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AIRWAY BIOLOGY

Pharmacologic Effects of Grain Weevil Extract on Isolated Guinea Pig Tracheal Smooth Muscle

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Abstract The grain weevil, an insect (pest) that infects grain, is a frequent contaminant of processed wheat, and its presence may contribute to respiratory abnormalities in grain workers. We studied the in vitro effects of an extract of grain weevil (GWE) on airway smooth muscle. Pharmacologic studies included in vitro challenge of guinea pig trachea with GWE, in parallel organ baths, pretreated with mediator-modifying agents or a control solution. Dose-related contractions of nonsensitized guinea pig trachea (GPT) were demonstrated using this extract. Pharmacologic studies were performed by pretreating guinea pig tracheal tissue with drugs known to modulate smooth muscle contraction: atropine, indomethacin, pyrilamine, acivicin, NDGA, BPB, TMB8, captopril, and capsaicin. Atropine, pyrilamine, BPB, and capsaicin significantly reduced the contractile effects of the extract at most of the challenge doses ($p < 0.01$ or $p < 0.05$). Inhibition of GWE-induced contraction by blocking of other mediators was less complete. We suggest that GWE causes dose-related airway smooth muscle constriction of the GPT by nonimmunologic mechanisms involving a variety of airway mediators and possibly cholinergic receptors.

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Introduction

Agricultural dust is a complex mixture of different organic materials, including insects such as the grain weevil. Previous studies demonstrate that occupational exposure to grain dust of both industrial workers and agricultural workers is associated with the development of a variety of respiratory syndromes, including asthma, rhinitis, hypersensitivity pneumonitis, and chronic obstructive pulmonary disease [1–9].

The grain weevil (*Sitophilus granarius*) is a frequent contaminant of wheat grain. It is a small reddish brown insect, which destroys stored wheat and other grains by eating the grain's interior. It is regarded as a common pest of farm-stored grain. Farm workers' asthma and mill workers' asthma have been attributed to grain weevil sensitivity [5]. In a previous study we reported that farm workers demonstrated a high prevalence of respiratory symptoms accompanied by lung function deterioration [2]. Occupational asthma due to the inhalation of grain weevil was reported by Frankland and Lunn [10]. Similarly, Lunn [11] and Lunn and Hughes [12] reported that mill workers' asthma was an allergic response to grain weevil in workers handling grain infested by the grain weevil on a daily basis.

Our previous experiments with water-soluble extracts of agricultural dust such as grain wheat, rye, barley, and hops have demonstrated a dose-related constrictor effect on isolated guinea pig tracheal smooth muscle in vitro produced by these extracts [13–15]. Since grain weevil is frequently present in farm materials inhaled by agricultural workers, the present study was undertaken to explore the

possible effect of grain weevil extract on isolated guinea pig tracheal smooth muscle.

Methods

Grain Weevil Extract Preparation

Grain weevil extract (GWE) was prepared from the whole body of grain weevils collected at a grain-processing mill near Zagreb, Croatia. The GWE was prepared in a weight-to-volume ratio of 1:10 by the standard method of Sheldon [16] at the Institute of Immunology in Zagreb. Briefly, the extracts were prepared by defatting the raw allergen material 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 freeze-dried immediately. The vials were then stored at −20°C [16]. This procedure provided a standardized, sterile extract with reproducible properties. The GWE extracts were reconstituted in sterile water before injection into the organ bath.

Protein Analysis

Gel electrophoresis was performed to determine the amount of protein in grain weevil extract. Analytical sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out according to the method Laemmli [17] in a 12% polyacrylamide gel using a vertical slab gel apparatus. The gel was stained with Coomassie Blue.

Endotoxin Assay

The amount of endotoxin (EU/mg) in grain weevil extract was determined by using the Limulus Amebocyte Lysate assay [18].

Guinea Pig Trachea Preparation

The tracheas of 35 young albino Hartley male guinea pigs (300–400 g) purchased from Charles River Labs (Wilmington, MA) were used. The animals were killed by CO_2 asphyxiation for 5 min and the tracheas were removed within 3 min of death. 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–Hanseliet 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, 11.0; and Na_2EDTA, 0.03, in glass-distilled water. Organ chambers were maintained at 36.5 ± 0.5°C and 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 this 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 GWE 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 GWE was performed, a challenge with carbachol 10^{-4} M was run. A dose–response curve with GWE was obtained by adding increasing volumes of extract or Krebs (used as a control) into the tissue bath with progressive half log increments of 10, 30, 100, 300, and 1000 μl. The potency of the GWE was determined by comparing its biological activity 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. In each experiment the responsiveness to maximal stimulation with carbachol 10^{-4} M was initially established. This was followed by washing, reestablishment of the baseline, and a dose–response challenge. Concentration–response curves were plotted using Kaleidagraph software (Synergy Software, Reading, PA) on the Power Macintosh (Cupertino, CA). Data points were fit by iteration to the logistic function:

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

where E is the observed muscle tension (grams above baseline), [A] is the concentration of the agonist, EC_{50} is [A] that elicits one-half of the maximal response, and n is the slope of the curve.

Statistical Methods

Mean values were compared between controls and drug-treated tissue using matched tracheal rings and the paired t

test. Thus, we compared control and drug-treated tissue responses for the same trachea. Comparison of the dose–response characteristics of GWE was performed using the unpaired *t* test. Statview software (Brain Power Inc, Calabasas, CA) for Macintosh was used to perform this analysis. The Statview v4.1 was used for the statistical analysis. Similarly, response parameters (E_{max} and EC_{50}) were characterized for individual tissues and compared between treatment protocols by the paired *t* test.

Drug Treatment

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. A specific blocking agent or a control solution (Krebs buffer) was added to the organ bath and incubated with the tissue for 30 min. A dust extract 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 added to the organ bath, e.g., atropine 10^{-6} M (anticholinergic; $n = 11$), pyrilamine 10^{-6} M (antihistamine H1 blocking agent; $n = 11$), indomethacin 10^{-6} M (prostaglandin synthesis inhibitor; $n = 11$), TMB8 10^{-5} M [3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester] (inhibitor of intracellular calcium mobilization; $n = 12$), NDGA 10^{-5} M [(nordihydroguaretic acid) (arachidonic acid pathway inhibitor); $n = 12$], acivicin 10^{-5} M (leukotriene synthesis inhibitor; $n = 12$), BPB 10^{-5} M [(bromophenacyl bromide) (phospholipaze-PLA2 blocking agent)]; $n = 12$], captopril (10^{-5} M, angiotensin-converting enzyme (ACE) inhibitor; $n = 12$), and capsaicin 5×10^{-6} M (8-methyl-*n*-vanillyl-6-nonenamide, an agent which stimulates the release of neuropeptides). All chemical agents were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Guinea Pig Trachea Assay

The average dose–response curve for GWE performed on 35 guinea pig tracheas and expressed as a percent of the response to the maximal carbachol contraction (10^{-4} M) is presented in Fig. 1 (curve labeled grain weevil extract [krebs]). As can be seen, GWE produces a dose-related constriction of smooth muscle, increasing from 12% at 10 µl to 132% at 1000 µl.

Comparisons of the modification of the contractile response of GWE in 11 guinea pig tracheas following pretreatment with atropine (10^{-6} M), pyrilamine (10^{-6} M), and indomethacin (10^{-6} M) are also shown in Fig. 1. The dose-related constriction of tracheal smooth muscle was significantly larger in comparison with that following pretreatment with atropine (10^{-6} M) and pyrilamine (10^{-6} M) at low and at high concentrations ($p < 0.05$ or $p < 0.01$). Indomethacin (10^{-6} M), however, did not have any significant protective effect on the constriction caused by GWE (NS).

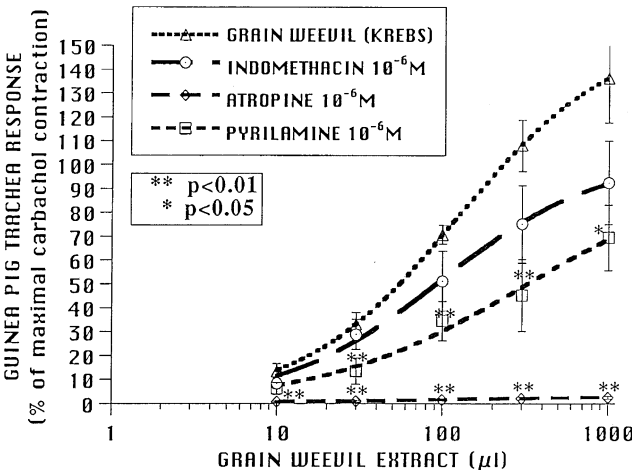

Fig. 1 The contractile response of guinea pig tracheal smooth muscle ($n = 11$) to grain weevil extract (GWE) following pretreatment with Krebs (control), atropine (10^{-6} M), pyrilamine (10^{-6} M), and indomethacin (10^{-6} M) (mean ± SE)

Figure 2 shows the dose-related contractile activity of GWE in 12 guinea pig tracheas following pretreatment with Krebs, acivicin (10^{-5} M), NDGA (10^{-5} M), and BPB (10^{-5} M). Acivicin significantly decreased the contractile activity of GWE ($p < 0.05$ or $p < 0.01$) from 100 µl to 1000 µl, while BPB significantly decreased the constriction starting at 30 µl to 1000 µl. NDGA did not have any significant protective effect throughout the study dose range.

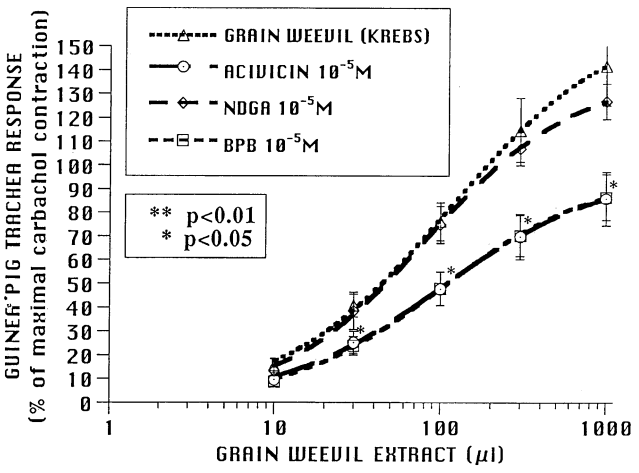

Fig. 2 The contractile response of guinea pig tracheal smooth muscle ($n = 12$) to grain weevil extract (GWE) following pretreatment with Krebs (control), NDGA (10^{-5} M), BPB (10^{-5} M), and acivicin (10^{-5} M) (mean ± SE)

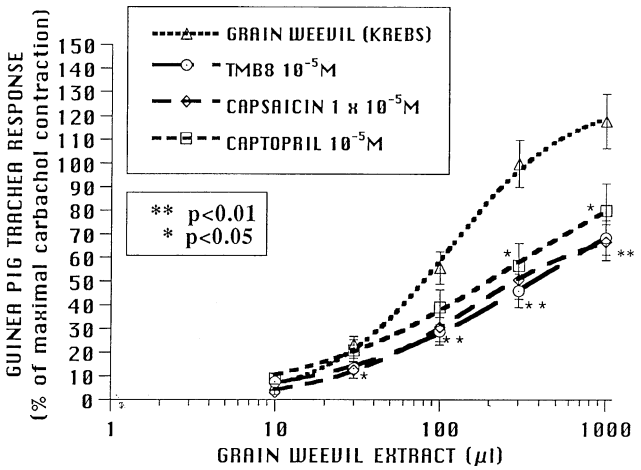

Fig. 3 The contractile response of guinea pig tracheal smooth muscle ($n = 12$) to grain weevil extract (GWE) following pretreatment with Krebs (control), captopril (10^{-5} M), TMB8 (10^{-5} M), and capsaicin (5×10^{-6} M) (mean ± SE)

Figure 3 demonstrates the dose-related contractile activity of GWE in 12 guinea pig tracheas following pretreatment with Krebs, TMB8 (10^{-5} M), captopril (10^{-5} M), and capsaicin (5×10^{-6} M). Dose-dependent contractions of guinea pig tracheal smooth muscle were significantly decreased by capsaicin (5×10^{-6} M) starting at 30 µl to 1000 µl ($p < 0.05$ or $p < 0.01$). For TMB8 (10^{-5} M) the protective effect was significant from 100 µl to 1000 µl ($p < 0.01$) and for captopril (10^{-5} M) starting at 300 µl to 1000 µl ($p < 0.05$).

Table 1 gives the E_{max} (measured as a % of carbachol) and EC_{50} (µl) for GWE for the 35 tested guinea pigs as well as for the animals tested with Krebs and corresponding drugs. The data show statistically significant differences for E_{max} between control (no drug pretreatment) and atropine, BPB, acivicin, TMB8, and capsaicin ($p < 0.01$ or $p < 0.05$). There was no significant difference for EC_{50} between control-treated guinea pig tracheas and drug-pretreated animals (NS) except for captopril ($p < 0.01$) and capsaicin ($p < 0.05$). Statistical analysis of E_{max} between different drugs revealed a significant difference between atropine and pyrilamine ($p < 0.01$), between atropine and indomethacin ($p < 0.01$), and between NDGA and acivicin ($p < 0.01$). For EC_{50} there was no significant difference between the different drug treatments (NS).

Table 1 E_{max} and EC_{50} for grain weevil extract

Drugs	*N*	Grain weevil extract	
		E_{max} (% carbachol)	EC_{50} (µl)
Krebs	35	146.44 ± 10.43	102.40 ± 9.87
Krebs		149.53 ± 20.96	99.06 ± 18.27
Atropine	11	5.39 ± 1.55**	44.03 ± 4.91
Pyrilamine		88.44 ± 21.16	56.80 ± 17.57
Indomethacin		112.90 ± 54.03	65.40 ± 6.87
Krebs		157.82 ± 18.46	74.76 ± 11.88
NDGA	12	134.10 ± 8.18	83.15 ± 14.35
BPB		100.96 ± 10.50*	72.37 ± 7.45
Acivicin		95.36 ± 13.12*	82.01 ± 7.85
Krebs		130.94 ± 14.93	135.90 ± 17.17
Captopril	12	110.25 ± 18.41	67.33 ± 8.75**
TMB8		77.96 ± 7.63*	112.84 ± 21.07
Capsaicin		74.60 ± 7.88**	78.14 ± 35.42*

Data are presented as mean ± SE

Difference between Krebs and corresponding pharmacologic agent statistically significant: * $p < 0.05$; ** $p < 0.01$

Protein Analysis

Protein analysis of GWE showed that it contained 480.00 µg/ml (54.98 µg/mg) of protein.

Endotoxin Assay

Endotoxin analysis demonstrated that GWE contained 17,578.13 EU/ml (2013.53 EU/mg) of endotoxin.

Discussion

Our data demonstrate that aqueous GWE causes a dose-related constriction of isolated guinea pig tracheal smooth muscle. The average dose–response curve was similar to those previously seen with other organic agricultural dust extracts such as rye flour, soy, wheat grain, barley, and hops [13–15, 19, 20]. The modifying effect of different drugs on guinea pig tracheal smooth muscle constriction caused by GWE suggests a complex interaction between GWE and guinea pig tracheal tissue.

By analyzing the patterns of response to different drugs used in our experiments with GWE, we conclude that the blockade of specific receptors has a modifying effect on the induced constriction. In particular, the muscarinic blocking agent atropine significantly reduced the constrictor response to the GWE extracts.

TMB8, an inhibitor of calcium mobilization, also significantly suppressed GWE effects. The agent limits free intracellular calcium levels. An increase in intracellular Ca^{2+} occurs in many smooth muscle preparations induced to constrict by receptor and nonreceptor mechanisms [21, 22]. Since elevation of cytosolic calcium is involved in the sequence leading to smooth muscle constriction, the effects of the calcium-modifying agents is not unexpected. A possible role for intra- and extracellular calcium blocking agents in the prevention of dust-related airway obstruction remains to be explored.

Pyrilamine, an H1 blocking agent, reduced the effect of GWE. In a study by Lunn and Hughes [12], bronchoprovocation inhalation with grain weevil protein produced

immediate responses characterized by a decrease of FEV_1 of 25% or more. Antihistamine drugs partly inhibited this bronchospasm as it did in our organ bath system.

Captopril, an ACE inhibitor, was associated with the enhancement of kinin-induced (e.g., bradykinin) contraction of airway smooth muscle [23, 24]. Paradoxically, in our system this enzyme inhibitor (weakly) reduced the contractile effect of the GWE. One possible interpretation of this finding is that the extracts may contain peptides that mediate relaxation and are sensitive to ACE and endopeptidase inhibitors.

Both acivicin and BPB reduced the effect of GWE on GPT. These agents block the leukotriene pathway of the arachidonic acid cascade, suggesting that leukotrienes exert a role in GWE constriction. NDGA, by contrast, inhibits the entire arachidonic acid cascade. Its lack of effect may suggest that GWE's influence on the arachidonic acid pathways may balance constrictor and relaxant effects (e.g., ehancement of relaxant prostaglandins and constrictor leukotrienes). Indomethacin had no significant effect on the constriction caused by GWE, suggesting that the role of prostaglandins in this response may not be important.

GWE contains measurable amounts of endotoxin. Fedan et al. [25] have shown that endotoxin alone does not cause constriction of guinea pig tracheal smooth muscle in vitro. Therefore, we believe the constrictor effects of GWE, seen in this model, are due to components other than bacterially derived contaminants.

The effect of capsaicin and captopril on EC_{50} (Table 1) may indicate shared affinity for receptor sites activated by GWE and these drugs. The effects of the drugs on E_{max} may indicate their potential to reduce the bronchoconstrictor effect of GWE.

Our experimental data with GWE suggest that the acute clinical effects seen in exposed workers may in part be related to a nonimmunologic (non-IgE) mechanism similar to that seen with other organic dust extracts such as animal food, spices, and swine confinement agents [20, 26, 27].

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