Cold weather exercise and airway cytokine expression

Michael S. Davis, Jerry R. Malayer, Lori Vandeventer, Christopher M. Royer, Erica C. McKenzie, and Katherine K. Williamson

Department of Physiological Sciences, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma

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EXERCISE-INDUCED ASTHMA is a syndrome in which strenuous exercise, particularly while breathing cold air, can trigger airway obstruction in subjects with hyperreactive airways (31). The term “exercise-induced asthma” is something of a misnomer, as it is not the exercise itself that causes asthma in these subjects. Rather, the subjects have preexisting airway disease, and the airway cooling and drying that occur during periods of increased ventilation trigger reflex bronchoconstriction that spontaneously resolves within 30–60 min. However, subjects that routinely perform strenuous exercise in cold conditions have a high prevalence of chronic airway inflammation and hyperreactivity [termed “ski asthma” because of its original description in cross-country skiers (39, 40)], suggesting that such activity actually may be capable of de novo induction of an asthmalike syndrome.

We have developed an equine model of ski asthma to study the effects of strenuous exercise while breathing cold air on pulmonary physiology and to determine whether exercise while breathing cold air can cause chronic airway disease. Horses have greater proportional increases in cardiopulmonary function during maximal exercise compared with humans (26, 35), but in this model the exercise intensity of the horses is controlled to match typical human cardiopulmonary responses to exercise and to minimize exercise-induced pulmonary hemorrhage (a condition common in maximally exercising horses). Previous studies with this model have confirmed the failure of the upper airways to fully warm inspired air, leading to exposure of lower airways to heat and water loss (8). These studies also confirmed that this stimulus is associated with airway mucosal damage, a feature also reported in humans after exercise while breathing cold air (37). In the present study, we used the equine model of ski asthma to test the hypothesis that exercise while breathing cold air causes airway inflammation by upregulating proinflammatory cytokines.

MATERIALS AND METHODS

All experiments were reviewed and approved by the Oklahoma State University Institutional Animal Care and Use Committee. Eight healthy adult horses were housed in individual stalls and fed grass hay supplemented with commercial grain mix throughout training and participation in these experiments. Training consisted of walking, trotting, and cantering three times weekly on a motorized high-speed treadmill in an indoor climate-controlled facility (ambient temperature 25°C) for 12 wk. A single graded exercise test was performed after 12 wk of training to determine the speed and slope that corresponded to a heart rate of 170 beats/min for each horse, and this speed and slope were used for all subsequent exercise tests.

Horses were assigned to either cold air or warm air exercise tests in a randomized crossover design with a minimum 1-wk washout between exercise tests. Each exercise test consisted of walking (1.8 m/s, 0° slope, 5 min), trotting (4 m/s, 0° slope, 5 min), and cantering (6.8–9.5 m/s, 2.5° slope, 5 min), differing only in the temperature and relative humidity (RH) of the inspired air: 25°C and 55% RH (12.6 g H2O/m3 air) for warm air exercise tests and −5°C and >95% RH (3.5 g H2O/m3 air) for cold air exercise tests. The cold air was produced by drawing ambient air through a high-volume air chiller connected to a loose-fitting face mask worn by the horse during exercise. The volume of air delivered (3,800 l/min) was in excess of the reported maximal inspiratory rate for horses exercising at submaximal levels (26) to prevent the horses from inhaling unchilled ambient air during exercise.

Five hours after completion of each exercise test, horses were sedated with intravenous xylazine (0.5 mg/kg) and butorphanol (0.025 mg/kg), and bronchoalveolar lavage was performed using a cuffed tube and 240 ml of warmed Hanks’ phosphate-buffered saline. The recovered fluid was pooled to determine overall recovery, and a 3-ml aliquot was removed for determination of total and differential cell counts. Slides were prepared by cytocentrifugation and stained with a

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modified Wright-Giemsa stain. The remaining fluid was centrifuged at 770 g for 10 min, and the supernatant was removed. Sixty milliliters of the supernatant were passed through a C-18 solid-phase extraction cartridge (Water), which was subsequently eluted with 4 ml of methanol. Aliquots of the extract were evaporated to dryness and analyzed for leukotriene B4 and cysteinyl leukotrienes by using commercially available kits (Cayman Chemical). The pelleted cells were resuspended in 1 ml of a proprietary phenol-guanidine isothiocyanate mixture (Trizol, Invitrogen), transferred to a 2-ml microcentrifuge tube, and homogenized by using a disposable pestle. An additional 1 ml of Trizol was added to the homogenate, and the sample was frozen at −80°C until RNA extraction and cytokine message quantitation (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ, and TNF-α) using real-time quantitative RT-PCR and species-specific primers and probes. At the time of analysis, samples were thawed and mixed with an equal volume of chloroform, and the aqueous phase was transferred to a new microcentrifuge tube. RNA was precipitated with −1 ml of isopropanol and centrifuged at 13,000 g, and the supernatant was discarded. Precipitated RNA was washed with sequential mixing and centrifugation of ethanol and water. Total RNA was quantified spectrophotometrically on the basis of 260-nm-absorbance at 280-nm absorbance ratios. Cytokine gene expression (IL-1

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
<th>Primer/Probe Reaction Concentrations</th>
<th>Total RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>TGGAAGAATCCAGGCCGCTCTA</td>
<td>CGGTCACTTTGCGGAAATAAC</td>
<td>ACTACCTGAAATGCGTGGCCCACCTAAGCCT</td>
<td>900/900</td>
<td>100 ng</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGGAAGAATCCAGGCCGCTCTA</td>
<td>CGGTCACTTTGCGGAAATAAC</td>
<td>ACTACCTGAAATGCGTGGCCCACCTAAGCCT</td>
<td>700/700</td>
<td>50 ng</td>
</tr>
<tr>
<td>IL-2</td>
<td>TGGAAGAATCCAGGCCGCTCTA</td>
<td>CGGTCACTTTGCGGAAATAAC</td>
<td>ACTACCTGAAATGCGTGGCCCACCTAAGCCT</td>
<td>900/900</td>
<td>100 ng</td>
</tr>
<tr>
<td>IL-4</td>
<td>CGGACATGGCGTCTGCCGTTCA</td>
<td>CAGGGCTAGATTTGCTCTTC</td>
<td>ATGCCCTTGGCAGGAAAACAGACAGA</td>
<td>700/700</td>
<td>20 ng</td>
</tr>
<tr>
<td>IL-5</td>
<td>GTGCTGAGCTGGAGCTTGTGCTTA</td>
<td>CGGTCACTTTGCGGAAATAAC</td>
<td>ACTACCTGAAATGCGTGGCCCACCTAAGCCT</td>
<td>900/900</td>
<td>50 ng</td>
</tr>
<tr>
<td>IL-6</td>
<td>GTGCTGAGCTGGAGCTTGTGCTTA</td>
<td>CGGTCACTTTGCGGAAATAAC</td>
<td>ACTACCTGAAATGCGTGGCCCACCTAAGCCT</td>
<td>900/900</td>
<td>50 ng</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>AGGAATGATTCCAGCTGATCTGCTTC</td>
<td>AGGAAGAGAGTACAGACCAGCGT</td>
<td>TGAGTGTTGAGAACACAAAGAAATGCTACTTCA</td>
<td>900/900</td>
<td>50 ng</td>
</tr>
<tr>
<td>TNF-α</td>
<td>AGGAATGATTCCAGCTGATCTGCTTC</td>
<td>AGGAAGAGAGTACAGACCAGCGT</td>
<td>TGAGTGTTGAGAACACAAAGAAATGCTACTTCA</td>
<td>700/700</td>
<td>50 ng</td>
</tr>
</tbody>
</table>

CTT, threshold cycle. See MATERIALS AND METHODS for description of ΔΔCT calculations.

Table 1. Cytokines quantified in the study, primer/probe sequences, primer/probe reaction concentrations, and total RNA used to quantify ΔΔCT values.

Table 2. Purity and concentration of RNA used to evaluate airway cytokine expression.

Horse: Sample | A260 | A280 | A260/A280 | Concentration
--- | --- | --- | --- | ---
Bella: Warm Air | 0.552 | 0.290 | 1.9 | 1.1 μg/μl
Bella: Cold Air | 0.804 | 0.419 | 1.9 | 1.6 μg/μl
Blondie: Warm Air | 0.609 | 0.319 | 1.9 | 2.44 μg/μl
Blondie: Cold Air | 0.608 | 0.322 | 1.9 | 2.43 μg/μl
Coalie: Warm Air | 0.919 | 0.516 | 1.8 | 3.68 μg/μl
Coalie: Cold Air | 0.430 | 0.267 | 1.7 | 1.72 μg/μl
Ivan: Warm Air | 0.914 | 0.478 | 1.9 | 1.83 μg/μl
Ivan: Cold Air | 0.690 | 0.387 | 1.8 | 1.38 μg/μl
Major: Warm Air | 0.289 | 0.177 | 1.7 | 1.156 μg/μl
Major: Cold Air | 0.722 | 0.382 | 1.9 | 2.89 μg/μl
Poco: Warm Air | 0.736 | 0.386 | 1.9 | 2.94 μg/μl
Poco: Cold Air | 0.714 | 0.371 | 1.9 | 1.43 μg/μl
Skipper: Warm Air | 0.585 | 0.304 | 1.9 | 2.34 μg/μl
Skipper: Cold Air | 0.703 | 0.378 | 1.9 | 1.4 μg/μl
Xena: Warm Air | 0.452 | 0.269 | 1.7 | 0.904 μg/μl
Xena: Cold Air | 0.706 | 0.375 | 1.9 | 1.41 μg/μl

A260, absorbance at 260 nm; A280, absorbance at 280 nm.
The overall cytokine pattern is predominantly a TH2 profile, while breathing cold dry air alters the airway cytokine profile. Neutrophils; Eos, eosinophils; Epith, epithelial cells. PMN, Eos, and Epith are fluid (BALF) differentials. Macro, macrophages; Lymph, lymphocytes; PMN, Eos, and Epith are half of the differential cell counts, and in most cases they accounted for less than 1% of the total differential count. BALF leukotriene concentrations were not different after warm air exercise and cold air exercise (leukotriene B4, 191.5 ± 97.89 vs. 214.7 ± 105.6 pg/ml, respectively; cysteinyl leukotrienes, 54.38 vs. 37.62 ± 20.97 pg/ml, respectively). BALF samples yielded cellular RNA of uniform consistency and with RNA concentrations exceeding 1 μg/μl in all but one sample (Table 2).

Airway cytokines characteristic of the TH2 phenotype were significantly increased by exercise while breathing cold air (Fig. 2). Median IL-4 mRNA expression was 12-fold greater and median IL-5 mRNA expression was 9-fold greater after exercise while breathing cold air than after exercise while breathing warm air. Other cytokines with proinflammatory activity (IL-6 and IL-2) showed smaller, but statistically significant, increases in mRNA expression after exercise while breathing cold air (3- and 6-fold increase, respectively). The median mRNA expression of the immunomodulatory cytokine IL-10 was 10-fold greater after exercise while breathing cold air. No other measured cytokines were significantly different between exercise while breathing warm air and exercise while breathing cold air.

DISCUSSION

Results of this study support the hypothesis that exercise while breathing cold air alters the airway cytokine profile. The overall cytokine pattern is predominantly a TH2 profile, which is not only the profile associated with preferential production of antibodies and downregulation of cell-mediated immunity but is also characteristic of asthma (42). However, we failed to demonstrate concurrent airway inflammation in the form of increased airway leukocytes or increased concentrations of leukotrienes. This is not necessarily surprising, because cytokine production is presumed to precede the cellular inflammatory response. Nevertheless, our results support the novel contention that exercise while breathing cold air can actually contribute to the development of asthma.

There were no significant differences in the volume of recovered BALF (warm 130.7 ± 4.7 ml; cold 130.0 ± 6.2 ml) or concentrations of different nucleated cells in the recovered BALF (Fig. 1), although there was a strong trend (P = 0.0625) toward increased bronchial epithelial cells in the lavages recovered after cold air exercise. Mast cells were detected in only half of the differential cell counts, and in most cases they accounted for less than 1% of the total differential count. BALF leukotriene concentrations were not different after warm air exercise and cold air exercise (leukotriene B4, 191.5 ± 97.89 vs. 214.7 ± 105.6 pg/ml, respectively; cysteinyl leukotrienes, 54.38 vs. 37.62 ± 20.97 pg/ml, respectively). BALF samples yielded cellular RNA of uniform consistency and with RNA concentrations exceeding 1 μg/μl in all but one sample (Table 2).

Previous studies have identified potential mechanisms for the transduction of the initial stimulus (airway cooling and desiccation secondary to exposure to unconditioned air) to airway inflammation. Both mast cells (9, 10) and airway epithelial cells (20) are osmotically sensitive and are activated by local airway hyperosmolarity that may occur during exercise while breathing cold air. The known osmotically induced repertoire of cytokine production by airway epithelium is rather limited, with only IL-8 and RANTES (regulated on activation, normal T-cell expressed and secreted) identified in vitro (19, 20). Although we expected to find upregulation of IL-8 expression and influx of airway neutrophils, we detected neither in this study. On the other hand, mast cells are rich sources of cytokines (4, 5), and although there are no studies specifically detailing cytokine production by osmotically activated mast cells, it has been previously shown that mast cells degranulate in response to hyperosmolar stimuli both in vitro (9, 10) and in vivo (18, 36). Furthermore, mast cell products are increased in blood after strenuous exercise while breathing cold air (24, 25), and drugs that inhibit mast cells are useful in blocking exercise-induced bronchoconstriction (13–15). Other sources of cytokines, such as T lymphocytes and macrophages, are not known to respond to nonantigenic stimuli and are thus unlikely to be the source of initial stimulus. Thus it is likely that mast cell activation is a key cellular event leading from airway cooling and desiccation to the expression of TH2 cytokines.

Although mast cells appear to be involved in the initial signal transduction and are capable of producing cytokines, they are unlikely to be the source of cytokine mRNA measured in this study. There were minimal numbers of mast cells recovered in the BALF from any of the horses (Fig. 1). Another step leading from mast cell activation to expression of TH2 cytokines in BALF cells is needed. The BALF recovered from the horses contained ample numbers of lymphocytes, and these cells are capable of autocrine TH2 amplification after exposure to TH2 cytokines (42), even if those cytokines were initially released by other cells. Thus we believe that peripheral airway cooling and desiccation during exercise while breathing cold air led to mast cell activation and release of TH2 cytokines, a response that was subsequently amplified by resident airway lymphocytes. Other investigators have pinpointed cellular expression of cytokine mRNA using in situ hybridization (23), and this technique would be appropriate for further studies of the equine airway responses to cold air to determine the cell populations active during these responses.
An alternative (or perhaps additional) mechanism for transducing the initial airway stimulus to preferential secretion of Th2 cytokines is through the release of neurokinins. Neurokinins have been shown to induce secretion of some Th2 cytokines is through the release of neurokinins. Neurokinins from T lymphocytes, even when those lymphocytes have been committed to a Th1 phenotype (27), and selective inhibition of neurokinin type 2 receptors can block the development of eosinophilia and expression of Th2 cytokines after allergen challenge in allergic mice (30). Neurokinins are released into the airway after hyperpnea challenge in a number of animal models, either as a primary event or secondary to leukotiene release (17). If the latter is true, then the lack of detectable change in BALF leukotrienes argues against a role for neurokinins in the induction of Th2 cytokines in equine airways after cold weather exercise. However, more direct studies are required to confirm or exclude this phenomenon.

A limitation of this study is that although we have described the effect of inspiring cold air while exercising, we have no information regarding the effect of the exercise challenge itself. Suzuki et al. (41) have shown that the circulating lymphocyte population is biased toward Th2 profile of cytokine secretion after exercise, an effect that may be due to concurrent release of glucocorticoids during exercise (11, 12). A similar pattern has been described in mice (22). These findings raise the question of whether the pattern of cytokine production in the airway cells is simply a reflection of systemic predisposition toward a Th2 phenotype, secondary to exercise-induced cortisol secretion. Although we cannot completely refute this possibility, it is interesting to note that Ainsworth et al. (2) failed to demonstrate a shift in cytokine expression in peripheral blood after exercise in horses. Furthermore, we found no significant difference in circulating cortisol concentrations in a different group of horses performing similar exercise while breathing warm and cold air (unpublished data). These observations, combined with the tenable hypothesis for upregulation of cytokine expression after cold air exercise, support our contention that the findings described in this paper are caused by local airway conditions or events.

The potential effect of the cytokine expression shift described in this study and the resulting alteration in antigen responses are consistent with common pulmonary diseases of both equine and human athletes. Antibody-mediated pulmon ary hypersensitivity (“heaves”) is common in horses (3) and has been linked to increased expression of Th2 cytokine expression (6, 23). The most prominent antigens identified in this syndrome are mold spores found in hay and straw bedding (32, 33), thus closely linking the development of heaves to the horse’s environment. Once a horse has developed such hypersensitivity, clinical exacerbations consisting of airway inflammation, recurrent airway obstruction, and airway mechanical hyperreactivity can be readily reproduced with environmental exposure to specific allergens, and remission can be achieved on removal of the horse from an environment conducive to allergen exposure (38). In this regard, heaves is quite similar to human asthma, including the fact that the initial cause of the hypersensitivity has not been elucidated. The data of this study provide a compelling possibility: that strenuous exercise followed by exposure to environmental antigens promotes overproduction of antibodies to those antigens. It would follow from this line of reasoning that heaves is more prevalent in athletic horses compared with sedentary cohorts and that heaves is more prevalent in colder climates compared with warmer climates. Unfortunately, neither of these possibilities has been critically explored in the existing literature.

The implications of these data can be extended to the syndrome of ski asthma. The precise nature of the inflammation characteristic of ski asthma, including the relative cytokine expression in the airways of affected subjects, has not been described. However, increased numbers of eosinophils in the airways of subjects with ski asthma (21) strongly implicate increased expression of IL-5, one of the hallmark cytokines of the Th2 phenotype (28) and one of the cytokines that were significantly expressed after exercise while breathing cold air (Fig. 2). Thus our data not only suggest the contention that some, if not all, of the abnormalities described for ski asthma result from exposure of airways to unconditioned air but also raise the question of whether other Th2-associated cytokines are increased in these subjects.

Our data further raise the possibility of local suppression of cell-mediated immunity through the increased expression of IL-10. The concept of exercise-induced immunosuppression is not novel, as many investigators have produced data supporting the concept of the “open window” of transient immune suppression after strenuous exercise (34). Furthermore, previous studies have demonstrated increased susceptibility to respiratory viruses in animals after strenuous exercise (3). However, we believe our data are the first to provide a specific mechanism for the exercise-induced open-window effect as a local pulmonary phenomenon. The potential deleterious effect of increased IL-10 expression is increased susceptibility to pathogens, particularly those that are normally cleared by cell-mediated immunity (29). On the other hand, it is possible that the increase in IL-10 is an appropriate response that moderates the net effect of the increased proinflammatory cytokine expression. These issues warrant further investigation, for which the equine model is ideally suited.

GRANTS

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REFERENCES


