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Pharmacological Studies of the Effect of Wheat Grain Extract

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Key Words
Wheat grain extract · Guinea pig trachea

Abstract
Background: Agricultural farm workers exposed to wheat grain dust are at risk of developing respiratory abnormalities. The pathogenesis of this injury is only partially understood. ***Objectives:*** To determine the effect of wheat grain extract on isolated guinea pig tracheal smooth muscle. ***Methods:*** In the current study, pharmacologic properties of wheat grain extract (WGE) were tested using guinea pig tracheas studied in vitro. Dose-related contractions of nonsensitized guinea pig trachea were demonstrated using these extracts. Pharmacologic studies were performed by pretreating guinea pig tracheal tissue with drugs known to modulate smooth muscle contraction: atropine 10^{-6} *M*, indomethacin 10^{-6} *M*, pyrilamine 10^{-6} *M*, acivicin 10^{-5} *M*, nordihydroguaretic acid (NDGA) 10^{-5} *M*, bromophenacyl bromide (BPB) 10^{-5} *M*, 3,4,5-trimethoxybenzoic acid-8-(diethylamino)-octyl ester TMB8 10^{-5} *M*, captopril 10^{-5} *M* and capsaicin 5×10^{-6} *M*. ***Results:*** WGE causes a dose-dependent constriction of guinea pig tracheal smooth muscle. Atropine, pyrilamine, TMB8 and acivicin significantly reduced the contractile effects of the WGE. Inhibition of contraction by blocking of other mediators was significant but less complete. ***Conclusion:*** We conclude that WGE causes a dose-related constriction of airway smooth muscle by nonimmunological mechanisms involving a variety of airway mediators and possibly cholinergic receptors.

Introduction

Agricultural dust is a complex mixture of organic materials. Previous studies demonstrate that occupational exposure to grain dust in industrial workers as well as in agricultural farm workers is associated with the development of variety of respiratory syndromes, including asthma, rhinitis, hypersensitivity pneumonitis, and chronic obstructive airway diseases [1–7]. In addition to respiratory effects of grain dust, wheat products can also cause pulmonary injury in other settings; for example, wheat flour causes asthma, rhinitis, dyspnea, wheezing and increased bronchial reactivity in urban bakers, as described by Prichard et al. [8]. The data of James et al. [9] suggest that exposure to grain dust causes changes in lung function in most subjects, but more severe reactions may occur in workers with a history of asthma or increased bronchial responsiveness.

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Cereal proteins have been identified as the causative agents of adverse reactions to wheat flour. Theobald et al. [10] studied the biologic significance of the extracted cereal proteins. These investigators demonstrated the release of histamine in vitro by basophil leukocytes obtained from patients suffering from asthma by adding aqueous wheat extract. In their study, IgG and IgE binding to wheat flour proteins was demonstrated by immunological testing. Bjorksten et al. [3] demonstrated the allergenic capacity of wheat protein by showing that 43% of bakers with asthma showed an increased specific IgE to wheat flour proteins. Allergen-specific histamine release from whole blood following incubation with native wheat flour was also demonstrated in flour-sensitive bakers [11].

The toxicity of grain dust may not be limited to IgE-mediated mechanisms. Extracts of wheat dust can also induce histamine release by nonimmunologic mechanisms from rat peritoneal cells [12]. In their experiments, Warren et al. [12] showed that an extract of wheat dust, with a low endotoxin content, produced noncytotoxic histamine release from peritoneal cells but not from purified mast cells. Histamine release from the mucosa of the antrum, corpus and duodenum of patients suffering from various diseases was induced in vitro by wheat [13]. The authors suggested that a non-allergic phenomenon rather than an immediate-type of hypersensitivity was responsible for this effect.

Our previous experiments with extracts of agricultural dusts such as rye, barley and hops demonstrated a dose-related constrictive effect on isolated guinea pig tracheal smooth muscle in vitro [14, 15]. The present study was undertaken to further explore the effect of another grain dust, wheat grain, on isolated guinea pig tracheal smooth muscle.

Methods

Wheat Grain Extract Preparation

Wheat grain extract (WGE) was prepared from wheat grain collected at a farm located near Zagreb, Croatia, where farm workers were studied as part of an epidemiological survey of respiratory function in agricultural farmers [6, 7]. Since processing of the grain was not involved in their work, we were interested in knowing specifically whether extract of the whole grain could cause bronchoconstriction. The WGE was prepared in a weight to volume ratio of 1:10 by the standard method of Sheldon et al. [16] at the Institute of Immunology in Zagreb. The extracts were obtained by defatting the raw grain with diethyl ether (boiling point 34°C). A 1:5 w/v extract was prepared by stirring the defatted material in phosphate-buffered saline (PBS) for 72 h at 4°C. The extract was then centrifuged and the supernatant was dialyzed for 48 h against PBS and after that for 24 h against distilled water. Subsequently, the supernatant was filtered under sterile conditions. The filtered extract was divided into 7-ml aliquots in glass vials, and immediately freeze-dried. The vials were then stored at –20°C. This procedure provided a standardized, sterile extract with standardized properties. The WGE was reconstituted in sterile water prior to injection into the organ bath. The solution was prepared with 30 mg of extract/cm^3 of water.

Protein and Endotoxin Analysis

Gel electrophoresis was performed in order to determine the protein content of the WGE. Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [17] in a 12% polyacrylamide gel using a vertical slab gel apparatus. The gel was stained with Coomassie blue.

The amount of endotoxin (EU/ml and EU/g) in WGE was determined by using the Limulus Amebocyte Lysate assay [18].

Guinea Pig Trachea Preparation

The tracheas of 30 young albino Hartley male guinea pigs (300–400 g) purchased from Charles River Labs, Wilmington, Mass., USA were used. The animals were sacrificed by CO_2 asphyxiation for 5 min and the tracheas were removed within 3 min of sacrifice. The animal tissues were manually trimmed to remove connective and other tissues. Four segments (rings, each 4–6 mm wide) were cut from a single trachea. The tissues were then suspended between two L-shaped stainless steel hooks mounted in a 20-ml organ chamber containing Krebs-Henseleit buffer of the following composition (μM): NaCl, 110.0, KCl, 4.80; $CaCl_2$, 2.35; $MgSO_4$, 1.20; $KHPO_4$, 1.20; $NaHCO_3$, 25.0; dextrose, 110.0, and Na_2EDTA, 0.03, in glass-distilled water. Organ chambers were maintained at 36.5 ± 0.5°C, and were continuously aerated with 95% O_2 and 5% CO_2 to maintain pH = 7.5 ± 0.1. The tissue segments were initially set to 2 g of tension, and were allowed to stabilize for approximately 1.5 h before the experiment began. During that period, the tissue was washed at 15-min intervals. After the relaxation period, the tension in each tissue segment was readjusted to 2 g for all subsequent assays. Isometric contractions were recorded using a Grass FTO3C force displacement transducer attached to a Grass polygraph recorder.

Steady-State Characterization of the WGE Dose-Response Curve

After equilibration, each tissue segment was maximally contracted with carbachol (10^{-4} M). This response was measured in grams of tension and designated as the maximal carbachol response for that tissue (100%). All subsequent contractions of the segment were normalized to this maximal carbachol response and expressed as a percentage of maximal carbachol-induced contraction. Before the contraction-response assay with WGE was performed, a challenge with carbachol 10^{-4} M was run. A dose-response curve with WGE was obtained by adding increasing volumes of the extract or Krebs (used as a control) into the tissue baths with progressive half-log increments of 10, 30, 100, 300, and 1,000 µl. The potency of the WGE was normalized by comparing the biological activity (measured in grams of tension) with the maximal contraction induced by carbachol (10^{-4} M) on the same tissue. The data were expressed as a percentage of the initial maximal carbachol contraction. Concentration-response curves were plotted using the Kaleidagraph software (Synergy software, Reading, Pa., USA) on the Power Macintosh (Cupertino, Calif., USA). Data points were fit by iteration to the logistic function:

$$E = E_{max}/(1 + (EC_{50}/[A]^n),$$

where E = observed muscle tension (grams above baseline), [A] = the concentrations of the agonist, EC_{50} = the [A] eliciting one half of the maximal response, n = slope of the curve.

Statistical Methods

Mean values were compared between controls and drug-treated tissues using matched tracheal rings by the paired t test. In this way, we compared control and drug-treated tissue responses for the same trachea. Comparison of the dose-response characteristics of WGE evaluated with different pharmacologic agents was performed using the unpaired t test. Statview software (Brain Power Inc., Calabasas, Calif., USA) for Macintosh was used to perform this analysis. Similarly, response parameters (E_{max} and EC_{50}) were characterized for individual tissues and compared between treatment protocols by the paired t test.

Pharmacologic Studies

In a typical drug experiment, the tissue was washed and baseline reestablished after an initial contraction with carbachol (10^{-4} *M*) demonstrated tissue viability and established maximal contractile tension with this agent. A specific blocking agent or a control solution (Krebs buffer) was added to the organ bath in a 20-µl aliquot and incubated with the tissue for 30 min. A WGE dose-response challenge was then performed. After the dose response, the tissue was again washed and carbachol (10^{-4} *M*) was added to verify the viability of the tissue. In these drug experiments, different drugs were tested in the organ bath including atropine 10^{-6} *M* (anticholinergic; n = 9), pyrilamine 10^{-6} *M* (antihistamine H_1 blocking agent; n = 9), indomethacin 10^{-6} *M* (prostaglandin synthesis inhibitor; n = 9), 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester 10^{-5} *M* (TMB8; inhibitor of intracellular calcium mobilization; n = 9), nordihydroguaretic acid 10^{-5} *M* (NDGA; arachidonic acid pathway inhibitor; n = 9), acivicin 10^{-5} *M* (leukotriene synthesis inhibitor; n = 6), bromophenacyl bromide 10^{-5} *M* (BPB; phospholipase-PLA2 blocking agent; n = 12), captopril (10^{-5} *M*, angiotensin converting enzyme inhibitor; n = 12) and capsaicin 5×10^{-6} *M* (8-methyl-n-vanillyl-6-nonenamide), an agent which depletes the mediators stored in irritant nerves (n = 12).

All chemical agents were obtained from Sigma Chemical Co., St. Louis, Mo., USA.

Results

Guinea Pig Trachea Assay

The dose-response curve for WGE performed on 30 guinea pig tracheas and expressed as a percent of the response to the maximal carbachol contraction (10^{-4} *M*) is presented in figure 1. As can be seen, WGE produces a dose-related constriction of smooth muscle, increasing from 13.73% at 10 µl to 106.72% at 1,000 µl (measured as a percent of the maximal carbachol constriction in that tissue).

Comparisons of the modification of the contractile responses of WGE following pretreatment with atropine (10^{-6} *M*), pyrilamine (10^{-6} *M*) and indomethacin (10^{-6} *M*) are shown in figure 2. The dose-related constriction of tracheal smooth muscle was completely suppressed following pretreatment with atropine (10^{-6} *M*) and significantly reduced by pyrilamine (10^{-6} *M*) at all concentrations tested ($p < 0.05$ or $p < 0.01$). By contrast, indomethacin (10^{-6} *M*) did not have any protective effect against constriction at the doses of wheat extract tested.

Figure 3 illustrates the dose-related contractile activity of WGE following pretreatment with acivicin (10^{-5} *M*), NDGA (10^{-5} *M*) and BPB (10^{-5} *M*). Acivicin significantly decreased the contractile activity ($p < 0.05$ or $p < 0.01$) throughout the whole testing range with WGE, while two other drugs significantly decreased the constriction only at higher doses of the extract (NDGA starting at 30 µl) and BPB (starting at 100 µl).

Figure 4 studies the dose-related contractile activity of WGE following pretreatment with TMB8 (10^{-5} *M*), captopril (10^{-5} *M*) and capsaicin (10^{-6} *M*). Dose-dependent contractions of guinea pig tracheal smooth muscle were significantly decreased by TMB8 (10^{-5} *M*) at all concentrations ($p < 0.05$ or $p > 0.01$) but were reduced by capsaicin (10^{-6} *M*) and captopril (10^{-5} *M*) only at higher doses (starting at 100 µl; $p < 0.05$ or $p < 0.01$).

Table 1 shows the E_{max} (% of carbachol) and EC_{50} (µl) for WGE for the 30 tested guinea pigs as well as for control and corresponding drug-treated tissues. The data show statistically significant differences for E_{max} between control-treated tissue and TMB8-, captopril-, BPB-, and atropine-treated tissue ($p < 0.01$) as well as for control and pyrilamine-treated tissues ($p < 0.05$). There was no significant difference for EC_{50} between control-treated tissues and corresponding drug-treated tissues. Statistical comparisons between the E_{max} of the different drug-tested tissues revealed significant differences only between atropine and pyrilamine and atropine and indomethacin ($p < 0.01$). For the EC_{50} there were no significant differences between the different drug-tested tissues.

Protein and Endotoxin Content

Protein analysis showed that WGE contained 6,342.91 µg/ml or 337.39 µg/mg (weight of protein/ml or mg of standard solution).

Endotoxin analysis demonstrated that WGE contained 20,703.13 EU/ml or 1,101.23 EU/mg of the standard solution.

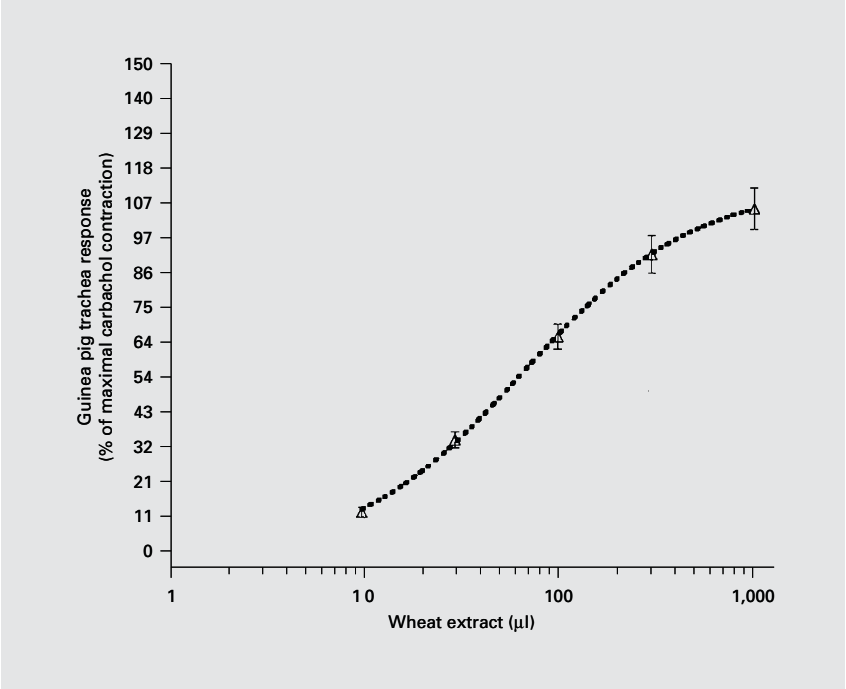

Fig. 1. Contractile response of isolated guinea pig tracheal smooth muscle to WGE (n = 30) expressed as a percentage of maximal carbachol (10^{-4} *M*) contraction (mean ± SE).

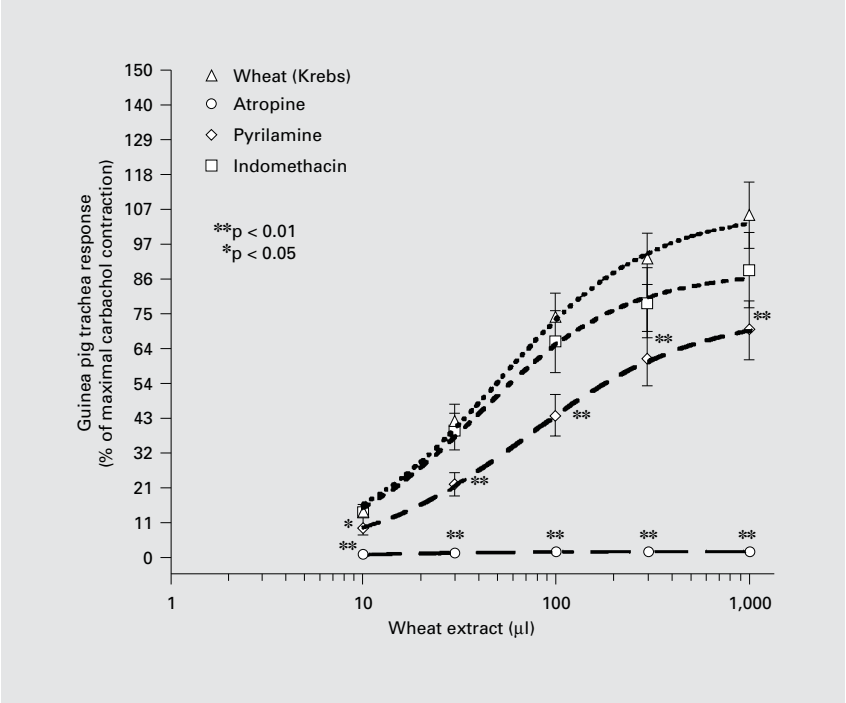

Fig. 2. Constrictor response of guinea pig trachea to WGE (n = 9) following pretreatment with atropine (10^{-6} *M*), pyrilamine (10^{-6} *M*) and indomethacin (10^{-6} *M*) compared to control (Krebs) (mean ± SE).

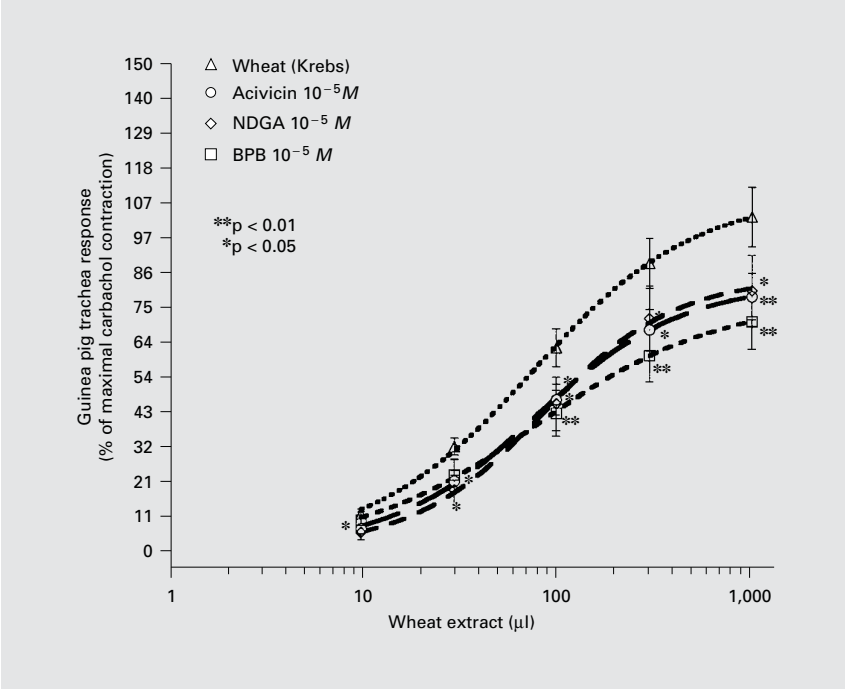

Fig. 3. Constrictor response of guinea pig trachea to WGE (n = 9) following pretreatment with NDGA (10^{-5} M), BPB (10^{-5} M) and acivicin (10^{-5} M) compared to control (Krebs) (mean ± SE).

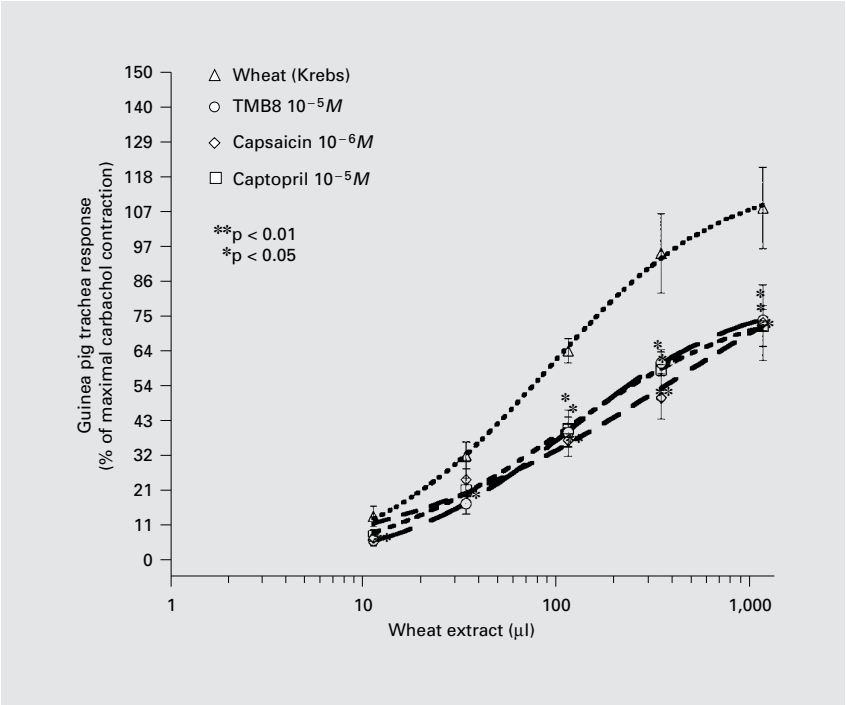

Fig. 4. Constrictor response of guinea pig trachea to WGE (n = 12) following pretreatment captopril (10^{-5} M), TMB8 (10^{-5} M) and capsaicin (10^{-6} M) compared to control (Krebs) (mean ± SE).

Table 1. E_{max} and EC_{50} values for wheat extract

Drug	Number	E_{max}, % of carbachol	EC_{50}, µl
Krebs	30	117.8 ± 7.7	73.5 ± 6.7
Group 1			
Krebs	9	109.0 ± 10.0	54.8 ± 5.7
Atropine	9	2.2 ± 1.2**	50.5 ± 22.7
Pyrilamine	9	74.5 ± 9.2*	82.1 ± 19.3
Indomethacin	9	91.8 ± 11.6	51.9 ± 10.5
Group 2			
Krebs	9	109.9 ± 8.8	80.9 ± 10.1
NDGA	9	83.0 ± 11.5	107.8 ± 24.8
BPB	9	75.6 ± 9.1**	101.3 ± 79.6
Acivicin	9	82.1 ± 8.0**	85.6 ± 10.1
Group 3			
Krebs	12	130.2 ± 16.3	82.6 ± 14.3
Captopril	12	86.9 ± 7.3**	97.8 ± 16.1
TMB8	12	84.3**	119.8 ± 31.3
Capsaicin	12	106.5 ± 31.2	71.3 ± 12.0

Four sets of data are presented. The first represents the mean E_{max} and EC_{50} for all 30 guinea pig control animals from all groups of experiments (Krebs); the following three groups consist of three sets of guinea pig tracheas pretreated with individual pharmacological agents. These three groups consisted of matched animals since each trachea provided four rings which received 1 of the 3 drugs or the Krebs. Data are presented as mean ± SE. Difference between Krebs-treated tissue and corresponding tissue pretreated with pharmacologic agent. * $p < 0.05$; ** $p < 0.01$.

Discussion

Our data demonstrate that aqueous WGE causes a dose-dependent constriction of isolated, nonsensitized guinea pig tracheal smooth muscle. WGE produced dose-response curves similar to those seen with other organic dust extracts such as soy [19], spices [20], animal food [21], barley, hops and rye [15] as well as flour and cocoa [14].

The pharmacological studies of WGE on guinea pig tracheal smooth muscle suggest a complex interaction between these airway irritants and guinea pig tracheal tissue. It appears that blocking specific receptors have a unique, characteristic modifying effect on WGE-induced constriction. In particular, the muscarinic blocking agent, atropine, has a striking effect on the response to wheat extract. Similarly, a considerable blocking effect was obtained with pyrilamine, a histamine-blocking agent. For agents affecting the lipoxygenase pathway of arachidonic acid (acivicin, BPB and NDGA), moderate effects were noted throughout the dose response suggesting a modest involvement of leukotrienes in this response. In our study, captopril, an angiotensin-converting enzyme inhibitor, which can enhance inflammatory peptides by preventing their degradation had only a minimal effect on the contractile response to wheat extract. Capsaicin, a compound that depletes peptides from irritant nerves in the airway, reduced the contractile effect of WGE, suggesting a neurogenic component to this inflammation. The action of TMB8 on WGE constriction is probably the result of its effect on smooth muscle contraction by modulation of intracellular calcium.

The response of the airway smooth muscle is probably not mediated by a single receptor or mediator mechanism but represents the simultaneous activation of several pathways. Complex organic dusts found in industrial and agricultural settings undoubtedly have many active agents that each contribute to the contractile response through different pathways. Additional experiments with crude extract will probably not clarify this issue which may require fractionation of WGE to separate out individual components of this extract.

Our experimental data on the pharmacologic effects of WGE suggest that the acute clinical effects of grain dust seen in workers may, in part, be related to a non-immunological mechanism similar to those seen with other organic dust extracts [14, 15, 19–21]. Our data with WGE in nonsensensitized animals are supported by the results of Brisman et al. [22] who suggested that nasal mucosal inflammation, in flour-dust-exposed bakers, may be non-allergic. This response was characterized by activation of neutrophils and fibroblasts. Similarly, Smith et al. [23] described that respiratory symptoms following wheat flour exposure are due to nonspecific irritant effects. DoPico et al. [24] performed inhalation provocation tests with extracts of durum wheat and durum wheat airborne dust in grain elevator workers and found 20% decrements of FEV_1. The bronchial reactions were immediate and/or late and were blocked by sodium cromoglycate. The authors sugested that these reactions were probably due to both specific and nonspecific mediator release (e.g. histamine) in tested subjects.

The WGE examined in this study contained large amounts of endotoxin. However, Buck et al. [25] and McFedan et al. [26] have shown that endotoxin, both in conjunction with organic dust extract and alone, does not cause direct constriction of guinea pig tracheal smooth muscle in vitro. Hence the effects of WGE in this study are probably not modulated by endotoxin.

Bellanti et al. [27] suggested that the measurement of plasma histamine following subcutaneous provocation with wheat antigens may provide a predictive marker for the diagnosis of food allergy. Siegel et al. [28] demonstrated that histamine can be found in a variety of agricultural dusts and the amount of histamine found in their samples of agricultural dusts that varied from 0.078 to 125.75 pmol of histamine. Our study did not directly measure histamine content of WGE, but the protective effect of pyrilamine suggests that this mediator is involved in the response. Lachance et al. [29] have demonstrated in a subject employed in a company producing biscuits, sensitivity to alkaline hydrolysis wheat glutein derivate, manifested by rhinoconjunctivitis and asthmatic symptoms.

De Zotti et al. [30–32] studied nonspecific bronchial responsiveness and blood eosinophils before and 24 h after a bronchoprovocation test with wheat flour. Fifty-five percent of the workers tested developed asthma following bronchoprovocation, which was accompanied by an increase in blood eosinophils 24 h after the specific exposure.

It would be of interest to extrapolate from our findings correlations that would translate into quantitative measurements of environmental pollution. However, several considerations limit this possibility. The health effects of exposure to grain dust may depend on the nature of the work involved in the handling of grain. This may include growing, harvesting and transporting the grain by farmers, management of the grain in storage facilities, further transport by such agents as dock workers or longshoremen, and finally processing of the grain into flour, feed and seed by mill workers before the use of the processed product for food production by bakers and food workers. Grain dust itself is complex and may include non-grain plant matter such as fungal spores, animal matter such as insect matter and animal particles, agricultural chemicals, as well as inorganic matter such as silica and soil. The current study focused on grain obtained directly from the farm setting. The components derived from the processing of this material such as flour were not studied. The extraction process eliminated many of the potential confounding components listed above but certainly not all. The establishment of a bronchoconstricting factor in the extract does, however, suggest a plausible mechanism for some of the clinical and epidemiological studies of farm workers.

Quantitative correlations between dust levels in the workplace and concentrations of WGE required to cause constriction of GPT would be of great practical interest but realistically such a correlation would be very speculative since the challenge method is so different (direct application to tissue versus inhalation of a suspension of dust particles). Furthermore, the expected concentration at any given site of the workplace varies. Concentrations of organic suspended particulates have been noted to vary by as much as several orders of magnitude [33].

We do note, however, that the endotoxin levels in this dust are about four times those commonly seen in similar cotton dust extracts. Cotton dust extracts cause contractions of smooth muscle in vitro, at concentrations similar to those of wheat grain extract. This might indirectly imply that if the in vitro contractile response mimics the in vivo response, one would expect to see dust responses in grain workers at levels similar to or lower than those elicited in cotton textile workers (current standard: 200 μg/m^3).

Our study confirms the bronchoconstrictor potential of wheat grain. Dust from this agricultural product has been associated with airway disease in a wide variety of occupational settings. Characterization of this extract indicates that, in the absence of sensitization, WGE causes guinea pig tracheal smooth muscle to contract as a result of cholinergic receptor as well as other mediator-related mechanisms. These findings suggest that in addition to specific allergic reactions, inflammatory mechanisms may be involved in this irritative response that do not depend on humoral immunity. Future investigations of wheat and other organic dusts may help to better understand the pathogenesis of occupational airway diseases.

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