. Figure 8 shows the combined results from the analysis of M2R and β -actin mRNA from VAS rats, VAD rats, and VAD rats that received RA. Similar to the observation from Western analysis, exposure to RA for 4 days did not fully restore M2R mRNA to the level that is observed in VAS rats. These results indicate that retinoids have a pretranslational effect on M2R gene expression in the bronchial wall. However, these biochemical analyses do not distinguish between M2R expression in prejunctional nerves and M2R expression in the bronchial smooth muscle. Therefore, we assessed prejunctional nerve M2R function physiologically.

RA restores the responsiveness of M2R to pilocarpine. To assess M2R function, we evaluated the ability of pilocarpine to decrease the airway responsiveness to vagal stimulation. Pilocarpine stimulates M2R activity and lessens the incremental increase in airway resistance that is observed after vagal

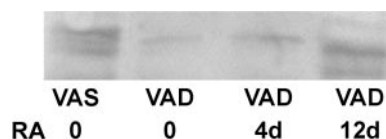


Fig. 5. Representative immunoblot for muscarinic receptor-2 (M2R). Bronchial proteins from VAS and VAD rats (that did not receive RA, 0) and from VAD rats that were supplemented for 4 or 12 days with RA were subjected to immunoblotting. Equal quantities of protein were loaded in all lanes. M2R migrated at ~ 65 kDa.

stimulation (8). Therefore, in situations where M2R autoinhibitory activity is high, pilocarpine will produce a larger lowering of resistance after vagal stimulation than when the autoinhibitory activity is low. As we have shown previously, VAD produces a decrease in pilocarpine responsiveness with a significantly higher resistance at $19 \mu\text{g/kg}$ pilocarpine (Fig. 9 and Ref. 28). A higher concentration ($38 \mu\text{g/kg}$) of pilocarpine reduced resistance after vagal stimulation in VAD rats. These data indicate that VAD lowers the sensitivity to pilocarpine, consistent with a reduction in M2R function. RA administration for 4 days did not restore the responsiveness to pilocarpine to a level that was similar to that observed in VAS rats. In contrast, treatment for 12 days completely restored the responsiveness to pilocarpine to a level that is similar to that observed in VAS animals. The time course of the restoration of M2R function is similar to that observed for the restoration of the steady-state levels of M2R mRNA and protein.

DISCUSSION

Our findings provide insights into the mechanisms that may be responsible for our previous observation that VAD influences airway reactivity to cholinergic agents and decreases M2R function. Administration of RA for 12 days restores M2R expression in bronchial tissue, prejunctional M2R function, and pulmonary elastance and resistance to the levels that are observed in VAS rats. Although bronchial epithelial and smooth muscle thickness were not affected by retinoid status, VAD was associated with a significant decrease in subepithelial elastic fibers. The administration of RA did not alter gas-exchange surface area or airway wall density; however, it did significantly replete subepithelial airway elastic fibers. Therefore, alterations in M2R expression and airway wall compliance may contribute to the cholinergic hyperresponsiveness in VAD.

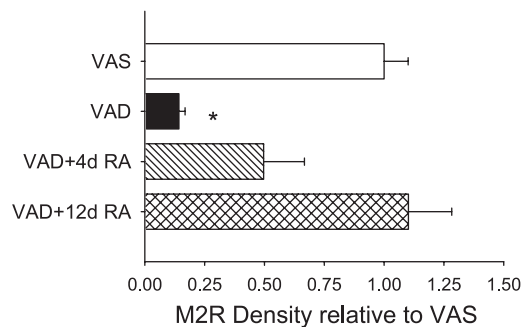


Fig. 6. Summary of data from three M2R immunoblots. Bronchial proteins from rats (3/treatment group) that were VAS, VAD, or VAD and treated with RA for 4 or 12 days were subjected to Western immunoblotting. Bars show the mean and brackets one SE of densities for M2R protein. * $P < 0.05$ compared with VAS by one-way ANOVA on ranks, Dunn's test.

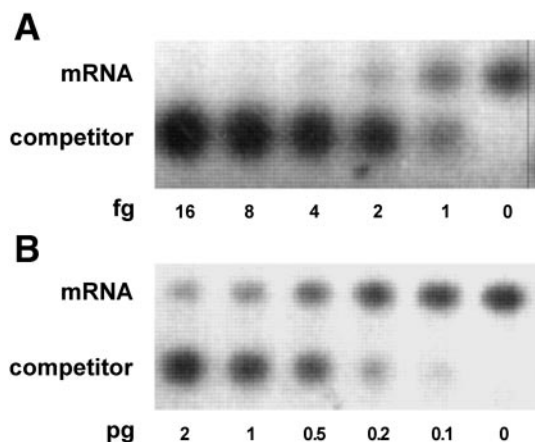


Fig. 7. Representative competitive PCR assay of M2R and β -actin mRNA. RNA was extracted from bronchial tissues of a VAD rat. The PCR reactions (using the same amount of RT product in all lanes) were performed in the presence of decreasing amounts of cDNA competitor that was amplified using the same primers that were used to amplify the RT mRNA. The quantity of competitor used is shown in fg (A) or pg (B) below each gel. The native M2R PCR product migrated at 266 bp, and the truncated competitor M2R cDNA migrated at 174 bp. The native β -actin PCR product migrated at 550 bp, and the truncated competitor β -actin cDNA migrated at 374 bp.

To our knowledge, these studies are the first to demonstrate that VAD results in a decrease in alveolar surface area (Table 1). Others have found that VAD is associated with patchy air space enlargement in rats, but morphometric analyses were not performed to define the magnitude of the histological differences (6, 41). Another novel conclusion from our data is that retinoids are not only required for normal alveolar development, but they are also required for maintenance of alveolar architecture after the alveoli have formed. Because our animals were not VAD during the period when most alveoli form (before *day 28*) it appears that deficiency influences events that occur beyond the period of maximal alveolar septation. Others have suggested that alveolar surface area in rats and mice increases after *day 28*, primarily by the enlargement of previously formed alveoli. Our data indicate that alveolar wall volume density is decreased in VAD lungs compared with equally inflated VAS lungs (Table 1). This suggests that VAD alters the volume density of alveoli and alveolar ducts through

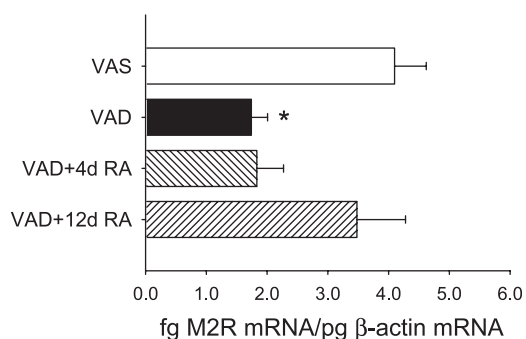


Fig. 8. Summary of data from M2R competitive PCR. RNA from bronchial tissues of rats that were VAS ($n = 4$), VAD ($n = 4$), or VAD and treated with RA for 4 ($n = 3$) or 12 ($n = 4$) days. The RNA was subjected to RT-PCR as described in the legend to Fig. 7. The quantity of M2R mRNA was normalized to the quantity of β -actin mRNA in the same sample. Bars show the mean and brackets the SE. * $P < 0.05$ compared with VAS by 1-way ANOVA, Student-Newman-Keuls test.

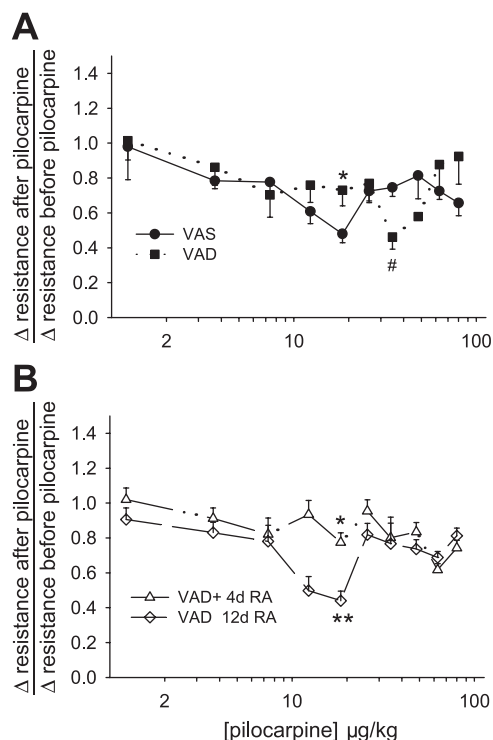


Fig. 9. Mean attenuation of vagal-mediated bronchoconstriction in response to various concentrations of pilocarpine in rats that were VAS (\bullet , $n = 4$) or VAD (\blacksquare , $n = 4$) or VAD rats that were exposed to RA for 4 days (VAD + 4d RA, \triangle , $n = 3$) or 12 days (VAD + 12d RA, \diamond , $n = 3$). The attenuation (ordinate scale) is expressed as a ratio of the increase in airway resistance after vagal stimulation, after pilocarpine administration, relative to the increase that was observed before pilocarpine administration. The abscissa shows the cumulative dose of pilocarpine. Error bars are 1 SE. $P < 0.05$, 1-way ANOVA, comparing VAS with VAD (#); VAS with both VAD or VAD + 4d RA (*); or VAD with VAD + 12d RA (**).

events that occur after the maximal period of septation, which is completed by 21 days. It is unclear how VAD alters the structure of alveoli and alveolar ducts after the period of maximal alveolarization.

VAD has important physiological consequences regarding airway reactivity to a cholinergic stimulus. We have not observed a difference in resistance or elastance in the absence of methacholine in VAD rats compared with VAS rats (Fig. 1). Therefore, it is unlikely that VAD-related structural changes in the lung parenchyma are solely responsible for our observations. Similarly, VAD did not increase the area of the epithelial or smooth muscle layers (Table 1), so it is unlikely that anatomic changes in the airways are solely responsible for the AHR. By altering M2R expression and function, retinoids may alter the intensity (as shown in Fig. 1) of the contraction that is induced by the cholinergic agent, which would in turn alter the degree of smooth muscle contraction.

The methacholine concentration-related increases in resistance and elastance, which are shown in Figs. 1 and 2, demonstrate that there is an increase in the sensitivity to and the response from the bronchoconstrictor at 8 mg/ml in the VAD rats. This suggests that there may be more than one mechanism that accounts for the VAD-related increase in responsiveness to methacholine. Sterk and Bel (39) have distinguished between prejunctional and postjunctional mechanisms for bronchial hyperreactivity (39). Prejunctional mechanisms include,

among others, an increase in the access of the constrictor stimulus to the neuromuscular junction or an increased sensitivity of the neuromuscular junction to the cholinergic stimulus. These account for a leftward shift in the dose-response curve, which signifies that lower concentrations of the constrictor are required to increase resistance (Fig. 2). An increase in the resistance and elastance at 8 mg/ml methacholine in VAD rats suggests that this concentration of methacholine produces a stronger bronchial muscular contraction in VAD. Sterk and Bel (39) suggested that this stronger contraction reflects postjunctional mechanisms, such as increased smooth muscle contractility, increased wall thickness, or decreased tethering by the pulmonary parenchyma. Our data indicate that VAD rats have a decrease in airway elastic fibers and a decrease in alveolar wall density, which could both contribute to a reduction in the restraint by parenchymal tethering effects on bronchial muscle contraction.

Consistent with our previous studies, we observed a decrease in M2R mRNA and protein in bronchial tissue from VAD rats (Figs. 5–8). Supplementation with RA for 4 days did not fully restore the mean levels of M2R mRNA and protein. Further increases occurred by extending the RA treatment to 12 days, which restored the mean levels to those observed in VAS rats. Our previous studies have shown that VAD does not alter the amount of muscarinic receptor-3 protein in bronchial tissue, indicating that the effect of VAD on M2R is not generalized to another muscarinic receptor that is expressed in the bronchi (28). Because these analyses involved tissues from the entire bronchus, one cannot distinguish M2R gene expression in the prejunctional nerves from that in the postjunctional smooth muscle. However, the studies (Fig. 9) of the pilocarpine responsiveness of vagally induced bronchoconstriction specifically target the prejunctional nerves, suggesting that RA supplementation alters prejunctional M2R expression.

Our data indicate that the defect in M2R function is likely not the only abnormality that is responsible for the increase in methacholine responsiveness that is observed in VAD rats. The vitamin A-related differences in the density of subepithelial elastic fibers, which we observed, probably also contribute to differences in the airway wall elastance. Others have described two populations of airway elastic fibers. The first, termed superficial, is decreased in severe asthma and is located immediately beneath the epithelial basement membrane and bridges between the epithelium and the underlying smooth muscle (22, 25). The second is intermingled within the smooth muscle, extends to the adventitia, and is thereby probably connected to the elastic fibers in the surrounding alveolar walls. The diminution in superficial elastic fibers and the attendant decrease in airway elastance are associated with an increase in methacholine responsiveness. Others have used excised airways that are free from surrounding lung tissue to demonstrate that ACh-induced airway narrowing is influenced by tethering forces that are applied to the airway epithelial and smooth muscle layers (29, 30). Our observation that VAD is associated with a decrease in the number of subepithelial elastic fibers suggests that elastic loading may be decreased in VAD bronchi. This may contribute to the AHR by decreasing the forces that exert outward traction on the noncartilaginous airways (24). Others have shown that a decrease in the elastic load correlates with an increase in the velocity of airway narrowing in response to

methacholine, suggesting that the airway responsiveness is influenced by the elastic impedance (14).

Although VAD is not a public health problem in the United States, asthma remains an important cause of mortality among children and young adults. AHR is a hallmark of asthma, and asthma may be affected by nutritional factors (21). Others have recently reported that the severity of asthma is negatively correlated with the plasma level of retinol in children, and children who were deficient had more frequent and severe exacerbations (3). Therefore, understanding the effects of retinoids on lung structure and AHR may provide novel insights into the mechanisms of AHR in asthma that occur in the absence of clinical or subclinical VAD.

GRANTS

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