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Vitamin A Deficiency Increases Airway Resistance Following C-fiber Stimulation

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Abstract

Vitamin A deficiency (VAD) remains an important problem in the developing world where environmental air pollution is increasing. Because the coincidence of these factors could increase the prevalence of asthma in these regions, airway reactivity to methacholine, capsaicin, and sulfur dioxide was studied in VAD rats. The impedance to a small volume oscillation was analyzed to determine airway resistance (R_{aw}) and lung elastance (H). VAD rats demonstrated larger increases in R_{aw} and H after the administration of methacholine, and in R_{aw} after administration of capsaicin or sulfur-dioxide. The muscarinic receptor-2 (M2R) selective antagonist AFDX-116 enhanced the effect of capsaicin in vitamin A sufficient (VAS) but not VAD rats and retinoic acid administration partially restored this enhancement. These data indicate that diminished auto-inhibitory muscarinic receptor-2 (M2R) function contributed to this increased airway responsiveness to pulmonary C-fiber stimulation in VAD rats. If children with VAD also have diminished M2R function, they may be more prone to develop asthma, particularly in the presence of environmental co-factors such as sulfur dioxide.

Keywords

retinoids; capsaicin; sulfur dioxide; asthma

1. INTRODUCTION

The efferent vagus nerves have been implicated in the pathogenesis of asthma exacerbations because they are targets for direct viral effects and for inflammatory eosinophils (Evans et. al., 1997; Jacoby et. al., 1998). During exacerbations, airway smooth muscle contraction is hyperresponsive to vagally mediated stimulation, because there is a decrease in autoinhibitory muscarinic2-receptor (M2R) function in the vagal pre-junctional terminals. Most of the experimental work has been conducted by stimulating the efferent vagus nerves of guinea pigs or rats following central vagal denervation (Belmonte et. al., 1997; Fryer et.

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al., 1984). In these preparations, eosinophilic cationic proteins, viral neuraminidase, or viral double-stranded RNA diminish M2R-autoinhibition, resulting in increased airway smooth muscle contraction following vagal stimulation. Whereas it is clear that M2R modulate this neurally mediated contractile response, it is less clear whether M2R modulate smooth muscle contraction that is initiated by other mechanisms that increase acetylcholine at the airway smooth muscle neuromuscular junction. Others have shown that M2R dampen smooth muscle contraction following methacholine (MCh) treatment of the ovine trachea, which is not innervated by the vagus nerve (Wagner et. al., 1999). Mice bearing a deletion of the M2R gene exhibit abnormal cardiac chronotropic and airway constrictor responses to MCh and exhibit premature relaxation of bronchial smooth muscle after muscarinic-induced contraction (Fisher et. al., 2004; Struckmann et. al., 2003). We have found that vitamin A deficient (VAD) rats exhibit a greater increase in total pulmonary resistance than do vitamin A sufficient (VAS) rats after exposure to aerosolized MCh (McGowan et. al., 2002). VAD rats exhibited diminished M2R gene expression and function, compared to VAS rats, and we suggested that this diminished M2R function contributes to the increased responsiveness to MCh in vitamin A deficiency (VAD).

The studies that are reported here address the hypothesis that VAD diminishes M2R function, which reduces airway contraction that is initiated by a C-fiber mediated reflex loop. Others have shown that pulmonary C-fibers contribute to airway contraction through two mechanisms. The first involves stimulation of nociceptive receptors in the airway epithelium, transmission of the impulse to the brain by the afferent C-fibers and then transmission to the airway smooth muscle by the vagal efferents (Lee et. al., 2003). The second pathway (referred to as the axon reflex) is localized to the airway wall and smooth muscle contraction is initiated by tachykinins or neurokinins that are released from the afferent C-fiber nerve terminals (Taylor-Clark et. al., 2006). We reasoned that diminished M2R function would render VAD rats more sensitive to airway contraction that is mediated through the efferent vagus nerves. We have studied the effects of agents that stimulate the afferent C-fibers in VAD and VAS rats on airway resistance, and how the response to stimulation is modified by interrupting efferent vagal transmission at the neuromuscular junction. We chose capsaicin as a model stimulant because it has relatively specific effects on the transient receptor potential channel, vanilloid subfamily member 1 (TRPV1) and others have characterized the afferent and efferent neural pathways in rats that are activated by exposure to capsaicin (Coleridge et. al., 1984; Gu et. al., 2003). We have used sulfur dioxide (SO₂) as an alternative stimulant of afferent C-fiber stimulation because it is known to contribute to asthma exacerbations in humans (Sunyer et. al., 2003). Sulfur dioxide exposure may be relevant to VAD in humans because ambient SO₂ levels are elevated in regions of the world where VAD is also common (Bruce et. al., 2000). Others have shown that the acute effects of SO₂ on ventilation in rats is primarily exerted through C-fibers, although rapidly adapting receptors also make a small contribution (Wang et. al., 1996).

Vitamin A deficiency is still prevalent in sub-Saharan Africa, South and Southeast Asia, and parts of Latin America (Underwood et. al., 1999). Children between ages 2 and 8 and pregnant or lactating females are at the highest risk for developing VAD (Underwood, 1994). Biomass with high sulfur content is more commonly used as fuel for cooking and heating in developing countries where VAD is prevalent (Bruce et. al., 2000). Thus children who live in

these countries are at risk for both VAD and exposure to high levels of SO₂. This is particularly a concern in rural China where approximately 30% of children have sub-clinical, 12% have clinical vitamin A deficiency and more than two-thirds of the homes use coal with an elevated sulfur content for heating and cooking (Jiang et. al., 2006; Qian et. al., 2004a). Therefore understanding the relationship between VAD and airway responsiveness to SO₂ may have significant clinical ramifications.

2. MATERIALS AND METHODS

2.1 Animal Care and production of vitamin A deficiency

Specific pathogen-free female Lewis rats were obtained from Harlan-Sprague Dawley (Madison, WI). All animals were maintained in HEPA-filtered cages, given food and water *ad libitum*, 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 Iowa City Veterans Affairs Medical Center and the University of Iowa. The rats were weaned at postnatal day 21 and placed on a VAD diet-modified (catalog number 96022, ICN Corp., Aurora, OH), for 7 to 10 weeks to achieve vitamin A deficiency (Smith, 1990). Vitamin A sufficient rats were age-matched females were purchased from Harlan-Sprague Dawley. The onset of VAD was identified by the cessation of weight gain which occurred earlier than in females who were fed the control diet. When the VAD rats stopped gaining weight, they received 25 µg of retinyl acetate that was administered orally at weekly intervals to prevent weight loss and a generalized nutritional deficiency. Twenty-five micrograms of retinoic acid (RA), in safflower oil, were administered orally daily for 12 days to some rats to determine whether this reversed the effects of VAD (McGowan et. al., 2005). Supplementation of VAD rats with RA for 12 days is sufficient to restore the expression of retinaldehyde dehydrogenase, a retinoid responsive gene (Bhat et. al., 1995).

2.2 Delivery of methacholine, capsaicin, or SO₂

Methacholine (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.145 M NaCl, 0.0015 M KH₂PO₄, 0.0027 M KCl, 0.0086 M Na₂HPO₄, pH 7.4 (PBS) at a concentration of 12 mg/ml on the day of the experiment and kept on ice. The MCh was administered for 30 s as an aerosol, which was generated by a DeVilbiss Ultrasonic Nebulizer and delivered into the inspiratory circuit using a Harvard 683 volume cycled rodent ventilator (Harvard Apparatus, Holliston, MA) with a V_T of 8.7 ml/kg. The nebulizer was activated for 30 s to prime the circuit and a stopcock was used to allow the Harvard 683 to deliver the aerosol through the tracheostomy tube for 30 s. In all experiments PBS was administered in the absence of a bronchoconstrictor at the beginning of the protocol to establish the baseline resistance and elastance.

Capsaicin (Sigma-Aldrich, St. Louis, MO) was dissolved in 70% ethanol and a stock 20 mM solution was kept at -20° for no more than two weeks. Capsaicin was delivered as an aerosol containing 0.2 mM capsaicin and 0.7% ethanol in PBS, using the same nebulizer that was used to deliver MCh. The stock solution of capsaicin was diluted immediately prior to generating the aerosol.

Sulfur dioxide (verified to be 500 ppm in N₂) was obtained from Matheson Tri-Gas (Montgomeryville, PA). The SO₂ was metered through a high-precision rotameter (FL-3013SA, Omega Engineering, Stamford, CT) and diluted in humidified air that was also precisely delivered (metered by a separate high-precision rotameter) to a mixing chamber, which served as a reservoir for the gas mixture that entered the endotracheal tube. Anesthetized, paralyzed, tracheotomized (see below) rats were placed inside a whole-body plethysmograph (PLY3114, Buxco, Wilmington, NC) with the endotracheal tube exiting from the plethysmograph. A Harvard Inspira ventilator was used to rapidly evacuate the closed plethysmograph. The negative plethysmographic pressures were adjusted to expand the thoracic cage and achieve V_T of 10 ml/kg with a respiratory rate of 45 bpm. Sulfur dioxide was delivered for 10 minutes immediately prior to the assessment of airway resistance. A large tidal breath of air was delivered by positive pressure every 2 minutes to minimize alveolar collapse. To maintain oxygenation during the transfer from the plethysmograph to the FlexiVent™ (Scireq, Montreal, QC, Canada), 8 ml of 100% O₂ were delivered immediately prior to transfer.

2.3 Mechanical ventilation and assessment of airway resistance

Rats were anesthetized with an intraperitoneal injection of 60 mg/kg of ketamine and 12 mg/kg of xylazine and the level of analgesia was assessed by pinching the foot pad. A tracheostomy was performed and a 2.0 mm internal diameter metal tracheal cannula was inserted and tied firmly in place. The rats were paralyzed with 7.5 mg/kg pancuronium bromide to eliminate extraneous respiratory movements during the resistance measurements and to permit negative pressure ventilation.

Airway resistance was assessed using a FlexiVent™ ventilator (Scireq, Montreal, Quebec, Canada), which delivered air immediately following delivery of aerosolized PBS, the test aerosol, or SO₂. The pressure transducers in the FlexiVent™ were calibrated at 0 and 20 cm H₂O and the tube calibration was performed using the same cannula that was used to ventilate the animal. Quasi-sinusoidal ventilation was delivered with a V_T of 8.7 ml/kg at 75 breaths per minute with 3 cm H₂O of positive end-expiratory pressure (PEEP). The FlexiVent was programmed to follow a sequence of events: deliver 3 successive breaths without exhalation to reset the volume history, ventilate for 12 sec, perform a maneuver to assess single breath respiratory system resistance and elastance, and perform a maneuver to assess oscillatory mechanics. Then the aerosol was delivered. Following aerosol delivery, 16 cycles of the oscillatory maneuver were performed. One cycle included 20 sec of ventilation followed by 8 s of oscillation, and then returns to the beginning of the cycle (the 16 cycles required 8 minutes). To assess single breath respiratory system resistance and elastance, a single breath was administered and the volume delivered and the pressure in the ventilator cylinder were assessed and used to calculate total respiratory resistance and elastance using the single compartment model. For the oscillatory maneuver an 8 s perturbation consisting of randomly generated mutually prime oscillations ranging from 0.5 to 20 Hz was delivered by the ventilator while the cylinder pressure and the volume of the oscillation were continuously monitored. The impedance to the oscillatory wave was calculated after subjecting the P vs frequency spectrum to fast Fourier transformation. The impedance was partitioned into the airway resistance (R_{aw}), tissue resistance (G) and tissue elastance (H)

components using the constant phase model (Hantos et. al., 1987). Data were exported to Microsoft Excel spreadsheets for further analysis. Because neither capsaicin nor SO_2 substantially altered H or G under these experimental conditions, only airway resistance (R_{aw}) is reported for these experiments. The sequence for administering the various experimental agents and concentrations are shown in the respective figures and legends.

For the protocols in which only MCh was aerosolized, the functional residual capacity was adjusted by changing the level of positive end-expiratory pressure between 1 and 5 cm H_2O . This enabled us to assess the ability of the tethering forces of the pulmonary parenchyma to counter the bronchoconstriction that was induced by MCh. Heart rate was monitored using three surface electrodes, a DP301 differential amplifier (Warner Instruments, Hamden, CT), and Biobench software (National Instruments, Austin, TX).

2.4 Administration of atropine or AFDX-116

To confirm that cholinergic neurotransmission mediated the capsaicin induced rise in airway resistance 100 $\mu\text{g}/\text{kg}$ of atropine-sulfate (Sigma-Aldrich) was administered as a single bolus into the femoral vein 5 minutes prior to capsaicin exposure. To more specifically assess the contribution of M2R autoinhibition to capsaicin-induced airway contraction, we studied how pre-treatment with 11-[[2-diethylamino-O-methyl]-1-piperidinyl]acetyl)-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AFDX-116) modified the response to capsaicin in VAS, VAD and VAD rats that had received RA for 12 days. AFDX-116 is a selective inhibitor of the M2R (Billard et. al., 1995). A pilot study using doses ranging from 15 to 130 $\mu\text{g}/\text{kg}$ established that the optimal dose of AFDX-116 (Tocris Bioscience, Ellisville, MO) was 60 μg per kg. A higher dose (130 $\mu\text{g}/\text{kg}$) did not specifically block M2R function and appeared to also inhibit M3R function. The AFDX-116 was administered in the same fashion as atropine.

2.5 Statistical analysis

Mean $\pm$ 1 SEM of data obtained from rats within each vitamin-A treatment group are given. The numbers of animals used are shown in the legends to the figures. Analysis of variance (ANOVA) tests were conducted and followed by the Student-Newman-Keuls post-hoc test. The area under the curve (AUC) for the R_{aw} or H versus time plots was calculated using the algorithm available in Prism 4 (GraphPad Software, San Diego, CA) and used to evaluate the effects of vitamin A status and PEEP. The initial R_{aw} or H, prior to MCh administration was subtracted from all post-MCh values for each subject when calculating the AUC. The data obtained from the studies using MCh and various levels of PEEP were analyzed using a 3-way ANOVA, to assess the overall effects of vitamin A deficiency of RA-administration and the level of PEEP. The data were also analyzed to assess the effect of vitamin A treatment at each level of PEEP using a 1-way ANOVA. The effects of capsaicin and AFDX-116 on R_{aw} among the three vitamin A-treatment groups were compared using a 2-way ANOVA. The effects of varying concentrations of SO_2 or RA between VAS and VAD rats were compared using a 3-way ANOVA. Statistical analyses were performed using SigmaStat (Jandal Scientific, San Rafael, CA). The Differences were considered significant if P was less than 0.05 (Rosner, 1990).

3. RESULTS

3.1 VAD increases airway resistance and lung elastance after methacholine administration

Figure 1 shows the time course of the R_{aw} and H in VAS, VAD, and VAD rats that had received RA for 12 d (VAD + 12 RA). Panel A suggests that R_{aw} was higher in VAD following MCh administration (the times of MCh administration are indicated by the arrows) than in VAS rats. To verify this impression the area under the curve (AUC) of the plots of R_{aw} vs. time was calculated for each rat and the results were compared among the three vitamin A-treatment groups (Table 1). A 3-way ANOVA, using subject, vitamin A status, and PEEP as the three independent variables, showed that vitamin A status accounted for significant differences ($p < 0.001$). Comparisons were also made at each level of PEEP, and VAD rats had significantly higher R_{aw} at 3 and 5 cm H₂O of PEEP, compared to VAS rats. In VAS rats, supplying either 3 or 5 cm H₂O of PEEP nearly completely ameliorated the rise in R_{aw} that followed MCh administration at 1 cm H₂O of PEEP. In contrast, in VAD rats, 5 cm H₂O of PEEP only partially restore R_{aw} to levels similar to those after PBS alone. After VAD rats were treated with RA for 12d (panel C), 3 or 5cm H₂O of PEEP significantly ameliorated the rise in R_{aw} compared to the untreated VAD rats (Table 1). These data indicate that RA was able to significantly reduce the rise in R_{aw} that was induced by MCh in VAD rats.

The elastance (H) after MCh administration to VAD and VAS rats and to VAD rats that had received RA for 12 days is shown in panels B and C respectively.. Similar to the R_{aw} , MCh increased H to a greater extent in VAD than in VAS rats. The AUC for the plots of H vs. time was calculated for each subject (Table 2). The results were compared using a 3-way ANOVA, with the same independent variables that were used to analyze the R_{aw} . As with the R_{aw} , vitamin-A status was a significant predictor of H ($p < 0.001$). A 1-way ANOVA was used to analyze the contribution of vitamin-A status at each level of PEEP. At 3 cm H₂O of PEEP H was significantly higher in VAD than in VAS rats, whereas at 5 cm H₂O of PEEP H was higher in both VAD and VAD + 12d RA rats than in VAS rats. Increasing FRC by supplying PEEP at either 3 or 5 cm H₂O reduced H in VAS rats to a level that was similar to that observed after PBS. However, in VAD rats, in the absence or presence of RA-treatment, raising PEEP to 3 or 5 cm H₂O did not afford as large a decrease in H as was observed in VAS rats. This indicates that the alveolar expansion, which was presumably achieved at higher levels of PEEP, was less effective in ameliorating the MCh-induced increase in H in VAD than in VAS rats, and this was not reversed by RA-administration.

3.2 VAD alters the ability of the M2R to inhibit the response to capsaicin

A pilot study was performed to determine the optimal concentration of aerosolized capsaicin which increased R_{aw} but did not produce tachyphylaxis. Based on these results, 200 μ M was chosen as the optimal capsaicin concentration, because higher concentrations (up to 500 μ M) did not produce greater increases in R_{aw} , and the response waned during successive administrations. Although the increase in R_{aw} after aerosolizing 200 μ M capsaicin was modest, this concentration yielded consistent results and the increase was observed after repeated dosing, if at least 20 minutes elapsed between doses. Figure 2 shows the responses of one representative VAS and one VAD rat to either MCh or capsaicin (caps). MCh

administration was used as a positive control to ensure that the VAD rats exhibited increased cholinergic responsiveness. VAD rats also exhibited a greater increase in R_{aw} after capsaicin administration than did VAS rats (Figure 3A). Atropine administration abolished the ability of capsaicin to increase R_{aw} , indicating that the response to capsaicin was mediated through cholinergic transmission. These findings are consistent with the hypothesis that diminished M2R-autoinhibition in VAD allows an increased response to capsaicin-induced efferent vagal impulses at the bronchial smooth muscle neuromuscular junction. In order to address this hypothesis more directly, we evaluated the effects of a selective M2R inhibitor (AFDX-116) on the capsaicin-mediated increase in R_{aw} . Figure 3A shows that whereas AFDX-116 increased the response to capsaicin in VAS rats it failed to do so in VAD rats. This indicates that M2R-autoinhibition is diminished in VAD rats, because release from autoinhibition by administering AFDX-116 produced a smaller enhancement in capsaicin-induced R_{aw} in VAD than in VAS rats. The magnitude of this enhancement is compared in Figure 3B, which clearly illustrates that administering RA to VAD rats restored the auto-inhibitory function of their M2R.

3.3 VAD rats are more responsive to SO₂ inhalation

Others showed that short-term administration of SO₂ increased bronchoconstriction through a pathway, which involves vagal efferent fibers (Wang et. al., 1996). Because others have shown that SO₂ inhalation results in C-fibers activation (Karlsson et. al., 1988; Wang et. al., 1996), we administered SO₂ gas for 10 minutes and assessed airway resistance immediately following SO₂ administration. Figure 4 shows that whereas SO₂ failed to increase R_{aw} at all of the concentrations that were administered to VAS rats, concentrations that were at 10 ppm or higher increased R_{aw} in VAD rats. Figure 5 compares the mean R_{aw} , which was significantly greater in four VAD compared to four VAS rats at 10, 26 and 50 ppm of SO₂. Increasing in SO₂ concentration to 125 ppm did not increase R_{aw} in VAS rats (data not shown).

4. DISCUSSION

Previous studies of M2R-mediated inhibition of airway contraction have primarily examined the effects of various manipulations on airway contraction that is induced by direct electrical stimulation of the efferent vagus nerve. Our study is among the first to use a selective inhibitor of M2R function in order to demonstrate that the M2R influences C-fiber mediated airway contraction. Others have shown that ipratropium (a non-selective inhibitor of M2R), but not a selective inhibitor of the muscarinic receptor-3 (M3R) diminished airway constriction after exposure to SO₂ (Hirose et. al., 2003). To our knowledge, this is the first report that M2R modify the response to capsaicin-induced airway contraction. Our findings are also unique because the VAD-related defect is reversible, indicating that M2R function is retinoid-dependent.

4.1 Explanation of exposure protocols

We adopted this particular SO₂-exposure protocol for several reasons. First, because we could not use the FlexiVent to deliver a corrosive gas, we chose negative pressure ventilation. Others have shown that using negative pressure ventilation to deliver SO₂

intratracheally is an effective method of administering corrosive gasses for short periods of time (10 to 15 minutes) (Halinen et. al., 2000). Second, the intratracheal route was chosen because the short duration of the effect of SO₂ required us to initiate the resistance measurements immediately after the exposure. Thus it was not possible to perform a tracheostomy or introduce an endotracheal tube quickly enough after the delivery of SO₂ to adequately assess the effects of SO₂. Third, the concentrations and duration of SO₂ exposure were chosen because they produced an increase in resistance but did not produce alveolar damage. Higher concentrations of SO₂ (more than 125 ppm) or exposure for more than 10 minutes produced transudation of fluid and red blood cells into the alveoli (observed on histological evaluation of the lungs at the completion of the study, data not shown) and increased the lung elastance. Because we wanted to use SO₂ to acutely stimulate C-fibers, we did not administer it chronically, by a trans-nasal route, as has been done in models of chronic bronchitis (Long et. al., 1999). Because of these limitations, our exposure differs from what humans would be expected to encounter. Children living in rural China are exposed to up to 0.3 ppm of ambient SO₂ in homes where coal is used for cooking and heating (Qian et. al., 2004b). Because this lower level of exposure is sustained for up to 24 h per day over many months, the mechanisms of airway responsiveness likely differ from those in our model. Despite these limitations, if VAD children in China (approximately 12% of children below age 6) (Jiang et. al., 2006), also have M2R-dysfunction, then VAD may amplify the injurious effects of SO₂.

Likewise, several factors influenced our adoption of the protocol for exposing rats to capsaicin. To study the effects of capsaicin on airway resistance, others have introduced capsaicin directly into the right ventricle or as an aerosol. Aerosolized capsaicin has been administered to guinea pigs and rats for 30 s during nasal breathing in concentrations ranging from 0.6 to 3 mM (Bergren, 2001; Bergren et. al., 2004). This produced a 20 to 60% increase in airway resistance in rats, which is similar to the magnitude of the increase (approximately 40%) that we observed aerosolizing 0.2 mM intratracheally. Thus although we observed only a modest increase in R_{aw} , it was similar to that which has been observed by others (Bergren et. al., 2004). We also limited the concentration of capsaicin because exposure to higher concentrations attenuates the response to subsequent administration (Mohapatra et. al., 2003). We did not use intraventricular delivery because this is associated with a significant transient decline in blood pressure and a minimal increase in airway pressure compared to aerosolized capsaicin (Gu et. al., 2003).

4.2 Factors that influence the response to methacholine in VAD

VAD rats exhibit a larger increase in R_{aw} and H following MCh-administration and PEEP less effectively minimized this increase, compared to VAS rats (Tables 1 and 2). Application of 3 or 5 cm H₂O of PEEP increased parenchymal tethering forces by promoting alveolar expansion. Greater expansion reduced H because the more expanded alveoli delivered greater outward tethering forces to the airways. This minimized collapse of the alveoli distal to airways that were narrowed by MCh. Minimization was less effective in VAD rats, suggesting that either the collapse was more complete or that the tethering forces were less effectively delivered, or both. Prior studies by others and our own laboratory have shown that VAD is associated with a decrease in the volume density of alveolar septa, as well as

alveolar interstitial elastin and collagen (Baybutt et. al., 2000; McGowan et. al., 2005). This rarefaction of parenchymal tissue would be expected to reduce the effectiveness of PEEP, because the outwardly retracting forces would be less effectively delivered to the airways because tethering structures are reduced. When 3 or 5 cm H₂O of PEEP enhanced outward tethering in VAS rats, this resulted in a significant restoration of the post-MCh R_{aw} to the level that was observed after administering only PBS. However in VAD rats R_{aw} remained significantly elevated at 3 or 5 cm H₂O of PEEP, relative to that observed in VAS rats after MCh. The administration of RA significantly restored ability of 3 and 5 cm H₂O of PEEP to normalize R_{aw} after MCh, but had relatively little effect on the ability of PEEP to normalize H. This suggests that RA may influence R_{aw} through a mechanism that does not involve alveolar tethering forces. Our earlier studies have shown that RA restores M2R gene expression and function in VAD airways (McGowan et. al., 2002; McGowan et. al., 2004). The observations that we have made using capsaicin and AFDX-116 further indicate that the M2R are involved in this restorative effect of RA.

4.3 Mechanisms whereby VAD may modify the airway response to noxious stimuli

VAD rats exhibited a significantly greater increase in R_{aw} after capsaicin than did VAS rats (Figure 3A). AFDX-116, a selective inhibitor of the M2R, increased the airway responsiveness of VAS rats to capsaicin, whereas AFDX-116 produced no significant enhancement in VAD rats (Figure 3B). These results suggest that the M2R serve an auto-inhibitory function and reduce the response to capsaicin in VAS rats. This auto-inhibitory function is less effective in VAD rats, promoting an increased response to capsaicin. RA-administration restored the ability of AFDX-116 to enhance the response to capsaicin, suggesting that RA restored M2R auto-inhibitory function.

We have not differentiated between the activation C-fibers that results in reflex activation of cholinergic vagal efferents and activation that results from the effects of locally released tachykinins (axonal reflex) in C-fibers. This would require direct measurement of impulse conduction by individual afferent C-fibers, or the use of specific inhibitors of tachykinins, neurokinins or calcitonin gene-related peptide. Our observations in rats differ from those of some other investigators which showed that capsaicin induces increases in R_{aw} by the axonal reflex in anesthetized guinea pigs (Bergren, 1988). Because there are significant species-related differences in the physiology of C-fibers, our findings may not apply to some other species.

We have found that intravenous atropine prevents capsaicin-induced airway contraction. Prior investigations have generally shown that capsaicin-induced airway contraction is mediated by cholinergic nerves. Administration of an atropine-aerosol reversed airway contraction that was induced by installation of capsaicin into the trachea but did not prevent contraction resulting from the inhalation of a capsaicin or metabisulfite aerosol by guinea pigs (Bergren, 2001; Yildirim et. al., 2005). We administered atropine intravenously, which may have provided better delivery of the drugs to the small airways and efferent nerves. Alternatively, our observation that atropine inhibited the capsaicin-induced increase of R_{aw} in rats, whereas others did not observe this in guinea pigs, may result from the larger increase in R_{aw} that is induced by capsaicin in guinea pigs (Karlsson et. al., 1988).

Our observations that atropine and AFDX-116 attenuate the effects of capsaicin indicate that cholinergic transmission is involved. However, we cannot exclude the possibility that VAD has other effects on reflex bronchoconstriction that is mediated by C-fibers. Other factors such as membrane hyperpolarization or an increase in potassium or calcium current can dampen the initial generation of impulses by TRPV1 (development of the generator potential). VAD could have effects on afferent, as well as efferent vagal fibers by decreasing the development or sustenance of the generator potential in the afferent nerves.

Others have shown that SO₂ administration causes airway afferent C-fiber stimulation, which results in bronchoconstriction (Hirose et. al., 2003; Wang et. al., 1996). Because VAD enhanced the response to capsaicin, one would predict that VAD rats would also be more responsive to SO₂. Figures 4 and 5 indicate that whereas VAS rats exhibited very little response to the intratracheal delivery of up to 50 ppm of SO₂ for 10 min, VAD rats showed significant increases in R_{aw} after inhaling as low as 10 ppm of SO₂. These data further suggest that VAD rats more readily develop bronchoconstriction after C-fiber stimulation, consistent with a loss of M2R auto-inhibitory function. The rapid recovery of the R_{aw} to basal levels is consistent with a transient effect of SO₂, as one would expect with stimulation of neural afferents.

Studies in humans and animal models indicate that SO₂ most likely induces bronchoconstriction through a neural reflex that involves non-cholinergic and/or cholinergic nerves. Asthmatics respond to lower concentrations of SO₂ than non-asthmatics and the onset and abatement of the response is similar to what we have observed in rats (Van Schoor et. al., 2000). Anticholinergic drugs have been shown to attenuate the response to SO₂ or metabisulfite in humans and guinea pigs (Mazzone et. al., 2002; Yildirim et. al., 2005). Therefore it is likely that the increase in R_{aw} that we observed, is also mediated, at least in part by cholinergic efferents.

4.4 Potential relevance to humans with VAD

Lower plasma retinol (vitamin A) is associated with more severe asthma exacerbations and lower FEV₁ during less acute phases of the illness (Arora et. al., 2002). Lower vitamin A intake is more prevalent among children with asthma and vitamin A deficiency is accompanied by diminished FEV₁ in children and adolescents (Huang et. al., 2001; Kassaye et. al., 2001). These data indicate that VAD alters lung function in asthmatics, who by definition demonstrate increased airway responsiveness. Our studies provide one possible mechanism for the effect of VAD in asthmatics. Our findings using VAD rats suggest that airway hyperresponsiveness should be studied in humans who are at risk for VAD.

Because SO₂ exposure can precipitate asthma exacerbations, VAD may interact with SO₂-exposure to increase the frequency or severity of exacerbations in children who are exposed to high ambient concentrations of SO₂. High ambient SO₂ is common in the homes of Chinese children living in rural areas, where coal is commonly used for cooking and heating (Qian et. al., 2004a). Vitamin A deficiency is also common in these same areas suggesting that asthma prevalence and/or severity could also be increased by VAD (West, 2002).

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Figure 1.

Methacholine produces larger increases in airway resistance (R_{aw}) and elastance (H) in Vitamin A deficient (VAD) rats. PBS was administered 30s prior to starting any measurements or methacholine was administered for 30 s at the times indicated by the arrows. R_{aw} and H were assessed at 30 s intervals for 8 min and the mean R_{aw} (panel A) or mean H (panel B) for 5 vitamin A sufficient (VAS, ○) and 5 VAD (▲) rats are shown. Error bars are 1 SEM. The response to methacholine was assessed at 3 different levels of positive end expiratory pressure (PEEP shown at the top of each panel). Panel C shows the R_{aw} (○, right axis) and H (▲, left axis) after exposure to PBS or methacholine at different levels of PEEP for 5 VAD rats that had been treated with retinoic acid (RA) for 12 days (d).

Figure 2.

Capsaicin (caps) produces a larger net increase in airway resistance (R_{aw}) in VAD than in VAS rats. Representative time course of the response to aerosolized PBS, methacholine (MCh) or caps for one VAS (panel A) and one VAD (panel B) rat. Arrows pointing upward indicate the times when an aerosol was delivered. Intravenous administration of atropine (arrows pointing downward) for 5 minutes prior to caps abrogated the increase in R_{aw} .

Figure 3.

The M2R-selective antagonist AFDX-116 accentuated the increase in airway resistance (R_{aw}) after capsaicin in VAS but not VAD rats. Panel A shows the mean $\pm$ SEM (n=4) maximal increase ($\uparrow$) in R_{aw} after caps in four VAS, four untreated VAD, and four RA-treated VAD rats without (■) and with (▲) prior administration of AFDX-116. Error bars are 1 SEM. (*) increase in R_{aw} in absence of AFDX-116 was significantly higher in VAD than in VAS rats (2-way ANOVA, $p < 0.02$). (#) increase in R_{aw} in absence of AFDX-116 was significantly lower in RA-treated (VAD+12d RA) than in untreated VAD rats (2-way ANOVA, $p < 0.02$). The same data were used to calculate the percent enhancement in the effect of capsaicin on R_{aw} (panel B). No enhancement was observed in VAD rats and this was significantly different from VAS and VAD+12d RA rats (*), 2-way ANOVA, $p < 0.02$.

Figure 4.

Airway resistance (R_{aw}) increases after inhalation of sulfur dioxide (SO₂) gas to a greater extent in VAD than in VAS rats. The time-course of R_{aw} after exposure to various concentrations (in parts per million, ppm) of SO₂ mixed with air is shown for one representative VAS and VAD rat. The arrows designate the different 10-minute exposure periods which are not shown to scale on the X-axis.

Figure 5.

The maximal increase ($\uparrow$) in airway resistance (R_{aw}) after sulfur dioxide (SO_2)-administration was greater in VAD ($\blacktriangle$) than in VAS ($\blacksquare$) rats. Mean $\pm$ 1 SEM R_{aw} for 4 VAS and 4 VAD rats after inhalation of different concentrations (ppm) of SO_2 in air. (*) R_{aw} was significantly higher in VAD than VAS rats at the concentrations of SO_2 shown, $p < 0.05$, 3-way ANOVA).

Table 1

Area under the curve (AUC) for airway resistance (R_{aw}) after exposure to methacholine

PEEP (cm H ₂ O)	3		1		5	
	mean	SEM	mean	SEM	mean	SEM
VAS	0.103	0.015	0.455	0.025	0.099	0.011
VAD	0.703 [*]	0.216	0.764	0.291	0.291 [*]	0.058
VAD + 12d RA	0.189 [†]	0.026	0.733	0.079	0.180 [†]	0.014

The AUC of the plots (arbitrary units) of airway resistance (R_{aw}) vs. time at each concentration of MCh was calculated for each animal shown in Figure 1 after subtracting the resistance prior to MCh administration. The overall effect of vitamin A treatment on AUC was compared using a 3-way ANOVA and was found to be significant $p<0.001$. Vitamin A-treatment groups were compared at individual levels of PEEP using a 1-way ANOVA with the Student-Newman-Keuls post-hoc test.

^{*}, $p<0.05$, VAD vs. VAS.

[†], $p<0.05$ VAD + 12d RA vs. VAD.

Table 2

Area under the curve (AUC) for elastance (H) after exposure to methacholine

PEEP (cm H ₂ O)	3		1		5	
	mean	SEM	mean	SEM	mean	SEM
VAS	5.540	0.447	25.755	2.118	5.5215	0.250
VAD	20.968 [*]	4.607	33.666	2.633	11.172 [*]	1.078
VAD + 12d RA	11.769	1.844	32.454	2.927	10.607 [‡]	1.290

The AUC (in arbitrary units) under the plots of H vs. time at each concentration of MCh was calculated for each animal shown in Figure 1 and analyzed in the same fashion as the R_{aw} . The overall effect of vitamin A treatment on AUC was compared using a 3-way ANOVA and was found to be significant $p<0.001$. Vitamin A-treatment groups were compared at individual levels of PEEP using a 1-way ANOVA with the Student-Newman-Keuls post-hoc test.

^{*}, $p<0.05$, VAD vs. VAS,

[‡], $p<0.05$ VAD + 12d RA vs. VAS