Vitamin A deficiency promotes bronchial hyperreactivity in rats by altering muscarinic M\textsubscript{2} receptor function

STEPHEN E. McGOWAN,\textsuperscript{1,2} JENNIFER SMITH,\textsuperscript{2} AMEY JO HOLMES,\textsuperscript{1} LORI A. SMITH,\textsuperscript{2} THOMAS R. BUSINGA,\textsuperscript{2} MARK T. MADSSEN,\textsuperscript{3} ULLA C. KOPP,\textsuperscript{1,2} AND JOEL N. KLINE\textsuperscript{2}

\textsuperscript{1}Department of Veterans Affairs Research Service and Departments of \textsuperscript{2}Internal Medicine and \textsuperscript{3}Radiology, University of Iowa College of Medicine, Iowa City, Iowa 52242

Received 10 August 2001; accepted in final form 25 November 2001

McGowan, Stephen E., Jennifer Smith, Amey Jo Holmes, Lori A. Smith, Thomas R. Businga, Mark T. Madsen, Ulla C. Kopp, and Joel N. Kline. Vitamin A deficiency promotes bronchial hyperreactivity in rats by altering muscarinic M\textsubscript{2} receptor function. Am J Physiol Lung Cell Mol Physiol 282: L1031–L1039, 2002. First published December 7, 2001; 10.1152/ajplung.00319.2001.—Vitamin A deficiency (VAD) remains an important health problem among children in developing countries. Children living in these areas have a higher mortality from respiratory infections, which likely results in part from suboptimal nutrition, including VAD. Bronchial hyperreactivity can follow viral respiratory infections and may complicate the recovery. To investigate whether VAD promotes bronchial hyperreactivity, we have assessed methacholine-induced bronchoconstriction in VAD and vitamin A-sufficient rats. Bronchial constriction developed at lower concentrations of inhaled methacholine in VAD than in vitamin A-sufficient rats. This did not result from an increase in the bronchial wall thickness or the clearance of a small molecule (with a size similar to methacholine) from the air space. The function and abundance of the muscarinic M\textsubscript{2} receptors in bronchial tissue were reduced in VAD rats, suggesting that this receptor may contribute to these animals’ diminished ability to limit cholinergic-mediated bronchoconstriction. A similar reduction in muscarinic M\textsubscript{2} receptor function has been observed in asthma. Vitamin A (retinol) and its congeners (retinoids) may be required to regulate bronchial responsiveness in addition to maintaining a normal bronchial epithelium.

VITAMIN A DEFICIENCY (VAD) is still an important cause of childhood disease in the developing world, and the estimated annual worldwide attributable mortality is 200,000. Although VAD most commonly produces visual abnormalities, it may also contribute to respiratory illnesses. Vitamin A (retinol) is required for the maintenance of airway epithelial cells and their repair after damage (40). In individuals with VAD, development of squamous cell metaplasia, a decrease in ciliated and secretory respiratory epithelial cells, and a replacement by keratinized epithelial cells in the tracheobronchial tree have been reported; these results have also been observed in rodent models of VAD (8, 51). These changes appear to result from a decrease in differentiation of cells in the basal cellular layers to form function-specific ciliated or secretory cells (17). All these pathological changes can be reversed by the administration of retinol (vitamin A) or its active metabolite retinoic acid (8). VAD increases the morbidity and mortality from some childhood infections, such as measles and infectious diarrhea, which may be largely related to defective epithelial barrier functions (40). VAD also increases the incidence and morbidity of childhood respiratory illnesses (45). Because vitamin A supplementation during hospitalization has not reversed mortality during acute pneumonia, the deficient state could contribute to mortality from more protracted consequences of respiratory illnesses (31). This would be consistent with a reduction in the ability of an altered epithelial surface to recover normal function after injury (26).

Prolonged bronchial hyperreactivity (BHR) is one protracted consequence of acute respiratory tract infection. It is particularly important in individuals with asthma, but it is also apparent in individuals without asthma (49). BHR can follow infection of the lower airways (respiratory syncytial virus-induced bronchiolitis) or the rhinitis and pharyngitis that are produced by rhinovirus. We hypothesize that VAD could contribute to excess morbidity and mortality from respiratory tract infection by enhancing or prolonging BHR. To begin to address this hypothesis, we studied rats with VAD, in the absence of respiratory infection, to determine whether the VAD itself is associated with BHR.

Others have demonstrated that a decrease in muscarinic receptor function contributes to BHR during response to a sensitizing antigen or after viral respiratory infection (1). The muscarinic M\textsubscript{2} receptor (M\textsubscript{2}R) is an important regulator of airway resistance, and its function is altered by a variety of inflammatory conditions. M\textsubscript{2}R act as autoreceptors and suppress muscarinic signaling after cholinergic-mediated contraction of airway smooth muscle by diminishing the release of acetylcholine from prejunctional vesicles in the smooth

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
muscle (9). This limits the availability of acetylcholine, which can stimulate postjunctional muscarinic M3 receptors (M3R) on bronchial smooth muscle cells and induce airway smooth muscle contraction. M3R function is diminished in asthma, which results in a decrease in basal airway caliber and hyperresponsiveness to bronchoconstrictors (14).

The following studies demonstrate that rats with VAD are more responsive to methacholine, a muscarinic agonist, than rats that were maintained on a vitamin A-sufficient (VAS) diet. In accordance with this finding, rats with VAD demonstrate a decrease in bronchial M3R protein and function.

METHODS

Preparation of VAD rats. Specific-pathogen-free female Sprague-Dawley or Lewis rats were obtained from Harlan Sprague Dawley (Madison, WI) or were derived from breeding in the Animal Care Unit at the Department of Veterans Affairs Medical Center (Iowa City, IA). All animals were maintained in HEPA-filtered cages, and sentinel animals were used to establish that the colony remained specific-pathogen free. The rats were weaned at postnatal day 19 and placed on a modified VAD diet (catalog no. 96022, ICN, Aurora, OH) for 7–10 wk to achieve VAD (44). VAS rats were littermates of the VAD animals or were age-matched animals that were 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 euthanasia. Retinoids were extracted from the plasma or liver tissue as previously described (27). The extraction was done under yellow light to minimize photoisomerization, and the samples were sealed under argon and stored at −20°C until they were analyzed by HPLC. Retinol and retinyl esters were separated on a Waters Resolve C18 column using a continuous gradient of 85% acetonitrile-15% H2O (A) to 80% acetonitrile-20% dichloroethane-0.1% cyclohexene (B) over 15 min at a flow rate of 1.5 ml/min (15). The mobile phase was held at 100% B for 10 min and then returned to 100% A for the final 8 min to reequilibrate the column. The absorbance at 325 nm was analyzed using a Dynamax UV-M detector and compared with retinol, retinyl palmitate, and retinyl acetate standards (Sigma Chemical, St. Louis, MO).

Analysis of BHR. Unanesthetized rats were placed in a barometric whole body plethysmograph (Buxco Electronics, Sharon, CT) that also served as an exposure chamber. The signal from Buxco pressure transducers was conditioned by a Buxco Max II converter demodulator, and the digital output was analyzed using the Buxco Biosystem XA SFT3812 software. The plethysmograph was calibrated with an injection of air that approximated the tidal volume of the rats (1.5 ml). These changes were continuously monitored. These changes resulted from pressure fluctuations caused by compression of the air within the chamber during respiration. A pneumotachograph in the wall of the chamber served as a low-pass filter and allowed for thermal compensation. The pressure signal from the chamber was used to continuously define the duration of inspiration and expiration, the maximal and minimal pressures, and the pressures during early and late expiration. These parameters were used to calculate the enhanced pause (Penh), which has been described by Hamelmann et al. (19). The Penh combines several parameters, including the peak inspiratory and expiratory pressures, the duration of expiration, and a timing comparison of early and late expiration, termed the pause. The Penh has been shown to increase during airway obstruction that is induced by cholinergic stimuli (19). Increasing concentrations (0–100 mg/ml in 0.145 M NaCl, 0.0015 M KH2PO4, 0.0027 M KCl, 0.0086 M Na2HPO4, pH 7.4, PBS) of methacholine aerosol (methacholine chloride; Sigma Chemical) were delivered by an ultrasonic nebulizer (model 99, DeVilbiss, Somerset, PA). The aerosol was delivered into the chamber for 3 min, and, immediately thereafter, pressure was measured over a 3-min period and the rat was exposed to air in the absence of aerosol. After whole body plethysmography, the rats were returned to their cages until they underwent analysis of bronchial-alveolar clearance of 99mTc-labeled diethylentriaminepentaacetic acid (DTPA). 99mTc-DTPA clearance from the air space. DTPA has been used to assess paracellular permeability of the respiratory epithelium (24, 30). 99mTc-pertechnetate was obtained from the Nuclear Medicine Department of the Veterans Affairs Medical Center and was used to label DTPA in a kit preparation (Malinkrodt, St. Louis, MO). This produces an ~400-Da radiolabeled particle that is used clinically for pulmonary ventilation scanning. 99mTc-DTPA (0.2 mCi) was administered as an intratracheal bolus in 400 μl of PBS to lightly anesthetized rats. The rats were allowed to breathe spontaneously for 1 min while being held in a vertical position with rotation to enhance the uniform delivery to all five lobes. The animals were then deeply anesthetized with ketamine (80 mg/kg) and xylazine (15 mg/kg) and mechanically ventilated with a tidal volume of 8 ml/kg at 80 breaths/min. The rats were imaged with a Siemens gamma-scintillation camera using a high-resolution collimator with a spatial resolution of ~5 mm. Sequential images were acquired at 20 s/frame continuously over a 45-min period. At 30 min, a single injection of 0.2 mCi of 99mTc-DTPA was administered into the tail vein, and the clearance from the lung was analyzed again to calculate the effects of recirculation of the 99mTc on the clearance of the isotope from the air spaces (25). The radioactivity as a function of time in selected areas of the rat was obtained from regions of interest drawn on the images. Accumulation and clearance of radioactivity in the thorax, lungs, kidneys, and bladder were monitored. Clearance values from the lungs were calculated and corrected for the radioactive decay of the 99mTc that occurred during the course of the experiment (25).

Histological analyses of airway wall thickness. At the time of euthanasia, the left lung was lavaged and inflated with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.0, for 6 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, and 35-mm slides were prepared and scanned using a Nikon scanner. The digitized images were analyzed using the Image-Pro Plus program (Media Cybernetics, Silver Spring, MD). The bronchi that presented a closed circle or oval profile were selected to quantify bronchial wall thickness (2). The airways measured using the subepithelial basement membrane and the peripheral border of the outer wall of the 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 (33). This analytic 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 (6).

Physiological assessment of M2R function. Studies were conducted to compare the sensitivity of the M2R to pilocarpine in VAS and VAD rats. In the doses that were used (0.3–50 μg), pilocarpine acts as an M2R agonist and enhances the suppression of vagally induced bronchoconstriction (5, 47). Rats were anesthetized with urethane (1.2 g/kg ip), and the femoral vein and artery and the trachea were cannulated. The animals were placed on constant-volume mechanical ventilation using a minute ventilation of 525 ml/kg and a rate of 60 breaths/min. An intrapleural catheter was placed through the right anterior thoracic wall, and the animal was paralyzied with succinylcholine (80 mg/kg). Both vagal nerves were isolated, sectioned, draped over silver electrodes, and insulated with orthodontic cement. The sympathetic nerve activity was blocked with propranolol. Transpulmonary pressure was measured using a variable-reluctance differential pressure transducer (model DP45-28, Validyne) sampling the pressures on the inspiratory side of the ventilator circuit and the intrapleural pressure. Inspiratory flow was measured using a pneumotachometer (model 8430, Hans Rudolph; flow range 0–3 l/min) and a differential pressure transducer (model DP103-12, Validyne) sampling pressures on both sides of the pneumotachometer screen. The signals for transpulmonary pressure, inspiratory flow, blood pressure, and heart rate were conditioned by amplifiers (Grass, Quincy, MA) and sent to the polygraph (model 7D, Grass). Transpulmonary resistance was calculated from the peak inspiratory pressure and flow. Both vagus nerves were stimulated with square-wave stimulation using a stimulator (model S88, Grass). The optimal voltage and frequency were determined for each animal at the beginning of the experiment to give an increase in transpulmonary resistance of 0.3–0.4 cmH2O·ml–1·s (46). Voltage and frequency were usually 15–25 V and 15–20 Hz, respectively, with a 0.4-ms pulse duration. The nerves were allowed to recover for 3 min after each stimulation. Once the optimal stimulatory parameters were ascertained, two control stimulations (in the absence of pilocarpine) were performed. Then incremental doses (0.25–10 μg/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. Two series of experiments using VAD rats from different litters were performed, and each series included four VAS and four or five VAD rats. Two VAS and one VAD rat were subjected to the same stimulation protocol but were not treated with pilocarpine to establish that resistance did not decrease over time with successive stimulations.

Immunoblotting for M2R and M3R. Western immunoblotting was used to analyze M2R protein in bronchial tissues from VAS and VAD rats. Immediately after euthanasia, the trachea and distal lung tissues were removed and cooled on ice, and the large bronchi were teased away from the surrounding lung tissue with a scalpel. Although care was taken to isolate the bronchial tissue, some surrounding lung tissues and vasculature remained. However, M2R is limited to the airway smooth muscle and glands; thus the surrounding alveolar tissues should contain very little M2R. The tissue was frozen in liquid N2 and maintained at –80°C until used. While still frozen, the bronchial tissue was homogenized in 50 mM Tris·HCl, pH 8.0, 10 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and 1% Triton X-100 and held on ice for 30 min. After centrifugation, the protein content of the supernatant was assayed, and a portion was reduced with dithiothreitol, alkylated with iodoacetamide, and subjected to electrophoresis on a 7.5% acrylamide reducing gel. Proteins were transferred to nitrocellulose, and the immunoblots were developed using rabbit anti-human M2R (Chemicon, Temecula, CA) and anti-rabbit IgG-peroxidase (Roche Biochemicals, Indianapolis, IN) and enhanced chemiluminescence (Amersham-Pharmacia, Piscataway, NJ). The same tissues and procedures were used to analyze M3R protein by immunoblotting. The anti-M3R antibody was obtained from Biogenesis (Poole, England, UK) or Research and Diagnostic Antibodies (Bencica, CA).

To demonstrate that the antibodies were specific for their intended protein targets, blocking peptides were used. These were incubated with their respective antibodies before the antibody was added to the immobilized bronchial proteins. The peptides were unique to M2R or M3R and were immunizing peptides that were used to make the anti-M2R and anti-M3R antibodies, respectively. The conditions for the SDS-PAGE and immunoblotting were identical to those described above.

Statistical analyses. Values are means ± SE. The Penh at each concentration of methacholine or the change in airway resistance at each dose of pilocarpine was compared in VAS and VAD rats using a one-way analysis of variance (ANOVA). The abundance of M2R and M3R proteins in the bronchial tissue or retinoids in plasma and livers of VAS and VAD rats was compared using Student’s t-test for unpaired variables. Differences were considered significant when P < 0.05 (38).

RESULTS

Demonstration of VAD. By weaning rats onto a VAD diet at postnatal day 19, we were able to achieve a VAD state by approximately postnatal week 9 in Lewis rats and by postnatal week 12 in Sprague-Dawley rats. Sprague-Dawley and Lewis rats were evaluated to establish whether BHR was limited to a particular strain of rats or whether it was evident in outbred (Sprague-Dawley) and inbred (Lewis) strains. VAD was confirmed by analyzing the retinol and retinyl ester content of the plasma and liver after the physiological measurements were conducted. The retinyl ester content of the liver of Sprague-Dawley rats was 1,748.8 ± 289.9 and 25.8 ± 4.9 (SE) nmol/g for VAS and VAD animals, respectively (n = 6). Similar determinations were made using Lewis rats, and the results are shown in Table 1. The levels of retinyl esters in the liver were lower in VAS Lewis than in VAS Sprague-Dawley rats and are consistent with the findings of others (10, 37). This is consistent with the reduced duration of administration of the VAD diet that is required to achieve VAD in Lewis compared with Sprague-Dawley rats. These data also indicate that the VAD animals achieved a VAD state.

Table 1. Retinoids in plasma and liver from Lewis rats

<table>
<thead>
<tr>
<th>Vitamin A Status</th>
<th>Plasma Retinol, nmol/ml</th>
<th>Liver Retinyl Esters, nmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAD</td>
<td>23.43 ± 2.48 (10)</td>
<td>414.20 ± 47.82 (12)</td>
</tr>
<tr>
<td>VAS</td>
<td>1.30 ± 0.19 (10)</td>
<td>2.85 ± 0.58 (6)</td>
</tr>
</tbody>
</table>

Values are means ± SE of number of rats in parentheses. VAD, vitamin A deficient; VAS, vitamin A sufficient.
BHR in unanesthetized animals. The assessment of BHR in unanesthetized animals allows us to evaluate the response to methacholine in rats that could also be used for studies of $^{99m}$Tc-DTPA clearance. The Penh has been used by others to evaluate airway obstruction and is an accurate and useful tool to assess serial changes in airway function during a bronchial challenge protocol in small animals (11, 12, 35). Our data indicate that this parameter increases after methacholine administration in a concentration-dependent manner. The results from studies using Sprague-Dawley (Fig. 1) and Lewis (Fig. 2) rats are shown and in both cases demonstrate that Penh increases to a greater extent at a lower concentration of methacholine in VAD than in VAS rats. The differences are significant (1-way ANOVA and Student Newman-Keuls post hoc test). The results are representative of the three studies that were performed using Sprague-Dawley rats and three studies using Lewis rats.

$^{99m}$Tc-DTPA clearance from the air spaces. A disruption of the bronchial and alveolar epithelial barrier function by VAD could increase the delivery of methacholine that has been deposited to the subjacent smooth muscle. The molecular mass of DTPA (395 Da) is twice that of methacholine. Therefore, changes in barrier properties that increase the clearance of DTPA from the air space should also increase the clearance of methacholine. Analysis of DTPA clearance in a Sprague-Dawley rat (Fig. 3) indicates that the rate of clearance is slightly higher from the left than from the right lung. There was not a consistent difference between the clearances from the two lungs of a particular rat, so the clearance from the area subtending both lungs was used for comparisons of VAS and VAD animals. The amount of radioactivity that was initially present in the right lung was usually greater than that in the left lung, consistent with a greater proportion of ventilation provided by the right than by the left lung. The combined data from two experiments conducted in six VAS and six VAD rats (Fig. 4) indicate that the
clearance of $^{99m}$Tc-DTPA in VAD rats was decreased rather than increased. When data from three experiments were combined, the percentage of the initial $^{99m}$Tc that is cleared per minute was $0.992 \pm 0.094$ and $0.51 \pm 0.089$ (mean ± SE, $P < 0.05$, 2-way ANOVA) for VAS and VAD, respectively. Therefore, an increase in the transfer of methacholine through the epithelial cell layer is unlikely to account for the increase in bronchial responsiveness that is observed in VAD rats.

**Analysis of bronchial diameter in VAD and VAS rats.** Because no vitamin A-related differences in DTPA clearance were observed, other parameters were analyzed to determine whether they could account for the increased BHR in VAD rats. Others have demonstrated that bronchial diameter is decreased during inflammatory conditions that produce BHR in rats and in humans with asthma (33, 48). Bronchial diameter was analyzed using morphometric techniques and was not significantly altered by VAD. The ratio of bronchial wall area to circumference of the epithelial basement membrane was calculated: 3.78 ± 0.16 and 3.61 ± 0.22 (mean ± SE, not significant, unpaired t-test) for VAS (19 airways from 5 animals) and VAD (18 airways from 5 animals), respectively. Thus a decreased basal bronchial diameter, which contributes to an increased airway resistance, did not account for the greater responsiveness to methacholine that was observed in VAD rats.

**Analysis of $M_2R$ function.** Using several different rodent models that produce BHR, others observed that a decrease in inhibitory $M_2R$ function correlates with an increased responsiveness to methacholine (46). Therefore, we analyzed $M_2R$ function using a protocol similar (including stimulus voltage, frequency, and duration) to that used by others to study rats (5). We analyzed changes in resistance (which combined changes in pressure and flow), rather than only changes in pressure. This increased the sensitivity of our ability to detect the small changes in airway dynamics that are produced during these experimental manipulations. The magnitude of the change in resistance that was induced by vagal stimulation was $0.2–0.4 \text{ cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{s}$, similar to that observed by others (46). We used two VAS rats and one VAD rat to demonstrate that successive stimulation of the vagus nerves does not extinguish the bronchoconstrictive response. For the three rats, $10.7 \pm 1.1$ (SD) stimulations were performed at $16.7 \pm 2.9$ (SD) V at $15 \text{ Hz}$. The vagally induced increase in airway resistance was determined after each stimulation. The change in resistance during the last stimulation was $102 \pm 20\%$ (mean ± SD) of the change in resistance during the first stimulation. These data indicate that 10 stimulations, the number required to complete the pilocarpine protocol, do not significantly attenuate the airway resistance response. Figure 5 illustrates that VAD rats are less responsive to pilocarpine than VAS rats, indicating a reduction in the activity of $M_2R$ in the airway walls to dampen the bronchoconstriction that is induced by vagal stimulation. The findings from the group of VAS and VAD rats that are shown in Fig. 5 are representative of those obtained using a second group of four VAS and five VAD rats that were studied (data from the second group are not shown).

**Analysis of $M_3R$ protein expression.** Bronchial smooth muscle contains $M_3R$ and $M_3R$, but not muscarinic $M_1$ or $M_4$ receptors. Therefore, we used Western analysis to analyze muscarinic receptor proteins in membrane fractions from airway homogenates. Figure 6A demonstrates that the antibodies appropriately recognized the $M_3R$ or $M_3R$ proteins and that the immunoreactive bands are not visualized after the antibodies were preadsorbed with the immunizing peptides. Representative immunoblots are shown in Fig. 6B and illustrate that $M_2R$ protein is decreased in bronchial tissue from VAD rats while $M_3R$ is not. The $M_3R$ and $M_3R$ migrated at 65 and 94 kDa, respectively, similar to the findings of others (28, 36). The densities of three immunoblots for $M_3R$ and $M_3R$ protein are shown in Fig. 6C. Vitamin A status uniquely alters the abundance of the $M_3R$ in bronchial wall tissue, since the abundance of the $M_3R$ did not change. The Western analysis does not distinguish between prejunctional $M_3R$ and postjunctional $M_3R$ located on the bronchial smooth muscle cell membrane. However, when considered together with the data shown in Fig. 5, the decrease in sensitivity to pilocarpine (a property of pre-synaptic $M_3R$) is likely, at least in part, to reflect a decrease in the abundance of the prejunctional $M_3R$ protein.

**DISCUSSION**

These investigations indicate that VAD increases airway responsiveness to an aerosolized cholinergic stimulus and that this increase in responsiveness involves an alteration in the expression and function of the $M_2R$. In VAD rats, the ability to develop $M_2R$-mediated suppression of bronchoconstriction is diminished, because there is a nearly twofold reduction in $M_2R$ protein in their bronchial tissue compared with VAS rats. The increased airway responsiveness was
observed in Sprague-Dawley and Lewis rats. The airway hyperresponsiveness did not appear to result from an increase in the permeability of the epithelial barrier to small molecules or from a decrease in the diameter of unstimulated airways. These findings demonstrate a novel effect of VAD on the lung and suggest that retinoids may be involved in the regulation of airway responsiveness.

Airway hyperresponsiveness was assessed in unanesthetized rats using whole body plethysmography. This method has been used by several other groups of investigators who have studied airway responsiveness to cholinergic agents (5, 46, 47). The primary data are elapsed time and fluctuations in chamber pressure that result from compression of the air in the closed space during the respiratory cycle. Flow is not mea-

---

Fig. 6. A: anti-muscarinic type 2 receptor (M2R) and anti-muscarinic type 3 receptor (M3R) antibodies are able to uniquely detect the proteins containing their unique immunizing (blocking) peptides, and preincubating the antibodies in the presence (+) and absence (−) of the immunizing peptides prevented detection of the expected proteins. Lanes 1 and 3, VAD rats; lanes 2 and 4, VAS rats. Molecular radii of the proteins are shown at right. B: representative immunoblots for M2R and M3R proteins in bronchial tissue. C: density (mean ± SE, n = 3) of M2R and M3R protein from the immunoblot in A and 2 other similar immunoblots for M2R and M3R. *P < 0.05 (Student’s t-test for unpaired variables).
sured, so resistance is not assessed. Although box pressure 
is analyzed during multiple phases of the respira-
tory cycle, one measurement, the \( P_{\text{enh}} \), has been most 
widely used to assess airway responsiveness. The \( P_{\text{enh}} \) 
has been shown to correlate with airway resistance in 
anesthetized, mechanically ventilated mice and with 
airway inflammation in several models of asthma (12, 
19). \( P_{\text{enh}} \) does not correlate with airway resistance 
when there is concomitant parenchymal lung disease 
(35). However, in our VAD rats, the architecture of the 
lung parenchyma was normal (data not shown), and the 
\( P_{\text{enh}} \) was always similar in VAS and VAD rats in 
the absence of methacholine. Therefore, \( P_{\text{enh}} \) is an 
appropriate and reliable surrogate of airway resistance 
in our studies of airway responsiveness in animals 
with normal pulmonary parenchyma. The magnitude 
of the increase in \( P_{\text{enh}} \) that we observed in rats, after 
delivery of a methacholine aerosol, is similar to that 
observed by others (11).

Our studies suggest that VAD may decrease the 
clearance of \(^{99m}\)Tc-DTPA from the pulmonary air 
space. The mechanism(s) responsible for this apparent 
decrease remains unclear. \(^{99m}\)Tc-DTPA is hydrophilic 
and is cleared by diffusion from the airways and the 
alveoli, although the alveoli provide a much larger 
surface area for transfer into the pulmonary circula-
tion. In both cases, it is cleared by a paracellular 
pathway, most likely through incomplete epithelial 
intercellular junctions. Because epithelial junctions 
are much less permeant than endothelial junctions, the 
epithelium is the rate-limiting barrier to diffusion and 
also overshadows the effects of circulatory or lymphatic 
flow. The effects of VAD on the alveolar epithelium are 
poorly understood, although retinoids are required for 
alveolar epithelial repair after injury, and they influ-
ence surfactant protein production (29). Retinoid de-
ciency has been shown to decrease the transcellular 
clearance of small molecules across a monolayer of 
cervical epithelial cells in vitro (16). The retinoid-defi-
cient state promotes the development of a squamous 
phenotype of cervical epithelium in vitro and in vivo. 
Because VAD also produces squamous metaplasia of 
the bronchiolar epithelium, this could produce a decrease 
in paracellular transport of a small molecule such as 
DTPA, as it does in the cervical epithelium.

Studies by other investigators suggest that VAD 
could alter cholinergic nerve activity. Retinoic acid 
promotes the development of a cholinergic phenotype 
in neural tissues and increases the expression of cho-
linc acetyl transferase and vesicular acetylcholine 
transporter, which increases the availability of acetyl-
choline at the presynaptic vesicle (20). Retinoic acid 
also increases the functional activity of the muscarinic 
receptors in neuroblastoma cells, including the \( \text{M}_2 \text{R} \) 
(4). A marked decrease in choline acetyl transferase 
activity in the internucleons of the brain striatum has 
been reported in mice that have undergone a deletion 
of the retinoid-X receptor (RXR)-\( \gamma \) gene (42). This 
is associated with an alteration in the response to dopa-
mine receptor antagonists. Others have found abnor-
malities in hippocampal long-term potentiation that 
interfere with spatial learning and memory in mice 
with gene deletions of RXR-\( \gamma \) or retinoic acid receptor-\( \beta \) 
(7). Similar abnormalities in learning and memory 
have been observed in rats with traumatic brain injury 
to the hippocampus. The traumatic brain injury pro-
duced a loss of \( \text{M}_2 \text{R} \) mRNA and protein in the hippoc-
campus (43). Thus there is circumstantial evidence 
that retinoids may influence cholinergic nerve function 
in the brain. Therefore, VAD may alter the activity of 
cholinergic nerves. This appears to be the case in the 
proximal and mid-large intestine of VAD rats, which 
develops a stronger secretory response to bethanachol 
than the corresponding intestinal segments in VAS 
rats (32). Similar to the tracheal-bronchial tree, the 
colon contains \( \text{M}_2 \text{R} \) and \( \text{M}_3 \text{R} \) (18, 41). Others have 
suggested that a decrease in \( \text{M}_2 \text{R} \) inhibitory activity 
could contribute to the diarrhea that is observed in 
VAD animals (32). These studies in the brain and colon 
are consistent with our observation that bronchial \( \text{M}_2 \text{R} \) 
function is altered in the VAD state.

Others have investigated the effects of VAD in mice 
and vitamin A supplementation of rats on ozone-
duced airway inflammation and BHR (21, 34). In mice 
and rats, vitamin A had a salutary effect on the ozone-
duced neutrophil influx. However, vitamin A supple-
mentation did not alter bronchial responsiveness to 
acetylcholine in rats (21). Similar to the study of Pa-
quette et al. (34), we did not observe that VAD by itself 
increased inflammatory cells in the airways or distal 
lung (data not shown). Although we observed that VAD 
increases BHR, Hisada et al. (21) found that vitamin A 
supplementation did not ameliorate the ozone-induced 
increase in BHR. Vitamin A supplementation may not 
alter the availability of vitamin A in animals with 
replete liver stores (most commercial diets contain 
retinol in higher amounts than the daily requirement) 
and, therefore, would likely not alter the availability of 
retinol to the airways. However, in a deficiency model, 
where hepatic stores and plasma retinol levels are 
reduced, airway stores are also reduced (27). Thus one 
is more likely to observe a change in bronchial reactiv-
ity in a deficiency model than in a supplementation 
model.

Airway hyperresponsiveness to cholinergic stimuli 
has been studied in a variety of animal models and is 
influenced by several conditions that are relevant to 
humans with asthma. Models of airway hypersensitiv-
ty to sensitizing antigens such as ovalbumin have 
shown that eosinophilic major basic protein can antag-
onize the binding of acetylcholine to the \( \text{M}_2 \text{R} \) and that 
this results in a decrease in the inhibitory function of 
the receptor (13). Others have shown that parainfluen-
za-1 viral infection of rats results in an increase in 
airway hyperresponsiveness that persists for up to 2 
wk after the infection (46). Parainfluenza-1 virus also 
increases BHR in guinea pigs during the acute phase of 
the infection (1). A decrease in \( \text{M}_2 \text{R} \) inhibitory function 
is responsible for the airway hyperreactivity in both 
models. These and other observations have clearly es-
established that the \( \text{M}_2 \text{R} \) plays a dominant role in regu-
lating the concentration of acetylcholine that is pre-
sented to the M₃R on the airway smooth muscle. There is no evidence that M₃R number or function is altered in these models. Thus the supply of acetylcholine to the smooth muscle cell, rather than the receptor responsiveness, appears to be the determining factor.

The structural and functional characteristics of the postganglionic M₂R are well established and have been reviewed (3, 18). The receptor is a member of the seven-transmembrane domain, G protein-linked receptor family. Although the genomic organization is understood, little information about the regulation of the M₂R gene is available. Jacoby and associates (22, 23) showed that interferon and parainfluenza viral infection reduce M₂R mRNA in cultured guinea pig airway neurons and that glucocorticoids alter M₂R function and gene expression in vitro and in vivo. Others have shown that interleukin-1β, tumor necrosis factor-α, and transforming growth factor-β₁ decrease M₂R gene expression in cultured HEL299 human embryonic lung fibroblasts (3). Portions of the 5’-regulatory regions of the chicken and human M₂R genes have been cloned and sequenced, and although some potential retinoic acid receptor/RXR element half-sites are present, there is no information to indicate whether VAD has a direct or indirect effect on M₂R expression. Additional studies of M₂R expression must be conducted in VAD rats to indicate whether M₂R mRNA is reduced and how quickly the physiological responsiveness to pilocarpine can be restored after the administration of retinoic acid. Likewise studies must be undertaken to determine whether VAD results in a loss of prejunctional nerve fibers or an alteration in their function without neuronal loss.

We thank J. Snyder (Department of Anatomy, University of Iowa) for making available the Image-Pro Plus program.

These investigations were supported by the Department of Veterans Affairs Research (S. E. McGowan and U. C. Kopp), National Heart, Lung, and Blood Institute Grants HL-53430 (S. E. McGowan) and HL-59324 (J. N. Kline), National Institutes of Health O'Brien Kidney Disease Center Grant DK-52617, and American Heart Association Grant-in-Aid 0150024N.

REFERENCES


