Pharmacologic Effects of Tobacco Dust Extract on Isolated Guinea Pig Trachea*

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Study objective: To determine the effects of tobacco dust extract (TDE) on isolated guinea pig tracheal smooth muscle.

Design: A controlled, in vitro smooth-muscle study of the effect of pharmacologic agents on TDE.

Methods: The effect of TDE on isolated guinea pig tracheal smooth muscle was tested using water-soluble extracts of dust obtained from machines in a cigarette manufacturing plant. Dose-related contractions of nonsensitized guinea pig trachea were demonstrated using these extracts. The dust extracts contained significant quantities of bacterial components (eg, endotoxin). Pharmacologic studies were performed by pretreating guinea pig tracheal tissue with drugs known to modulate smooth-muscle contraction: atropine, indomethacin, pyrilamine, nordihydroguaretic acid, acivicin, bromophenacyl bromide, 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester, captopril, and capsaicin.

Results: Atropine strikingly reduced the contractile effects of these extracts. Inhibition of contraction by blocking other mediators was less complete. There was no inhibition of contraction by hexamethonium (10^-4 mol/L, 10^-5 mol/L, 10^-6 mol/L), suggesting that nicotine was not the major contractile mediator of TDE. A separate analysis using different molecular weight fractions of TDE indicated that the constrictor activity appears to be primarily in the fraction with a molecular weight < 10 kd. Additionally, the constrictor effect resided entirely in the nonlipid fraction of the extract. We suggest that TDE causes dose-related airway smooth-muscle constriction by nonimmunologic mechanisms involving a variety of airway mediators and possibly cholinergic receptors.

Conclusions: The bronchoconstrictor activity of TDE resides primarily in its low molecular weight, nonlipid fraction, and hexamethonium studies suggest that this agent is not nicotine.

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Key words: isolated guinea pig trachea; pharmacologic testing; tobacco dust extract

Abbreviations: ACE = angiotensin-converting enzyme; BPB = bromophenacyl bromide; EC50 = concentration of the agonist eliciting one half of the maximal response; Emax = maximal response to tobacco dust extract; EU = endotoxin units; NDGA = nordihydroguaretic acid; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulfate; TDE = tobacco dust extract; TMB8 = 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester

Respiratory disorders among tobacco workers have been described by several authors. As early as 1948, McCormick et al1 reported that because of the nature of the manufacturing operations in the tobacco industry, certain potential health hazards exist for tobacco workers. Several investigators describe an increased prevalence of respiratory findings including asthma and chronic obstructive bronchitis among tobacco workers when compared with control subjects.2–9

In addition to clinical and epidemiologic studies demonstrating the respiratory effects of tobacco dust, several investigators have looked at in vitro models to study the toxicologic effects of this agent. York et al10 demonstrated that (cigarette) tobacco extract affected the in vitro oxygen consumption of pulmonary macrophages. Tobacco extract produced a biphasic effect on macrophage respiration: a stimulation at low concentrations and an inhibition at higher concentrations. Bernal-Madrazo and Ham-Carrillo11 studied leukocyte migration inhibition factor in healthy individuals (both smokers and non-
smokers) using an extract of tobacco. They found that an antigen contained in tobacco is able to sensitize a high proportion of the exposed population, independent of smoking status. Additional experimental studies with tobacco extracts demonstrate that application of such extracts on rat lip mucosa causes irritational hyperplasia that is related to the amount of irritant. It was greatest in those rats subjected to the longest period of application of the tobacco extract and was independently related to the concentration of tar in the extract.12

Review of the literature suggests that there are no available data on the direct effects of tobacco dust extracts (TDEs) on isolated guinea pig tracheal smooth muscle. Based on our previous experience with organic dusts, we hypothesize that tobacco extract contains agent(s) that cause nonimmunologically mediated bronchoconstriction. The present study was undertaken to evaluate this potential constrictor response and to characterize it pharmacologically. In addition, we further analyzed the airway effects of several subfractions of crude TDE by measuring the intensity of their constrictor activity on the tracheal tissue.

**Materials and Methods**

**TDE Preparation**

TDE was prepared from tobacco dust collected from the contents of bales of tobacco leaf used to manufacture cigarettes in a tobacco processing plant located in Zagreb, Croatia. In this same industry, the workers had been previously studied as part of an epidemiologic survey of respiratory health. The TDE was prepared in a weight/volume ratio of 1:10 by the standard method of Sheldon et al11 at the Institute of Immunology in Zagreb. In brief, the extract was prepared by defatting the raw material with diethyl ether (boiling point 34°C). A 1:5 weight/volume extract was prepared by stirring the defatted material in phosphate-buffered saline solution for 72 h at 4°C. The extract was then centrifuged and the supernatant was dialyzed for 48 h against phosphate-buffered saline solution 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.14 This procedure provided a sterile extract with standardized properties. TDE extract was reconstituted in sterile water prior to injection into the organ bath.

The TDE was subsequently fractionated into two components: the first had a molecular weight > 10 kd, and the second had a molecular weight < 10 kd. This fractionation was accomplished by passing the extract in solution through a Centricon 10 filter (Amicon; Billerica, MA). Since the preliminary studies on the isolated guinea pig tracheal tissue with both extracts demonstrated that most of the constrictor activity for smooth muscle was found in the second extract (molecular weight < 10 kd), the latter was further separated into two fractions: one containing lipid, and one from which all lipids were removed. The material was rendered lipid free with butanol-di-isopropyl ether (40:60 volume/volume) by the method of Cham and Knowles.14

**Protein Determination and Endotoxin Assay**

The protein content of the TDE was determined by the method of Lowry et al.15 The amount of endotoxin (endotoxin units [EU] per milligram) in TDE was determined by using the Limulus Amebocyte Lysate assay (BioWhittaker; Walkersville, MD).10

**Protein Analysis**

Gel electrophoresis was done to determine the presence of proteins in TDE for the TDE fractions of low (< 10 kd) and high (> 10 kd) molecular weight. Analytical sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli17 in a 12% of polyacrylamide gel using a vertical slab gel apparatus. The gel was stained with Coomassie blue. The choice of 10 kd as a cut-off point is arbitrary but is dictated by the work of previous investigators and technical considerations.

**Guinea Pig Trachea Preparation**

We used the tracheas of young albino Hartley male guinea pigs (300 to 400 g) purchased from Charles River Labs, Wilmington, MA. The animals were killed by carbon dioxide 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 to 6 mm wide) were cut from a single trachea, and each was suspended between two L-shaped stainless steel hooks mounted in a 20-mL organ chamber containing Krebs-Hansellet buffer of the following composition: NaCl, 110.0 mmol/L; KCl, 4.80 mmol/L; CaCl2, 2.35 mmol/L; MgSO4, 1.20 mmol/L; KHPO4, 1.2 mmol/L; NaHCO3, 25 mmol/L; dextrose, 11 mmol/L; and Na2 ethylenediaminetetraacetic acid, 0.03 mmol/L in glass distilled water. Organ chambers were maintained at 36.5 ± 0.5°C and were continuously aerated with 95% oxygen and 5% carbon dioxide 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 the period of stabilization, 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 (Grass Instrument; Quincy, MA).

**Steady-State Characterization of the TDE Dose-Response Curve**

After equilibration, each tissue segment was maximally contracted with carbachol (10−4 mol/L). This response was mean
sured 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. The dose-response effect on smooth-muscle contraction was determined using TDE fractions in increasing volumes added into the tissue bath with progressive half-log dose increments of 10, 30, 100, 300, and 1,000 L. Isometric contractions induced by TDE were measured to the sequential cumulative dose increments. Concentration response curves were plotted using Kaleidograph software (Synergy Software; Reading, PA) on the Power Macintosh (Macintosh; Cupertino, CA). Data points were fit by iteration to the logistic function:

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

E is the observed muscle tension (grams above baseline), \( E_{\text{max}} \) is the maximal response to TDE, A is the concentrations of the agonist, EC\(_{50} \) is the A elicting one half of the maximal response, and n is the slope of the curve.

**Statistical Methods**

Mean values were compared between control and drug-treated tissue using matched tracheal rings, by the paired t test. In this way, we compared control and drug-treated tissue responses in the trachea of the same animal. Comparison of the dose-response characteristics of different TDE fractions was performed using the unpaired t test. Similarly, response parameters (Emax and EC\(_{50} \)) were characterized for individual tissues and compared between treatment protocols by the paired t test. The Statview version 4.1 software (Brain Power; Calabasas, CA) for Macintosh performed the statistical analysis.

**Drug Treatment Protocol**

In a typical drug experiment, the tissue was washed and baseline reestablished after an initial contraction with carbachol 10\(^{-4} \) mol/L. A specific blocking agent (or a control solution) was then added to the organ bath and incubated with the tissue for 30 min. A TDE dose response challenge was then performed. After the dose response, the tissue was again washed and carbachol 10\(^{-4} \) mol/L was added to verify the viability of the tissue. In the drug experiments, different drugs were added to the organ bath, such as atropine 10\(^{-6} \) mol/L (anticholinergic, n = 6), pyrilamine 10\(^{-6} \) mol/L (antihistamine-blocking agent, n = 6), indomethacin 10\(^{-6} \) mol/L (prostaglandin synthesis inhibitor, n = 6), 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester (TMBS) 10\(^{-5} \) mol/L (inhibitor of intracellular calcium mobilization, n = 6), nordihydroguaretic acid (NDGA) 10\(^{-5} \) mol/L (arachidonic acid pathway inhibitor, n = 6), acivicin 10\(^{-5} \) mol/L (leukotriene synthesis inhibitor, n = 6), bromophenacyl bromide (BPB) 10\(^{-5} \) mol/L (phospholipase A\(_2 \) blocking agent, n = 6), captopril 10\(^{-5} \) mol/L (angiotensin-converting enzyme [ACE] inhibitor, n = 6) and capsaicin 5 \times 10\(^{-6} \) mol/L (N-methyl-n-vanillyl-6-nonenamide, an agent that stimulates the release of neuropeptides, n = 6).

Since nicotine evokes atropine sensitive contractions of tracheal smooth muscle by a direct action on cholinergic nerves, we also studied the effect of pretreatment with hexamethonium (10\(^{-4}, 10^{-5}, \) and 10\(^{-6} \) mol/L; an anionic blocking agent of nicotinic neurotransmission, n = 6). All chemical agents were obtained from Sigma (St. Louis, MO).

**Results**

**Guinea Pig Tracheal Assay**

TDE produced dose-dependent contractions of guinea pig tracheal smooth muscle with an Emax of 100.18% (as percentage of control) and an EC\(_{50} \) of 53.96 \( \mu \)L. Comparisons of the modification of the contractile responses to TDE by atropine (10\(^{-6} \) mol/L), pyrilamine (10\(^{-6} \) mol/L), and indomethacin (10\(^{-5} \) mol/L) were presented in Figure 1, left, A; for NDGA (10\(^{-5} \) mol/L), BPB (10\(^{-5} \) mol/L), and acivicin (10\(^{-5} \) mol/L) in Figure 1, center, B; and for captopril (10\(^{-5} \) mol/L), TMBS (10\(^{-5} \) mol/L), and capsaicin (5 \times 10\(^{-6} \) mol/L) in Figure 1, bottom, C.

Atropine (10\(^{-6} \) mol/L) completely blocked the

**Figure 1.** Left, A: The contractile response of guinea pig trachea to TDE following pretreatment with Krebs solution (control), atropine (10\(^{-6} \) mol/L), pyrilamine (10\(^{-6} \) mol/L), and indomethacin (10\(^{-6} \) mol/L) [mean ± SE]. Center, B: The constrictor response of guinea pig trachea to TDE following pretreatment with Krebs solution (control), NDGA (10\(^{-5} \) mol/L), BPB (10\(^{-5} \) mol/L), and acivicin (10\(^{-5} \) mol/L). Right, C: The constrictor response of guinea pig trachea to TDE following pretreatment with Krebs solution (control), captopril (10\(^{-5} \) mol/L), TMBS, and capsaicin (5 \times 10\(^{-6} \) mol/L).
constrictor effects of TDE at low concentrations as well as at high concentrations (p < 0.01). The constriction by TDE extract was significantly blocked by NDGA (10−5 mol/L), BPB (10−5 mol/L), acivicin (10−5 mol/L), and TMB8 10−5 mol/L. Pretreatment with other drugs (indomethacin, capsaicin, captopril) only partially inhibited the constriction of guinea pig smooth muscle caused by TDE. Pretreatment with pyrilamine had no significant effect on TDE-induced constriction. These findings suggest a complex interaction of this airway irritant, with airway cells and smooth-muscle receptors leading to the release of mediators from airway tissue and the enhancement of their effect on smooth muscle. Hexamethonium pretreatment at three separate concentrations (10−4 mol/L, 10−5 mol/L, and 10−6 mol/L) had no effect on the TDE dose-response curve.

The dose-response curves of TDE extract (n = 21) and the different TDE fractions (<10 kd and >10 kd) are presented in Figure 2, left, A, and compared to unfractionated TDE extract (n = 21). Similarly, the unfractionated TDE is compared with the <10-kd (delipidated) fraction and <10-kd (lipid) fraction in Figure 2, right, B, as a percentage of maximal carbachol contraction (10−4 mol/L). The bulk of the contractile effect is seen in the low-molecular-weight fraction ( < 10 kd). The high-molecular-weight fraction (> 10 kd) had a lesser effect. The low-molecular-weight, delipidated fraction had an effect similar to that of the unfractionated sample, while the lipid-containing fraction was essentially without constrictor effect.

The TDE fraction of molecular weight < 10 kd responded in the presence of hexamethonium in a manner identical to the whole TDE extract. Specifically, there was no difference in contractile effects of TDE < 10 kd in the presence of hexamethonium (compared to control without hexamethonium).

The mean values for Emax (as a percentage of maximal carbachol) and EC50 for TDE, following pretreatment with control and pharmacologic agents, are presented in Table 1. The data are mean values for six guinea pigs. Most of the significant differences in tracheal smooth-muscle response between control (Krebs solution) and drug-treated tissue were seen for Emax. EC50 values were significantly different from control only for pretreatment with atropine and TMB8.

Table 2 presents Emax and EC50 values for different TDE fractions. The TDE fraction for the

<table>
<thead>
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<th>Table 1—Emax and EC50 Values for TDEs*</th>
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<td>Drugs</td>
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<td>Krebs solution</td>
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<tr>
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<td>Captopril</td>
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<tr>
<td>TMB8</td>
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<td>Capsaicin</td>
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*Experiments were run independently with sets of three drugs, and each independent experiment had its own control with Krebs solution.
†p < 0.01, statistically significant difference between Krebs solution and corresponding pharmacologic agent.
‡p < 0.05, statistically significant difference between Krebs solution and corresponding pharmacologic agent.

Figure 2. Left, A: The constrictor response of guinea pig trachea TDE (crude extract) and high- and low-molecular-weight fractions of TDE (<10 kd and >10 kd, respectively [mean ± SE]). Right, B: The constrictor activity of TDE (crude extract) and lipid and nonlipid fractions of TDE: <10 kd (delipidated [delip]) and <10 kd (lipid [lip]).
low-molecular-weight TDE (<10 kd) had the greatest constrictive effect on tracheal smooth muscle (Emax, 114.35%; EC50, 49.50 μL), followed by the low-molecular-weight, delipidated fraction of <10 kd (Emax, 102.31%; EC50, 30.97 μL). The lipid-containing fraction with molecular weight <10 kd did not have any constrictor effect whatsoever on tracheal smooth muscle. The constrictor effect of the TDE fraction of high molecular weight (>10 kd) was considerably less (Emax, 39.71%; EC50, 21.99; p<0.05) than that of the crude extract. It was also significantly less potent than that of the low-molecular-weight (<10 kd) fraction and the low-molecular-weight, delipidated fraction of the TDE (p<0.01 and p<0.05, respectively).

**Protein and Endotoxin Content**

The protein content was 520 μg/mL for the unfractionated (crude) TDE, 164 μg/mL for the low-molecular-weight TDE fraction, and 88 μg/mL for the high-molecular-weight TDE fraction. For the low-molecular-weight, delipidated TDE fraction, it was 96 μg/mL.

The endotoxin content measured in two separate samples of the crude TDE was 111,328 endotoxin units (EU)/mL (5,227 EU/mg) and 107,422 EU/mL (4,950 EU/mg), respectively. For reference, standard laboratory measurements of industrial waste cotton dust exhibits average values of 5,000 EU/mg of endotoxin (National Institute of Occupational Safety and Health biochemistry laboratory).

**Protein Analysis**

In the present study, the TDE fraction of molecular weight <10 kd demonstrated one prominent band of <7 kd on SDS-PAGE. In the fraction >10 kd, there were several high-molecular-weight visible protein bands. Figure 3 represents the SDS-PAGE of the low- and high-molecular-weight TDEs in a 12% gel.

**Discussion**

The in vitro challenge of guinea pig tracheal tissue by TDE produces dose-dependent contractions of this tissue similar to those seen with other organic dust extracts such as those of spices,18 latex,19 soy,20 swine confinement agent,21 animal food,22 as well as cocoa and flour.23 By analyzing the patterns of response to different drugs used in our experiments with TDE compared to other organic dusts which we have studied, we conclude that blocking specific receptors has a unique, characteristic modifying effect on TDE-induced constriction. In particular, the muscarinic blocking agent atropine has a striking effect on the maximal response to TDE (Emax), while the antihistamine pyrilamine does not have any measurable effect on TDE-induced constriction. For agents affecting the arachidonic acid pathway (eg, indomethacin, acivicin, BPB, NDGA), moderate effects were noted throughout the dose response. Since all these agents modify mediators that are potentially contractile agents (eg, leukotrienes, prostaglandins), it is possible that TDE enhances its cholinergic activity through the arachidonic acid cascade.

![SDS-PAGE of TDEs in a 12% gel](image)

**Table 2—Emax and EC50 Values for TDE and Tobacco Fractions**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Emax, % of Carbachol</th>
<th>EC50, μL</th>
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<tbody>
<tr>
<td>TDE</td>
<td>100.18</td>
<td>53.96</td>
</tr>
<tr>
<td>Tobacco fractions, kd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 10</td>
<td>114.35</td>
<td>49.50</td>
</tr>
<tr>
<td>&lt; 10 (delipidated)</td>
<td>102.31</td>
<td>30.97</td>
</tr>
<tr>
<td>&lt; 10 (lipid)</td>
<td>0*</td>
<td>0*</td>
</tr>
<tr>
<td>&gt; 10</td>
<td>39.71*</td>
<td>21.99†</td>
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</table>

*p<0.01, difference between TDE and each fraction.
†p<0.05, difference between TDE and each fraction.
Captopril, an ACE inhibitor, has been associated with the enhancement of kinin-induced (eg, bradykinin) contraction of airway smooth muscle.\(^{24,25}\) In our system, the ACE inhibitor had only a minimal effect on the contractile response to TDE.

TMB8, an inhibitor of calcium mobilization, suppressed considerably the TDE effects. This 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.\(^{26,27}\) The possible role for intracellular and extracellular calcium blocking agents in the prevention of organic dust-related airway obstruction remains to be explored.

The TDE examined in this study contained large amounts of endotoxin. Buck et al\(^{28}\) and Fedan et al\(^{29}\) 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. It is therefore unlikely that the constrictor effects of tobacco dust, seen in this model, are dependent on these bacterially derived contaminants. Nevertheless, it is plausible that these bacterial products are involved in sensitizing these tissues in vivo to the effect of organic dusts.\(^{30}\)

Tobacco dust is a complex mixture of different organic ingredients. The fraction of tobacco dust extract with molecular weight \(> 10,000\) d has a less pronounced constrictor effect on tracheal smooth muscle than did the low-molecular-weight fraction. This suggests that the tobacco extract fraction with molecular weight \(< 10,000\) d contains agents that are capable of directly constricting smooth muscle. Nicotine is a major pharmacologic agent contained in tobacco leaves and associated with the addictive properties of tobacco use. Our extraction process probably removed the nicotine in this dust, explaining the absence of a hexamethonium effect. This implies that the major contractile agent in this extract is not nicotine but some as-yet-undefined, low-molecular-weight substance(s).

The pharmacologic studies described with TDE in the guinea pig tracheal model are consistent with a complex interaction between airway irritants present in TDE and guinea pig tracheal tissue. Our experimental data with TDE suggest that the acute clinical effects of tobacco dust in workers may in part be related to a nonimmunologic (non-IgE) mechanism similar to that seen with other organic dusts extracts, such as spices,\(^{10}\) swine confinement dust,\(^{21}\) and animal food.\(^{22}\) The effect of atropine suggests that cholinergic nerves and/or receptors may be involved. The role of other mediators appears to vary with different organic extracts, but for TDE the arachidonic acid cascade appears to play a role. These findings may have clinical and therapeutic implications for the acute and chronic respiratory symptoms and lung function changes experienced by tobacco workers. The possible contributions of airway inflammation and allergic mechanisms to the development of chronic respiratory disease in tobacco workers will require further study using this model.

**References**

26 Alexandre MA, King AP, Fuero M. Effect of TMB-8 on alpha-adrenoreceptor agonist and KCL induced-contractions in isolated rabbit aorta. Gen Pharmacol 1993; 24:921–928