Study on bronchoconstriction induced by histamine or serotonin in "Precision Cut Lung Slices" of the horse

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Summary

The aim of this study was to determine the effect of histamine and serotonin, as well as the influence of their inhibitors, on bronchoconstriction using equine precision-cut lung slices (PCLS), which were prepared from 5 horses affected by various degrees of small airway disease (SAD) and a sixth horse free of respiratory disease. Over all horses, histamine caused a concentration-dependent bronchoconstriction, with a median effective concentration of 1820 nM. The histamine induced bronchoconstriction could be inhibited by incubation with the H1-receptor antagonist cetirizine before the addition of histamine. However, incubation with the H2-receptor-antagonist ranitidine, the H2 and H3-receptor antagonist thioperamid and the H4-receptor antagonist JNJ7777120 had no significant effect. Serotonin induced a concentration-dependent bronchoconstriction in the horse without evidence of airway disease only. The serotonin-induced reaction in this case was inhibited when the PCLS was previously incubated in ketanserin. The possibility that respiratory disease could have a negative effect on the serotonin-receptor or the serotonin-induced bronchoconstriction pathway is discussed.

Keywords: Bronchoconstriction / histamine / serotonin / PCLS / horse / pulmology / chronic airway disease

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Untersuchung der durch Histamin oder Serotonin induzierten Bronchokonstriktion in "Precision Cut Lung Slices" des Pferdes

Das Ziel der Studie lag in der Untersuchung des Effektes von Histamin und Serotonin sowie des Einflusses ihrer Inhibitoren auf die Bronchokonstriktion an equinen Precision-Cut Lung Slices (PCLS), die von 5 Pferden mit chronisch obstruktiver Bronchitis (COB) und einem lungengesunden Pferd gewonnen worden waren. Histamin führte zu einer konzentrations-abhängigen Bronchokonstriktion bei einer effektiven Konzentration von 1820nM im Median. Die Histamin induzierte Bronchokonstriktion konnte durch die Inkubation mit dem H1-Rezeptor-Antagonisten Cetirizin vor der Zugabe von Histamin gehemmt werden. Im Gegensatz dazu hatte die Inkubation mit dem H2-Rezeptor-Antagonist Ranitidin, dem H3 und H4-Rezeptor Antagonist Thioperamid und dem H4-Rezeptor Antagonist JNJ7777120 keinen signifikanten Effekt. Serotonin führte nur bei dem lungengesunden Pferd zu einer konzentrations-abhängigen Bronchokonstriktion. Diese Serotonin-induzierte Bronchokonstriktion konnte durch die vorherige Inkubation mit Ketanserin gehemmt werden. Ein möglicher negativer Einfluss chronischer Atemwegserkrankungen auf den Serotonin-Rezeptor oder die Serotonin-induzierte Bronchokonstriktion werden diskutiert.

Schlüsselwörter: Bronchokonstriktion / Histamin / Serotonin / PCLS / Pferd / Pulmologie / chronische Atemwegserkrankung

Introduction

Small airway disease plays an important role in equine medicine. Affected horses often present with exercise intolerance (*Deegen* 1981), coughing and dyspnoea (van der Haegen et al. 2005). Affected horses show an increased reactivity to aerosolised particles such as fungal hyphae found in mouldy hay or straw. This may be due to epithelial damage and the exposure of nerve endings in the airway wall (*Barnes* 1996). Histological findings include epithelial necrosis and goblet cell hyperplasia with a loss of ciliated cells. A reduction in dynamic compliance and increased respiratory resistance is seen as a result of hypertrophy of the smooth airway muscles (*Kaup* et al. 1990), and in combination with mucus accumulation, mucosal oedema and fibrotic remodelling leads to an obstruction at the bronchiole level. Bronchodilating agents such as clenbuterol (0.8 µg/kg BW) can be effective, however long term use leads to reduction in drug efficacy (*Abraham* et al. 2001). For the development of new therapeutics it is necessary to understand the inflammatory process and to avoid the development of irreversible changes.

The pathophysiology of small airway disease remains unknown, despite being the subject of many studies. Previous work has suggested the involvement of leucotrienes and histamine in the development of the disease (*Berger* et al. 1998, *Creese* et al. 1986, *Wohlsen* et al. 2001, *Olszewski* et al. 1999). The histamine inhalation provocation test (*Klein* and *Deegen* 1986) can be used to identify horses affected by nonspecific airway hypersensitivity. Decreases in dynamic compliance and increases in airway resistance, work of breathing and maximum intrathoracic pressure differences correlate to the severity of lung disease. Little is known about histamine-receptor-subtypes in the equine lung.

Serotonin acts as a hormone and a neurotransmitter on one of the serotonin receptors (5-HT). In the horse, much attention has been paid to the prokinetic effects of serotonin on the gastrointestinal tract (*Delesalle* et al. 2008) and its vasoconstrictive effects in laminitis (*Menzies-Gow* et al. 2008, *Zerpa* et al. 2007), whereas its effects on equine small airways has not been studied ex vivo. The PCLS technique was described by *Martin* et al. (1996) and *Wohlsen* et al. (2001) in mice and human lung tissue. *Vietmeier* et al. (2007) were the first to use the technique for equine lung, and it was used for several earlier studies by our group (*Schwalfenberg* 2007, *Barton* et al. 2010a, *Barton* et al. 2010b, *Fugazzola* et al. 2012). The PCLS technique enables ex vivo investigation of airway contraction in response to drugs. This technique also allows a number of drugs to be tested on the same lung tissue reducing the number of animals used.

Material and Methods

Animals

Fourteen horses, euthanized for reasons unrelated to respiratory disease, were included in the study. Horses were excluded from the study if they had cardiovascular abnormalities or had undergone general anaesthesia 72 hours prior to euthanasia. A clinical examination of the respiratory tract, arterial blood gas analysis, bronchoscopy and tracheobronchial cytology were performed on the day before euthanasia. Precision-cut lung slices of six horses were randomly assigned to be used for histamine and serotonin experiments and results are presented in this paper.

Precision-cut lung slices

Immediately following euthanasia with pentobarbital 300 mg/kg KGW IV the thoracic cavity was opened at the right 7th intercostal space. The accessory lobe was identified medial to the caudal vena cava and with the help of an extractor developed by *Vietmeier* et al. (35) was separated from the rest of the lung. Through a cannula the lobe was filled with a low melting-point agarose solution (1,5% final concentration) and placed on ice for 20 minutes to solidify the agarose for cutting. The lobe was then cut in 1 cm thick slices and intact bronchiolar rings were excised with the help of a sharpened metal tube. The tissue cores were cut in 400 μm-thick slices (PCLS) with a Krumdieck tissue slicer Model MD 4000 (Alabama Research and Development, A Division of Alabama Specialty Products, P.O. Box 739 Mundford, AL 36268).

Culture medium

PCLS were placed in 12-well plates and covered with 1 ml RPMI-1640-Medium. The RPMI-1640-Medium is a bicarbonate buffer and nutrition source containing amino acids and vitamins. The slices were incubated at 37°C during which time the medium was replaced three times initially to remove the agarose and cell debris and then at intervals of ten, twenty and thirty minutes.

Assessment of viability

Viability of the equine bronchioli was assessed by degree of bronchoconstriction induced by the potent bronchoconstrictor methacholine (10^{-5} mol/l). The bronchoconstriction of the slices was expressed as the percentage of contraction after addition of methacholine compared to the initial cross-sectional area. Bronchioli were considered viable and included in the study if they demonstrated at least a 50% reduction in cross-sectional area and relaxed following removal of the methacholine by serial replacement with the buffering-solution.

Studies on bronchoconstriction and its inhibition

Histamine acts on each of the four histamine receptors. It was used in rising concentration, from 10^{-7} to 10^{-4} mol/l, to induce contraction of the bronchioli. To see if the induced contraction could be inhibited, antagonists were tested. Each of the antagonists used inhibits one receptor, with the exception of thioperamid which acts both on the H3- and H4-receptors. Cetirizine was used to antagonise the H1-receptor, ranitidine for the H2-receptor and JNJ7777120 for the H4-receptor. The antagonists were used at a concentration of 10^{-5} mol/l. Additionally, to check for the presence of H4-receptors within equine lung, 4-methylhistamine, a H4-receptor agonist, was tested on the PCLS of three horses.

Serotonin was used in serial concentrations from 10^{-8} to 10^{-5} mol/l. A single serotonin receptor antagonist, ketanserin (10^{-5} mol/l), was used.

Serial solutions of increasing concentrations of histamine and serotonin were added to the PCLS to determine the maximum degree of contraction. This step was repeated a second time following a 30 minute incubation of the PCLS in RPMI-medium containing the specific receptor antagonist. The bronchiole lumen was photographed using an inverted microscope two minutes after each change in concentration of the bronchoconstricting agent. The area of the bronchial lumen was measured with a computer program ("scion image" version beta 4.0.2) after conversion of the image to grey scale tiff-format to increase contrast of bronchiole borders. The cross sectional area of each bronchiole before the addition of a mediator was defined as 100 %. Bronchoconstriction was then expressed as the reduction of bronchial lumen (cross-sectional area) from this initial state as described by *Barton* et al. (2010a).

Statistics

The statistical analysis utilised the original cross-sectional area size data and was analyzed by a non parametric ANOVA test and a Dunn's test in which the degree of bronchoconstriction induced by each mediator was compared to the initial state. In contrast to this, the graphics were constructed using the percentage data. In this way different airway sizes could be visually compared.

Results

Four of the 14 horses were found to have severe findings compatible small airway disease as defined by *Ohnesorge* et al. (1998). Nine horses had mild findings and only one horse had no signs of respiratory disease.

35 viable PCLS from the accessory lobe of 6 horses (1 severely affected, 4 mildly affected, 1 free of respiratory disease) were chosen to be included in the described subsequent tests. As described in former studies (*Vietmeier* et al. 2007, *Barton* et al. 2010a) PCLS remain viable for up to 72 hours allowing accurate and precise repetition of contractility analysis.

In the present study histamine caused a concentration-dependent bronchoconstriction with a median effective concentration (EC50) of 1820 nM (fig. 1a). A significant contraction (p=0.0005), compared to the initial cross-sectional area, could be seen after addition of 10^{-5} mol/l of histamine. This bronchoconstriction was inhibited by the addition of cetirizine, a H1-receptor antagonist (fig. 1b and fig. 2). In contrast, the H2-, H3- and H4-receptor antagonists had no effect (fig.

1c-e). Corresponding to this finding, the addition of 4-methylhistamine (10^{-5} mol/l), which acts on H4-receptors, did not induce a bronchoconstriction (fig. 1f).

Only PCLS from one out of six horses exposed to serotonin showed concentration dependent bronchoconstriction with a median effective concentration (EC50) of 82 nM occurring

between the addition of serotonin in a concentration of 10^{-6} and 10^{-5} mol/L (fig. 3b). A significant contraction (p=0.028), compared to the initial cross-sectional area, could be seen after addition of 10^{-5} mol/l of serotonin. This serotonin induced contraction was inhibited in this single horse by the serotonin receptor antagonist ketanserin (10^{-5} mol/L) (fig. 3c). It was the only horse in this study free of respiratory disease.
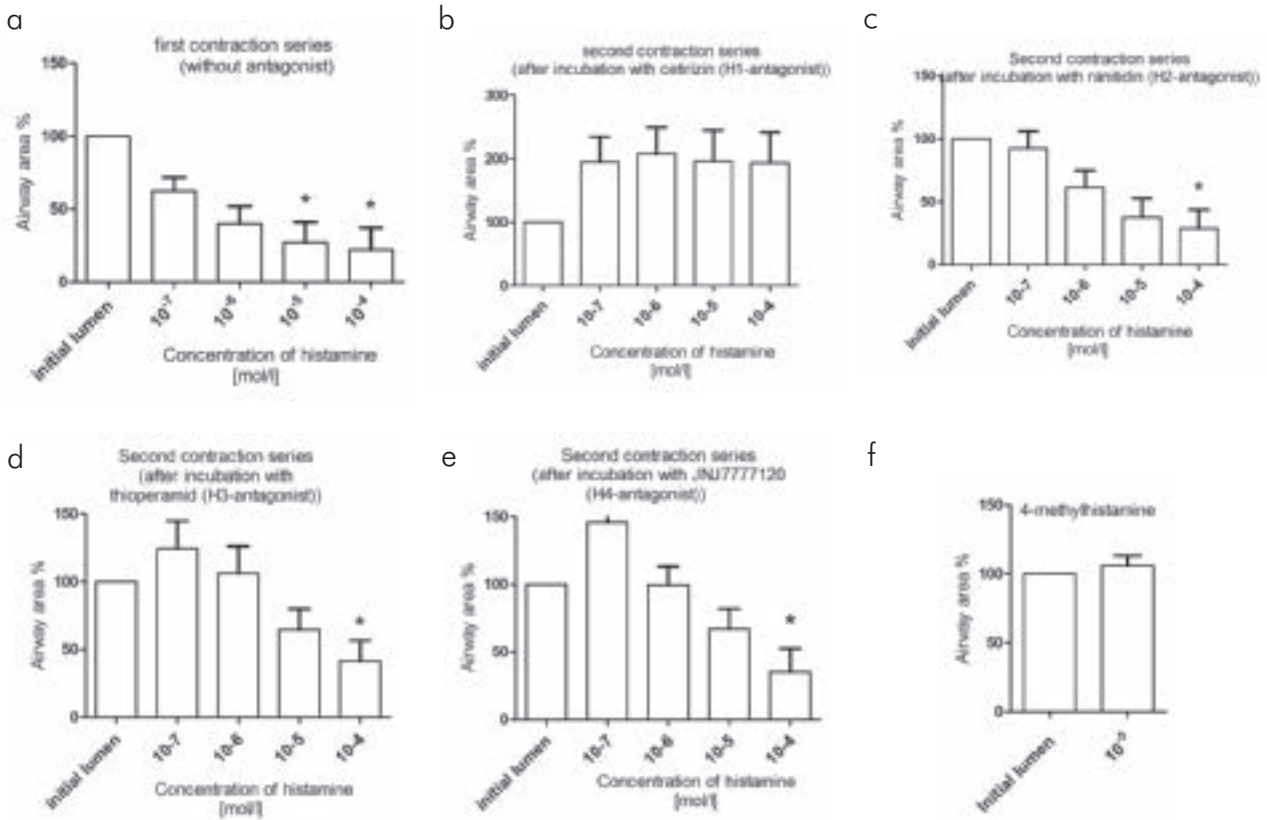

Fig. 1   Concentration dependent bronchoconstriction in equine precision-cut lung slices induced by histamine (fig. 1a), 4-methylhistamine (fig. 1f) and the effect of histamine-antagonists (fig. 1b-e). Values are expressed as a percentage of initial bronchiole lumen cross-sectional area.
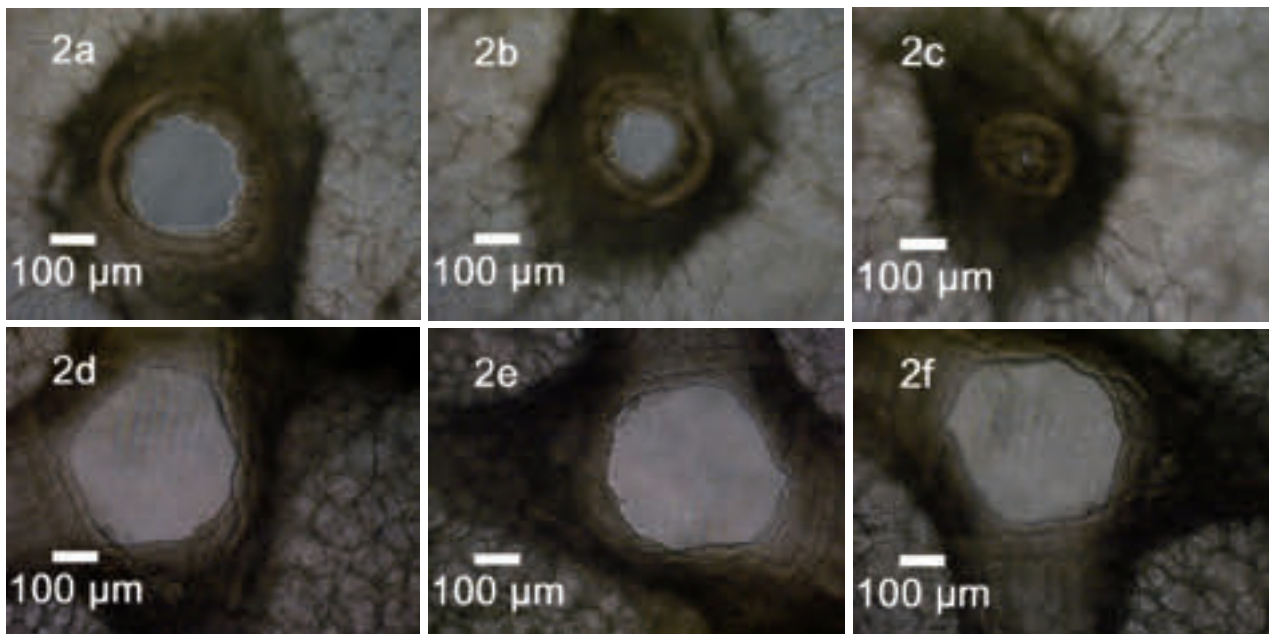

Fig. 2   Microscopic images of precision-cut lung slices (PCLS) before (2a) and after exposure to histamine in different concentrations (2b 10^{-7} mol/l and 2c 10^{-4} mol/l). In figures 2d-f the influence of the incubation with the H1-receptor antagonist cetirizin is shown (2d before exposure to histamine, 2e 10^{-7} mol/l and 2f 10^{-4} mol/l)
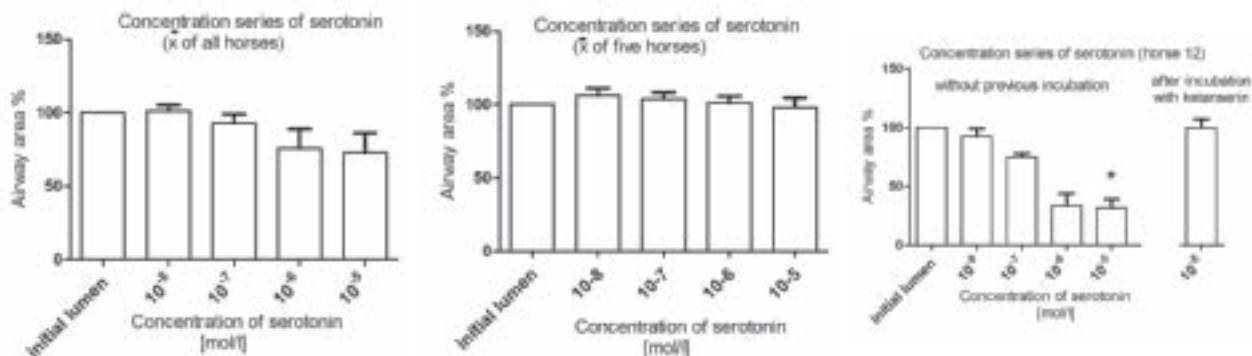
Fig. 3 Effect of serotonin on precision-cut lung slices (PCLS) from all 6 horses (fig. 3a) and from 5 horses affected by lower airway disease compared to the concentration depending contraction induced in one horse free of lower airway disease (fig. 3c). This contraction could be inhibited by a previous incubation with ketanserin (10^{-5} mol/l) (fig. 3c). Values are expressed as a percentage of initial bronchiole lumen cross-sectional area.

Discussion

Histamine induces bronchoconstriction in humans, guinea pigs and marmosets, a new world monkey. Previous work has demonstrated the high sensitivity of guinea pigs' (EC50=217 nM) (*Ressemeyer* et al. 2006) and marmosets' (EC50=425 nM) (*Seehase* et al. 2011) small airways to histamine. Studies on the horse provided variable results. *Vietmeier* et al. (2007) and *Barton* et al. (2010b) demonstrated a high sensitivity to histamine (EC50=200 nM) which is comparable to that described in guinea pigs (EC50=217 nM) (*Ressemeyer* et al. 2006). In contrast to former studies, horses appear to be less sensitive in the present study. This reaction could be compared to human small airways (EC50=2710 nM) (*Seehase* et al. 2010). Therefore, it is difficult to predict how histamine acts in the horse and to which other species they are comparable.

Bronchoprovocation by histamine inhalation has provided different results in former studies; therefore the effect of histamine in the pathogenesis of small airway disease remains controversial. Whereas *Klein* and *Deegen* (1986) demonstrated decreased dynamic compliance and increased respiratory resistance in horses suffering from small airway disease compared to healthy individuals with the Histamine-Inhalation-Provocation-Test (HIPT), this could not be confirmed by *Perkins* et al. (2008) using the same protocol. *Traub* (2005) also found only minimal bronchoconstriction in response to inhalation of histamine, whereas *Hoffmann* (2002) demonstrated increased reactivity to histamine inhalation even during clinical remission. In the current ex vivo study, histamine induced a statistically significant, concentration dependent bronchoconstriction independent of severity of disease and in the healthy horse, which could be inhibited by the H1-receptor antagonist cetirizine. This finding further supports the clinical study by *Olsen* et al. (2007) on horses, who also found cetirizine to be effective. Although pharmacokinetic studies demonstrated a short half-life of cetirizine in horses (*Olsen* et al. 2007), therefore requiring twice daily application, the ease of oral administration improves usability of cetirizine.

Antihistamines have been found to be ineffective in the treatment of small airway disease (*Barnes* 2001) because of a low receptor affinity and a pH-dependent receptor binding. During an allergic reaction the resultant drop in pH means leads to decreased receptor-binding of antihistamines (*Wellmann* 2007).

In the present study the H2- and H3-receptor antagonists ranitidine and thioperamid had no inhibitive effect on histamine-induced broncoconstriction, which could be explained by their lack of action on H1-receptors. Ranitidine is used in equine medicine to reduce gastric acids (*Löscher* 2006). The H2-receptor could be found in small airways of humans, sheep, pigs, guinea pigs and horses (*Chand* et al. 1979). Whereas *Johnson* (1998) declared the H2-receptor to be an inhibitory receptor which induced relaxation of small airways, *Olszewski* et al. (1999b) supposed no effect of it.

Thioperamid is an H3-receptor antagonist used in human medicine for sleep disorders, migraine and adiposity (*Leurs* et al. 2005). In horses, a H3-receptor antagonist was proven to increase tissue sensibility for histamine which led to airway relaxation (*Olszewski* et al. 1999b).

The H4-receptor was characterized for the first time in 2000 (*Nakamura* et al. 2000). In human airways it was found in low density (*Akdis* et al. 2006). In mice, the highly selective H4-receptor-antagonist JNJ7777120 (1-[(5-Chloro-1Hindol-2-yl)carbonyl]-4-methylpiperazine) leads to a reduced immigration of inflammatory cells into affected bronchial areas (*Fung-Leung* et al. 2004). The H4-receptor-agonist H4-methylhistamine induces relaxation of the trachea and small airways (*Chand* et al. 1979). So far, there is no evidence for the existence of a H4-receptor in equine small airways. In the

Table 1 Mediators are able to induce bronchoconstriction

	Horse	Man	Guinea pig	Marmoset	Mouse
Histanime	+	+	+	+	-
Serotonine	(+)	-	+	-	+

+: effective; -: ineffective; (+): partially effective

present study the absence of H4-receptors was confirmed on the one hand by the missing bronchoconstriction induced by 4-methylhistamine and on the other hand by the H4-receptor antagonist JNJ7777120, which had no bronchoconstriction inhibiting effect.

In conclusion, bronchoconstriction in horses can be induced by the action of histamine on the H1-receptor and can be inhibited ex vivo by the H1-antihistamine cetirizine. The H2- and H3-receptor may exist in equine small airways but does not induce bronchoconstiction. The H4-receptor is supposed to be absent, but further work needs to be done to prove this theory.

Previous work demonstrated that serotonin is able to induce a concentration dependent bronchoconstriction in rodents (*De Swert* et al. 2007). The existence of serotonin receptors in airway smooth muscles of these animals is proven. In the present study there was no evidence of serotonin-induced bronchoconstriction in five of six horses. In humans and marmosets serotonin does not induce a reaction either (*Seehase* et al. 2010). Guinea pigs show a high sensitivity for serotonin (EC50=69 nM) [17], which can be compared to the single horse which responded to its addition (EC50=82 nM). Serotonin did induce bronchoconstriction in murine airways but they seemed to be less sensitive than those of other species (EC50=2000 nM, *Held* et al. 1999).

In the single case, in which serotonin-induced bronchoconstriction occurred, the horse was found to be free of respiratory disease. This contraction was inhibited by the serotonin receptor antagonist ketanserin. Unfortunately only one horse free of respiratory disease was included in this study, so it remains speculative that serotonin receptors might be present in the equine lung and that bronchoconstriction can only be induced in healthy horses. It might also be possible that respiratory disease may have a negative effect on the density of serotonin-receptors.

It has been shown that horses affected by small airway disease do not react to the addition of an allergen with an immediate allergic reaction (early-phase response) but an exaggerated late-phase response. Only in healthy horses an immediate allergic reaction can be seen (*Deaton* et al. 2007). This suggests that the reaction of a healthy horse to an inhaled allergen is a protective mechanism which avoids the distribution of the allergen in small airways. This bronchoconstriction may be mediated by serotonin, but further studies involving greater case numbers are required. Nevertheless, it is seems that the serotonin effect or pathway might be disabled during the development of small airway disease.

Acknoledgements

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