Review Article

Deciphering the Role of Bronchial Hyper-Responsiveness in Equine Pasture Asthma

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ABSTRACT

Bronchial hyper-responsiveness (BHR) describes a lung abnormality in which airways are easily triggered to constrict in response to normally harmless inhaled stimuli, and is a key element of human asthma pathophysiology. BHR contributes to equine respiratory diseases including inflammatory airway disease and recurrent airway obstruction. Collectively these diseases account for over 80% of poor performance in equine athletes, and at least 10% of veterinary admissions. BHR is also a contributing factor in 'exercise induced pulmonary hemorrhage'. Increased sensitivity to airway constriction that characterizes BHR is a documented sequel to viral respiratory infections in several species, including horses and humans. Five respiratory viruses known to circulate extensively in equine populations place the horse at risk for BHR. Despite adverse effects of BHR on equine health, there remains a gap in our fundamental understanding of how gene products coordinate in the lung to cause BHR. Leveraging the equine genome sequence, we employ systems biology including proteomics and RNA sequencing to model the complex biology of BHR in the lungs of horses with pasture asthma. Using a self controlled experimental design, gene products that segregate with seasonal asthma exacerbation in diseased horses are being identified and their relevant physiology identified to address the need for better recognition and management of BHR in equine disease.

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1. Introduction

Bronchial hyper-responsiveness (BHR, aka airway hyper-responsiveness) is a key pathophysiologic feature of asthma that is characterized by increased airway sensitivity to inhaled agonists of bronchoconstriction [1]. This includes a steeper slope of the dose-response curve and a greater maximal response to these constrictors. Practically speaking, it is a state in which airway contraction is easily triggered in response to substances that do not normally elicit this response in healthy individuals. BHR is recognized as an important factor in airway diseases that severely limit the health, well-being, athletic potential, and financial value of horses in the United States and abroad [2–6]. BHR is also the keystone of bronchoconstriction in human asthma that drives the development of novel therapeutic agents [7,8]. Despite the clear importance of bronchial homeostasis to equine health and athletic potential, the contributions of BHR in equine diseases and the molecular mechanisms by which inhaled stimuli result in BHR and bronchoconstriction

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remain poorly characterized. Improved characterization of BHR will improve diagnosis, treatment, athletic potential, and genetic selection in horses. These findings also have strong translational relevance for human asthma.

2. Impact of BHR Diseases on Equine Health

BHR is a defining characteristic of the pathophysiology of two common respiratory conditions of the horse, inflammatory airway disease (IAD) [2] and recurrent airway obstruction (RAO) [3]. Reflecting the similarities of these diseases with human asthma, a new nomenclature has been advanced, using the names mild and/or moderate equine asthma for IAD [9], and severe equine asthma for RAO [10]. To prevent ambiguity in addressing two distinct severe equine asthma syndromes, the terms barn dust asthma and pasture asthma are used in the text to distinguish the two syndromes. The human condition is termed as human asthma, and IAD is distinguished as mild and/or moderate equine asthma.

Horses with mild and/or moderate equine asthma appear clinically normal at rest, but exhibit poor athletic performance and airway inflammation [3]. Mild and/or moderate equine asthma accounts for 60%–80% cases of poor performance in equine athletes [11,12] and has been shown to be a risk factor for exercise-induced pulmonary hemorrhage [13]. Horses with severe equine asthma, both barn dust asthma [2] and pasture asthma [14], exhibit exacerbations of acute airway obstruction that cause respiratory distress and result from bronchospasm, mucus accumulation, and airway inflammation. Severe equine asthma has been cited as one of the most frequent respiratory diseases in horses, constituting 11% of US veterinary admissions [15] and 14% of the general horse population in Great Britain [6]. In addition, a significant body of evidence indicates that viral infections lead to BHR and a propensity for bronchoconstriction [6,15–18]. Accordingly, there is ample evidence that BHR has a major impact on health worldwide [2–6].

Barn dust asthma is generally recognized as a disease that occurs in mature horses worldwide that are housed in stalls [2]. However, two severe equine asthma syndromes are described. Barn dust asthma occurs more commonly in continental climates [2], whereas pasture asthma affects horses maintained in pasture during summers in the southeastern United States and the United Kingdom [19,20]. Prevalence of these two BHR diseases, barn dust asthma and pasture asthma, are estimated to be 11% and 5% of veterinary admissions, in the United States and in Louisiana (unpublished survey), respectively [21,22]. Although the stimuli eliciting barn dust asthma in horses are likely complex and diverse, the disease is reliably triggered, and has been induced by challenge with organic molds that are found in hay [23,24]. Pasture asthma is also reported in mature horses, but differs from barn dust asthma because affected horses are housed primarily on pasture, where exacerbations are statistically associated with hot temperatures and high humidity (dew point temperature), fungal spores, and grass pollens [19,25]. Horses with pasture asthma experience remission of clinical signs during the winter. From a diagnostic, therapeutic, and etiopathologic standpoint, an important distinction between barn dust asthma and pasture asthma in the southeastern United

States is that the horses with pasture asthma in this region demonstrate marked improvement when removed from pasture-associated aeroallergens and maintained in a stall without pasture access. It is noteworthy that profound hypoxemia is not uncommon in horses with pasture asthma. Reversing prolonged hypoxic vasoconstriction and bronchospasm in these cases may necessitate oxygen therapy to achieve responsiveness to environmental change and bronchodilator therapy. Although horses with pasture asthma can progress to exhibit year-round respiratory impairment at rest, the severity of pasture asthma during the summer generally leads to humane euthanasia of affected horses before the onset of severe respiratory impairment in the winter.

3. BHR is a Complex, Multifactorial Disease

BHR has been extensively investigated as a central component of human asthma pathobiology. In their insightful review, Chapman and Irvin [26] summarize emergent knowledge that BHR reflects multiple mechanistic factors each with differing and fluid contributions across the human asthmatic population. Accordingly, BHR reflects a sum total of the dynamic interactions of inflammatory pathways, airway smooth muscle, airway epithelia, structural remodeling of the airways, and genetic factors, which are influenced by changing environmental factors as well as specifics of asthma therapy. From this understanding emerges a paradigm in which mechanisms of bronchoconstriction have the promise to explain clinical phenotypes and improve their treatment.

Airway smooth muscle is recognized as pivotal cell type mediating BHR and long-term lung function impairment in human asthma [27]. However, airway smooth muscle has emerged to have roles in BHR and bronchoconstriction that far exceed actin- and myosin-mediated muscle contraction. Changes in contractile responses, proliferation, cell migration, secretion of inflammatory mediators, receptor densities, and receptor sequence all contribute variably to BHR in human asthma patients [28]. Increased airway smooth muscle is a prominent feature of airway remodeling that correlates to worsening pulmonary function in human asthma [29]. Increases in airway smooth muscle are greater in subjects with severe human asthma as compared with less severe forms of the disease, indicating that increased airway smooth muscle is a key structural change in the progression from less severe to more severe BHR [29,30]. Increased airway smooth muscle mass and proliferation have been identified in the lung of horses with barn dust asthma in response to antigen exposure [31,32], with antigen avoidance decreasing airway smooth muscle in barn dust–affected horses [31–33]. Similarly, in the lungs of horses with pasture asthma, increases in smooth muscle mass have been identified [14,34].

Sympathetic and parasympathetic nervous system inputs moderate airway smooth muscle caliber. Parasympathetic innervation is considered the dominant neural pathway from which cholinergic stimulation of muscarinic receptors (M1–M5) elicits bronchoconstriction and mucus secretion. Acetylcholine stimulates airway smooth muscle contraction primarily via stimulation of muscarinic M3 receptors [35]. Differences in muscarinic receptor

expression and subtype distribution have not been detected in lung tissue from horses with barn dust asthma [36].

Muscarinic M1 receptors in the parasympathetic ganglia facilitate ganglionic neurotransmission and are found in peripheral equine lung, but not trachea or bronchi [37]. Muscarinic M2 receptors are the most plentiful muscarinic receptors in equine bronchi and peripheral lung [37]. Muscarinic M2 receptors located on postganglionic parasympathetic nerves have a major role in limiting acetylcholine release in an autocrine fashion, providing negative feedback control that is physiologically relevant and limits airway smooth muscle contraction [35]. Loss of M2 receptor function has been demonstrated in human asthma, animal models of BHR, and in barn dust asthma, leading to a vagal-mediated BHR [38,39]. Muscarinic M2 receptor dysfunction is of increasing interest as a mechanism of BHR induced by viral infection [40,41] and hyperinsulinemia [42]. Muscarinic M2 can also indirectly limit β_2-adrenoceptor–mediated sympathetic nervous system relaxation of airway smooth muscle through inhibition of adenylate cyclase [35].

Contrasting parasympathetic innervation, direct sympathetic innervation of airway smooth muscle has not been demonstrated. Instead, adrenergic stimulation of β_2-adrenoceptors (β_2-AR) on airway smooth muscle results in bronchodilation. Alpha$_2$-adrenoceptor (α_2-AR) stimulation inhibits acetylcholine release, accounting for improvement in pulmonary function that has been observed with the administration of α_2-AR agonists [43,44]. Alpha$_2$-adrenoceptor dysfunction has been described in airways of horses with barn dust asthma [39]. Finally, differences in β_2-AR density, attenuated coupling of β_2-AR to stimulatory G_s-protein, decreased high affinity β-AR in lung and bronchi, and decreased activation of adenylate cyclase activity have all been identified in horses with barn dust asthma [45].

In both homeostasis and disease, neurogenic regulation of bronchial tone is also moderated by a complex interplay of nonadrenergic noncholinergic cotransmitters, neuropeptides, and diverse inflammatory mediators [46–52]. Isolated effects of these mediators and receptors on bronchial tone continue to be characterized. Although this information reveals important facets of airway smooth muscle biology, compartmentalization of mediator effects is not reflective of the sum total of interactions that affect airway smooth muscle at the organism level.

4. Horses with Equine Pasture Asthma Demonstrate Clinical Features of Human BHR

To investigate the mechanisms of BHR in pasture asthma, defining characteristics of the contractile phenotype including clinical and pathologic facets of the disease were prioritized. In describing BHR, several distinctions are relevant. Quantifying BHR necessitates the ability to measure pulmonary function, restricting availability of testing for horses. BHR can be specific, or nonspecific, referring to whether the process of eliciting BHR diagnostically is antigen-specific [53]. Clinically and diagnostically, BHR is identified using agents that cause bronchoconstriction via direct effects on the airways (methacholine and histamine), or that work indirectly (antigen, mannitol, hypertonic saline) by inducing release and synthesis of contractile inflammatory mediators [54]. Controlled bronchoconstriction is induced using serial increasing doses by nebulization to achieve a threshold decrease in pulmonary function (bronchoprovocation) [55]. In human asthma, the magnitude of BHR as measured with bronchoprovocation is proportional to multiple measures of asthma severity including risk of exacerbation, declining lung function, and a need for increased treatment to control symptoms [56–60].

Although BHR has consistently been demonstrated to be proportional to human asthma severity, the severity of respiratory impairment during an isolated asthma episode reflects the magnitude of airway obstruction, not the magnitude of BHR. Lessons from bronchoprovocation testing to identify BHR in human asthmatics indicate that BHR is not stable, such that failure to detect BHR must be interpreted in the clinical context [53,61–63]. Corticosteroids and withdrawal of the inciting aeroallergen would be expected to decrease BHR, particularly to indirect bronchoprovocation [64,65]. Intercurrent pneumonia could be expected to worsen BHR. Thus, bronchoprovocation is useful in human asthmatics that report symptoms, particularly in those with normal baseline lung function [66]. This leads to the rationale that negative bronchoprovocation in the presence of active asthma symptoms indicates that diagnoses other than asthma should be considered in humans [26]. Variability in the nature of bronchoprovocation has been documented in horses using direct and indirect bronchoconstriction agents in horses with barn dust asthma [67]. This points to the utility of delineating the specific phenotypic characteristics of bronchoconstriction in BHR equine respiratory diseases to improve recognition, diagnostic testing modalities, and management of these conditions.

During a 6-year period, methacholine bronchoprovocation has been used to quantify BHR in horses with pasture asthma during seasonal disease remission. BHR of a magnitude that is diagnostic of moderate to severe human asthma has been consistently identified, indicating that BHR in this population persists between episodes of seasonal disease exacerbation. That pasture asthma is a lifelong disease indicates that BHR persists for life. In line with the severe, long-term, progressive, and debilitating nature of the condition in the southeastern United States, and inherent difficulty in isolating horses from pasture aeroallergens, methacholine bronchoprovocation is performed in our clinical equine population as a screening diagnostic during seasonal remission to identify horses at risk of having pasture asthma. Most horses require operant conditioning to accommodate the instrumentation for procedures. Similarly, BHR to methacholine and indirect bronchoconstriction have also been identified in horses with barn dust asthma, persisting for a period of days to weeks following removal of the inciting antigen [67–71]. Hyper-responsiveness to nebulized challenge with unique mold species has also been demonstrated in barn dust asthma [72–74].

5. The Equine Genome Sequence: A Bridge to Identifying Protein Signatures of Respiratory Health and Disease

To be able to leverage knowledge gained from investigations of BHR in human asthma, we sought evidence

that the collective *functions* of gene products directing lung biology during homeostasis are shared between horses and humans. To achieve this goal, functional annotation was provided for the Texas A&M 21,351 element equine whole genome oligonucleotide array [73]. Functional annotation assigns the biological functions of a gene product in a codified manner, using a universal hierarchical language termed gene ontology (GO) [74]. Genome-scale Omic technologies generate lists of hundreds to thousands of gene products, exceeding the ability of the human mind to coordinate information for each entry and extract its relevant biology. Gene ontology annotation and the associated analyses make it possible to analyze a data set of this size and identify, for example, among 45 proteins that broadly participate in "cellular carbohydrate metabolic processes" (assigned the identifier GO:0044262), the 10 proteins that exert this effect through "positive regulation of gluconeogenesis" (assigned GO:0045722). By comparing the way that GO annotations in large sets of gene products are enriched in one population relative to another, it is possible to identify over- and under-represented molecular functions, cellular components, and biological processes. In performing this GO annotation, a significant delay in annotation of peer-reviewed literature to the GO database was identified, across all species. This challenge is magnified in the horse, for which a dedicated, funded, manual biocuration effort does not exist. Accordingly, at this time, computational modeling is not necessarily reflective of the comprehensive knowledge of biology. To address this need, additional commercial and academic sources for information relative to gene function and interaction have arisen, for example, Ingenuity Knowledge Base, Pathways Studio (https://www.pathwaystudio.com/) and the Kyoto Encyclopedia of Genes and Genomes (http://www.kegg.jp/kegg/).

With this improved functional annotation of the equine genome sequence in place, we performed shotgun proteomics to identify and quantify proteins in cell-free bronchoalveolar lavage fluid (BALF) from six normal horses, using the equine genome sequence for peptide prediction [75]. We hypothesized that if the biologic functions of proteins that define homeostasis in lungs of horse and human were conserved, these functional groups of proteins would occur in the same relative abundance. We identified 582 unique proteins in normal BALF and compared them with published cell-free proteomes from mouse and human, as described. Gene ontology modeling identified conservation, between the normal BALF proteomes of human, mouse, and horse, of seven cellular component GO categories, eight molecular function GO categories, and four biological process GO categories among the top 10 categories in each ontology. Thus, GO modeling of the normal BALF proteome demonstrated conservation of protein functions in lung fluids across these species. We are now using this approach to model the effects of BALF proteins to understand their role in neutrophilic airway inflammation, a disease facet that distinguishes equine asthma from other animal asthma models. Building on previous and ongoing efforts to improve functional annotation for the equine genome, our effort demonstrates how functional annotation can be organized to identify the complex biology within genome scale expression data sets,

in a manner that is relevant to investigating disease pathophysiology.

6. Canonical Pathways Identify Signatures of Human Asthma in Horses with Equine Pasture Asthma

Decades of research have culminated in an understanding that disease pathogenesis, particularly that of complex diseases, reflects imbalances in a system of thousands of gene products that work in a coordinated fashion to achieve homeostasis [76]. Investigating these diseases is therefore best served by approaches that can identify and quantify changes in this coordinated network. Before the availability of the equine genome sequence, equine disease research relied on painstakingly quantifying individual gene products as messenger RNA or proteins [77–79]. Not only did this approach necessitate extensive and costly development and validation of equine-specific reagents, its reliance on quantifying individual gene products was poorly suited to identifying changes in the composition of networks of gene products that work in unison and are incompletely characterized. These limitations were forever changed with the emergence of Omics technologies [80]. These technologies, and in particular RNA sequencing, bring scalability to accurately quantify all RNA species across the range of expression that is characteristic of any biologic sample [81]. This tool has revolutionized disease pathogenesis and drug discovery research, and is particularly well suited to investigating the complex interactions of genetic and dynamic environmental factors that characterize asthma [80].

BHR, as a defining feature of human asthma, has been extensively investigated for decades. Still, the coordinated biologic pathways that prevent or cause variable severity of BHR across individuals, despite equivalent aeroallergen challenge to the respiratory epithelium, remain poorly characterized. To determine the suitability of the horse for identifying pathophysiologic mechanisms of BHR with the potential to impact novel asthma therapeutics in horses and humans, we characterized the lung transcriptome of horses with pasture asthma during disease exacerbation. In preliminary experiments, short-read RNA sequencing was performed on serial thoracoscopic lung biopsies acquired from the same individual pasture asthma-affected horses and their nondiseased herdmates, during seasonal disease exacerbation and remission. This experimental design provides a self-controlled model and a unique framework for simultaneously controlling individual and environmental variability, so that gene products segregating with disease can be refined. Furthermore, this approach can be expanded to incorporate variables that account for differences in disease severity in the analysis of differentially expressed genes. Rather than simply viewing disease as a dichotomous (diseased/nondiseased) variable, such an approach to analysis makes it possible to identify gene products that are associated with, and can predict relevant changes in clinical disease severity.

To this end, sequences were filtered in a standard manner, aligned to the equine reference genome (EquCab2.0), and nearly 200 unique transcripts were identified as differentially expressed by disease and season at a 5%
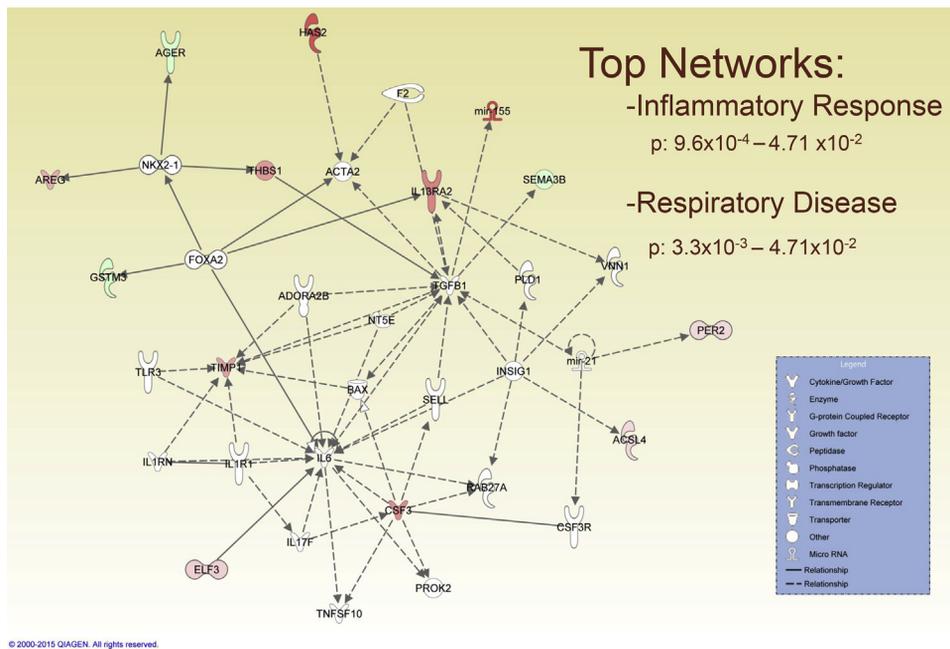
Fig. 1. Molecular signatures of asthma were identified by modeling 180 gene products that were differentially expressed by season and disease using Ingenuity Pathways Analysis. The canonical pathways, inflammatory disease and respiratory disease, were significantly over-represented in diseased horses during disease exacerbation.

false discovery rate. Orthologous proteins as predicted in human, mouse, and rat were identified (www.biomart.org). Consistent with the clinical disease phenotypes of pasture asthma and human asthma, canonical pathway analysis performed using Ingenuity Pathway Analysis (www.qiagenbioinformatics.com), identified "inflammatory response" and "respiratory disease" as the top molecular networks (Fig. 1) from gene products with documented roles in BHR and human asthma. This finding supports the relevance of using the pasture asthma–restricted lung transcriptome, in this self-controlled manner, for deciphering the pathophysiology of BHR in horses.

RNA sequencing provides a powerful tool to explore altered regulation in biological networks. Nonetheless, some challenges are particularly noteworthy. RNA sequencing provides a snapshot of the transcriptome at a point in time that may not be reflective of a larger period. Pervasive questions remain on how to best analyze and interpret the abundance of data this technology provides, particularly because different analysis pipelines do not yield identical outcomes. Also, characteristics of the sample influence the utility of this technology. For instance, our experimental design necessitates additional experiments to localize differential expression within individual cell types of lung tissue. Many gene products are also not transcriptionally regulated, such that the presence or quantity of mRNA does not assure proportional protein expression. Finally, although these data can be extremely useful to identify changes in gene networks that segregate with disease, and to identify novel therapeutic targets, comprehensive analysis of the thousands of gene products described remains cumbersome, particularly when identifying specific gene targets.

In characterizing the lung transcriptome of horses, a particular challenge occurs with the assignment of

significance when expression of a gene product is zero in any sample. Absence of expression may indeed reflect an absence of the transcript, or that the depth of sequencing was inadequate for detection. Several solutions to this problem have been proposed [82]. In analyzing data of this nature, we identify genes with zero expression data in remission and some level of expression in the exacerbation season, or the converse. Manual literature reviews are conducted to identify gene products with documented relevance to asthma, but also to search terms of relevance to the biologic question at hand. Among the gene products identified in this manner were endothelin [83], IL-6 [84], and TNF [85] which all have documented roles in the pathophysiology of human asthma, further supporting the translational relevance of our approach. In our pipeline, expression of targeted gene products is next documented at the protein level using immunohistochemistry, and the relevant physiology is documented by modulating each protein's effects using isolated airways in tissue baths and precision cut lung slices.

7. Summary

The equine genome sequence, coupled with the advent of genome-scale expression platforms, have facilitated equine genome research. However, harnessing the full potential of these technologies is hindered by limited assignment of function to equine gene products, and even greater limitation to the assignment of this information in a manner that is computationally accessible. Most experiments that use these Omics technologies rely on extrapolations of gene product functions from human, mouse, and rat. Building on strong clinical and histologic similarities between equine pasture asthma and human asthma, we enriched existing functional annotation, and used both

proteomics and RNA sequencing in the horse to further characterize the similarities between the two conditions at a network level. We have confirmed conservation of gene product functions in normal BALF across human, mouse, and horse, and demonstrated that the canonical gene pathways that mediate inflammatory disease and respiratory disease are conserved in the lungs of horses with pasture asthma and human asthmatics. Based on these findings, and with support from the Agriculture and Food Research Initiative Animal Health Program competitive grant no. 2015-67016-23172 from the USDA - National Institute of Food and Agriculture, we are characterizing differences in the lung transcriptome of horses with pasture asthma to identify novel aspects of BHR physiology as potential therapeutic diagnostic and targets.

Acknowledgments

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