

An Overview of Species Differences in the Effects of a Water Extract of Cotton Bract on Isolated Airway Smooth Muscle, and Effects of *E. coli* Lipopolysaccharide

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Our laboratory has been comparing the activity of a water extract of cotton bract (CBE) with the isolated trachealis smooth muscle of the dog, guinea pig, and cat. CBE induced contractions that were not mediated by 5-hydroxytryptamine (5-HT), histamine, or muscarinic receptors. The active agent(s) in CBE was dialyzable (< 14,000 molecular weight), and substantial activity was retained after low-temperature ashing. CBE potentiated contractions of dog trachealis to histamine and 5-HT and relaxation responses to isoproterenol, whereas it had no effect on responses to methacholine and KCl. In the guinea pig trachealis, CBE reduced responsiveness to KCl, potentiated relaxations to adenosine and ATP, and did not alter the responses to the remaining agents. Responses of cat trachealis to KCl and isoproterenol were potentiated by CBE, while those to 5-HT were unaffected. Neurogenic cholinergic contractile responses were potentiated by CBE in the trachealis of the dog, but not of the guinea pig, while neurogenic relaxations were potentiated by CBE in guinea pig trachealis but not in the dog trachealis. There are thus marked species differences in the acute effects of CBE on airway smooth muscle. Due to recent interest in the possible involvement of bacterial endotoxins in the etiology of byssinosis, we examined the effects of *E. coli* lipopolysaccharide (LPS) in guinea pig trachealis. An initial examination revealed that LPS potentiated responses to histamine, but not those to methacholine and isoproterenol. This effect vanished upon a second appraisal with a different batch of LPS. The effect of LPS in airway smooth muscle is thus, at present, equivocal.

Introduction

Byssinotic workers experience chest tightness on the first day of the work week. Bronchoconstriction appears to be involved in this symptom (1). In addition, inhalation of cotton bract extracts by healthy human subjects evokes bronchospasm (2,3). The agent(s) responsible for both the acute effects of inhaled cotton dusts, and dust and plant extracts, and for the symptoms accompanying chronic exposure of textile workers to the dust, is not identified. A large number of candidate agents and mechanisms have been proposed (4). There are many reports that indicate that extracts of cotton bracts and other plant parts, and dusts, cause contraction of a variety of respiratory and nonrespiratory smooth muscles (4-13). Smooth muscle, therefore, is a convenient system for the bioassay of ac-

tivity in extracts. It has been our premise that, while the bioassay experiments are useful *per se*, little information regarding mechanisms of byssinosis can be obtained using a nonrespiratory smooth muscle. The reason for this is that smooth muscles are as varied in their innervations and physiological and pharmacological characteristics as they are in their function and anatomical location. An additional complication is that there also exist marked species differences in the characteristics of homologous smooth muscles. An appreciation of some of these considerations would seem to be relevant to the development of a model for the disease using an animal, the airway smooth muscle of which resembles most closely the biological profile of human tissue.

This report contains an overview of results already reported (14, 15) on the effects of a water extract of cotton bract on dog and guinea pig trachealis smooth muscle. New information on similar experiments performed using feline trachealis muscle also is described. We have utilized extracts of cotton bracts (CBE) in our studies, since bract

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extracts evoke bronchoconstriction in humans (2,3) and since the principal contaminant in mill dust is derived from the bracts (16). As a starting point for our long-term studies, we have intentionally not purified the CBE. We have reasoned that the crudest preparation of cotton bracts is the most likely to contain etiologically important substances. This is in contrast to the acetone-treated bract extract preparation used in the study of Russell et al. (10), the only report in the literature using airway smooth muscle that is directly comparable to our studies.

There is recent interest in the possibility that endotoxin may contribute, in part, to the respiratory symptoms of byssinotics (17). Endotoxin initiates the formation of substances, such as products (e.g., C5a) of the complement system (18,19) that are active pharmacologically in smooth muscle (20, 21). Since it is important to understand whether endotoxin affects airway smooth muscle directly, or whether it modifies the reactivity of the muscle to other agents, we examined the interaction of *E. coli* lipopolysaccharide (LPS) with several agents in the guinea pig trachealis.

Methods

Preparation of Crude, Dialyzed, and Ashed CBE

Details of the preparation of crude CBE have been reported (14). CBE was a crude, water extract of frost-killed cotton bracts collected in 1977 near Idalou, TX. The water extract was filtered, lyophilized and reconstituted in sterile, nonpyrogenic saline.

Crude CBE was dialyzed through a 12,000–14,000 molecular weight cutoff dialysis tubing to obtain dialyzable and nondialyzable fractions (15). Ashed CBE was obtained by subjecting a lyophilized sample of crude CBE to a low-temperature ashing procedure (15).

Preparation of Isolated Trachealis Muscles

The experiments conducted were in adherence with the DHEW guidelines (22) and other national and international codes. Male mongrel dogs (14–21 kg) and male cats were obtained from a local pound. Male guinea pigs (350–500 g) were from Camm Research Institute (Wayne, NJ). The animals were anesthetized with 40 mg/kg sodium pentobarbital (IV in dogs, IP in guinea pigs and cats), and sacrificed by thoracotomy.

The preparation of dog trachealis (DT) and guinea pig trachealis (GPT) for organ bath studies has been described (14,15). Trachealis muscles from cat trachea (CT) were prepared as for the DT, with the exception that only one "strip" of muscle was obtained from each cartilage ring. The preparations were mounted to holders, placed in separate, water-jacketed organ chambers (3 mL for concentration–response studies; 8 mL for frequency–response studies) containing a modified Krebs-Henseleit solution at 37°C (14) and attached to force-displacement transducers for the recording of isometric tension re-

sponses. Each tissue was placed under its respective optimum resting tension (a 1-g resting tension was used for CT), and a 1-hr period of equilibration preceded the actual experiment. In experiments in which the tissues were stimulated with electrical field stimulation to elicit neurogenic responses, the tissues were placed between the platinum ring electrodes of special holders.

Concentration–Response and Frequency–Response Determinations

Concentration–response curves for agents that cause contraction or relaxation were obtained following the stepwise, cumulative addition of the agents to the bath. The conditions for evaluating relaxation responses have been described (14,15).

Frequency–response curves for neurogenic contractile or relaxation responses were evoked with stepwise–increasing frequencies of electrical field stimulation (10 sec trains of rectangular wave stimuli; 50 V, 0.5 msec duration) delivered at 7 min intervals. Additional details of experimental protocol are given in Fedan et al. (14,15). In several experiments using the DT and GPT, the contractile effects of cholinergic nerve stimulation were blocked with atropine (0.1 μ M; a muscarinic receptor antagonist), and the neurogenic contribution of adrenergic inhibitory nerves to responses was blocked by a mixture of drugs (referred to as PPG), which included propranolol (1 μ M, a β -adrenergic receptor antagonist), phentolamine (1 μ M, an α -adrenergic receptor antagonist), and guanethidine (10 μ M, an adrenergic neuron blocker).

Analysis of Data Used in the Present Experiments on GPT and CT

Each tissue was used for one concentration–response determination. Cat trachealis muscles were weighed at the end of the experiment to normalize contractile tension responses in terms of grams wet weight of tissue (gww), or "g tension/gww." Responses of the GPT are expressed in terms of grams, since the actual muscle which contributed to the responses is too small to be weighed accurately. The results are expressed as means \pm SEM. n is the number of separate experiments. Geometric mean EC₅₀ values (the concentration of agent which produces 50% of the maximum response) were determined from linear regression analysis of probit-transformed data. The data were evaluated for differences using Student's *t*-test. The 0.05 level of probability was considered significant.

Studies on the Effects of CBE in DT and GPT Preparations

The following discussion summarizes the salient findings, interpretations, and conclusions which have been reported earlier (14,15).

Direct Effects of CBE

CBE causes contraction of the DT and GPT. In high concentrations CBE is seen occasionally to relax the GPT, and a "bell-shaped" concentration-response curve results. The magnitude of contractions to CBE in the GPT and DT is small in comparison to responses elicited with methacholine, histamine, 5-HT, and KCl, but it may be sufficient to explain the bronchospasm observed when bract extracts are inhaled.

CBE-induced contractions of the DT and GPT are not antagonized by the muscarinic, histamine, or 5-HT receptor antagonists, atropine, pyrilamine, and methysergide, respectively. Responses to CBE are not, therefore, mediated by these receptors. A similar finding has not been made in other laboratories (14).

The active agent(s) in CBE which induces contractions of the DT and GPT is dialyzable through 12,000–14,000 molecular weight cutoff membranes. Virtually no activity remains in the nondialyzable fraction when tested in the DT, whereas some may be retained and contribute to responses of the GPT. This finding is in general agreement with observations made by Russell et al. (10) for DT.

Ashed CBE is capable of contracting the GPT, although some activity is lost. We have suggested that extractable minerals may be responsible for the activity of ashed samples, since many divalent cations induce responses or otherwise interfere with excitation-contraction coupling in smooth muscles. A role of minerals in byssinosis has been proposed for consideration in view of the knowledge that there are differences in the biological activities of botanical material obtained from plants grown in different geographical regions; these may correlate to local soil conditions in addition to cultivar type.

Modification by CBE of Responses of Tissues to Other Agents

We have reasoned that, in addition to its direct effect, CBE might alter the reactivity of airway smooth muscle to endogenous mediators and neurotransmitters. We have therefore examined in DT and GPT the influence of CBE on the concentration-response curves of several excitatory and inhibitory substances. The results of these experiments are summarized in Table 1, which also is useful for comparing the results of similar experiments in CT (*vide infra*). These observations indicate the following: (a) CBE modifies differentially the pharmacological characteristics ("reactivity") of airway smooth muscle; (b) this effect of CBE is not nonspecific, i.e., not all agents are influenced. The relative specificity of this CBE effect would indicate that it does not result from cellular toxicity; (c) for a given agent, the effect of CBE is not evident in both species (e.g., see histamine), or an opposite effect can be produced (e.g., see 5-hydroxytryptamine). There exist, therefore, species differences in the effect of CBE on the reactivity of the DT and GPT.

Modification by CBE of Neurogenic Excitatory and Inhibitory Responses

An additional way in which CBE might affect airway resistance could involve an effect on neurotransmission by the intrinsic excitatory and inhibitory nerves which participate in the regulation of airway smooth muscle tone. The innervation of the DT and GPT is similar in that both tissues contain cholinergic excitatory and adrenergic inhibitory nerves. The GPT has an additional, nonadrenergic inhibitory innervation, which is lacking in the DT, but which also is found in cats and humans. There is convincing evidence that the nonadrenergic inhibitory transmitter may be ATP, or its breakdown product, adenosine. It also has been suggested that vasoactive intestinal polypeptide is the transmitter from the nerves (15). Through the appropriate use of atropine and PPG, alone and in combination, it is possible to eliminate the cholinergic and/or adrenergic contributions to neurogenic responses. The nonadrenergic inhibitory influence is obtained by difference. The following effects of CBE on neurogenic responses have been observed (14,15).

In the DT, cholinergically induced contractions are potentiated by CBE. This appears to result from a prejunctional facilitation of cholinergic neurotransmission, since there is no postjunctional interaction between CBE and methacholine (Table 1), a stable analog of the transmitter acetylcholine. This effect of CBE is not blocked by methysergide. Adrenergic inhibitory responses of the DT are unaffected in the presence of CBE.

A different situation exists in the GPT. In this case, CBE has no effect either on cholinergic excitatory or adrenergic inhibitory responses. However, inhibitory responses mediated by the nonadrenergic system are enhanced in the presence of CBE. This effect is not blocked by methysergide. In consideration of the possibility that the release of ATP, or the postjunctional effect of released ATP (or adenosine), are potentiated by CBE, we evaluated the influence of CBE on ATP- and adenosine-induced responses. It may be seen in Table 1 that the reactivity of the tissues to ATP and adenosine was increased by CBE. The postjunctional effects of released ATP (or adenosine) may thus be enhanced by CBE. Whether CBE could facilitate the nonadrenergic neurotransmission process cannot be ascertained from these findings. We have not yet examined the influence of CBE on responses to vasoactive intestinal polypeptide to evaluate the possible involvement of this substance in the CBE effect.

We have drawn a number of conclusions from these observations. Aside from the obvious species differences in the effect of CBE on neurogenic responses of the DT and GPT, these findings indicate that the intrinsic nerves in airway smooth muscle are targets for the actions of CBE and, as such, this mechanism could be involved in the etiology of byssinosis if chronic symptoms are initiated by the acute effects. While the effect of CBE on parasympathetic responses seen in the DT is consonant with a bronchoconstrictive state, it is difficult to relate exacerbation of the influence of the nonadrenergic inhib-

Table 1. Influence of CBE (2.1 mg/mL) on reactivity of dog and guinea pig isolated trachealis to several agents.^a

Trachealis	Agent	Sensitivity to agent ^b	Maximum response ^c
Dog	Methacholine	Unchanged	Unchanged
	Histamine	Unchanged	Increased
	5-Hydroxytryptamine	Unchanged	Increased
	KCl	Unchanged	Unchanged ^d
	Isoproterenol	Unchanged	Increased
Guinea Pig	Methacholine	Unchanged	Unchanged
	Histamine	Unchanged	Unchanged ^e
	5-Hydroxytryptamine	Unchanged	Decreased ^e
	KCl	Decreased ^e	Unchanged
	Isoproterenol	Unchanged	Unchanged ^e
	Adenosine triphosphate	Increased ^f	Increased
	Adenosine	Increased ^f	Increased

^aThis concentration is at or slightly above threshold for inducing contraction.

^bAs determined from EC₅₀ values.

^cContraction for methacholine, histamine, 5-hydroxytryptamine, and KCl; relaxation for isoproterenol, adenosine triphosphate, and adenosine.

^dThe maximum response appeared increased, but the effect was not statistically significant.

^eThis superscript is used to highlight species differences in effects.

^fThe increase in sensitivity was small, but statistically significant.

itory innervation on muscle tone to bronchoconstriction. However, if the influence of this innervation on airway muscle tone were to be reduced, the loss of an important bronchodilating mechanism could lead to bronchoconstriction. This is a tenable working hypothesis insofar as the inhibitory innervation(s) of the guinea pig trachea are compromised following chronic sensitization of the animals to induce airways hyperreactivity (23), and a reduced role of the nonadrenergic inhibitory innervation in sensitized animals has been proposed (24).

Studies on Cat Trachealis

Because of the species differences observed in the effects of CBE on the DT and GPT discussed above, we were prompted to conduct similar studies using the CT. As in humans and in guinea pigs, the airway smooth muscle of the cat receives a nonadrenergic innervation which probably is of physiological significance (25).

CBE evoked concentration-dependent contractions, the maximum response occurring at ca. 30 mg/mL of CBE; the maximum response (~150 g developed tension/g wet tissue weight) was approximately 35% of that obtained

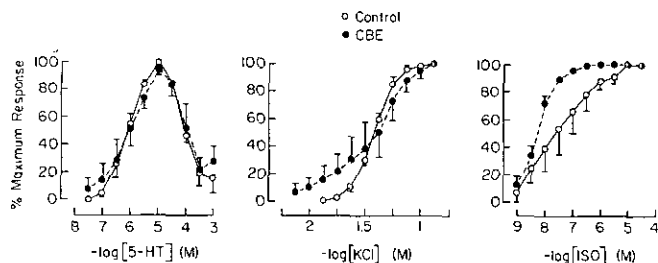


FIGURE 1. Effect of CBE (2.1 mg/mL) on the 5-hydroxytryptamine (5-HT), KCl, and isoproterenol (ISO) concentration-response relationships of CT. Relaxation responses to isoproterenol were obtained following the induction of contractile tone with 17.8 mM KCl. $n = 4$ for each agent. The vertical bars in this and the other figures indicate SEM.

with 120 mM KCl ($n = 4$; data not shown).

The effect of CBE on 5-hydroxytryptamine, KCl and isoproterenol concentration-response curves is shown in Figure 1. The concentration of CBE used in these experiments was 2.1 mg/mL, a concentration just above threshold for inducing contractions. CBE had no effect on the 5-HT concentration-response curve (i.e., the EC₅₀ and maximum response were not affected in the presence of CBE). This finding is different from the effect of CBE in the DT and GPT (see Table 1), in which the maximum responses were increased and decreased, respectively, by CBE.

CBE reduced the threshold for responses to KCl, and increased significantly responses to low concentrations of KCl. The EC₅₀ and maximum response for KCl were

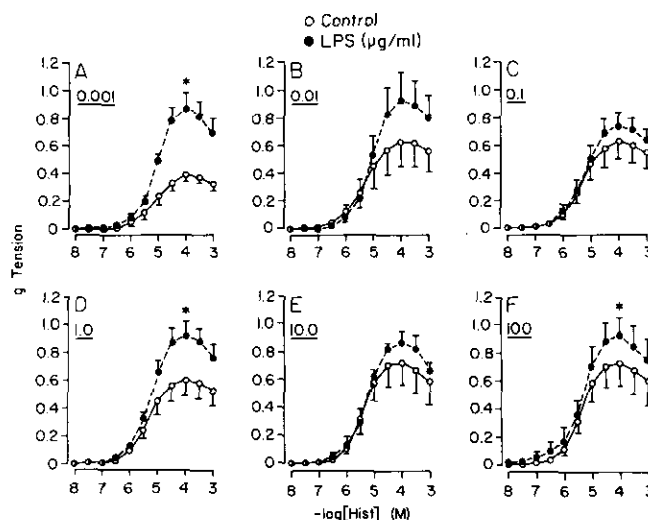


FIGURE 2. Effect of *E. coli* LPS on the histamine (Hist) concentration-response relationship of the GPT. The underlined number in each panel refers to the concentration of LPS which was present, in $\mu\text{g/mL}$. The asterisk (*) indicates significantly different from control (no LPS present). $n = 4-8$ (see Table 2).

not affected. Inspection of the results suggested that the KCl concentration–response curve obtained in the presence of CBE was shallower than the control curve. However, an analysis of the data indicated that the slopes of the KCl curves were not significantly different, perhaps because of the small size of the sample ($n = 4$). These changes in the KCl concentration–response curve of the CT are of a type not seen in the DT and GPT (Table 1).

CBE had no effect on the maximum response to isoproterenol. It produced some effects on the isoproterenol concentration–response curve which appeared real, but which, due to small sample size ($n = 4$), were nevertheless not significant. These included the seemingly increased sensitivity of the preparations, as judged by a 5.6-fold shift of the isoproterenol concentration–response curve to the left of control ($0.1 > p > 0.05$), and an increased slope of the concentration–response curve ($0.1 > p > 0.05$). If these trends in the data are supported with additional experiments to increase sample size, then the effects of CBE on responses of the CT to isoproterenol would provide another example of species-dependent effects of CBE.

Attempts were made to evaluate the effect of CBE on neurogenic responses of the CT, in order to determine whether the potentiating action of CBE on nonadrenergic inhibitory responses of the GPT might also be evident in feline tissue. Unfortunately, the preparations deteriorated markedly when stimulated electrically, both when under the influence of intrinsic, spontaneous tone, and when the tissues were made to develop a contrac-

tion with 5-HT or histamine; reliable frequency–response curves could not be obtained. Therefore, the possible effects of CBE on excitatory and inhibitory neurogenic responses could not be tested.

Effect of *E. coli* LPS on Responses of GPT

Concentration–response curves for various agents were evaluated in the presence of several concentrations of *E. coli* LPS (lipopolysaccharide B, *E. coli* 0127:B8; Difco Labs) following a preincubation period of 15 to 30 min. In this series of experiments, individual histamine and methacholine concentration–response curves were, for each concentration of LPS, generated in the same experiment; studies with isoproterenol were conducted separately. Figure 2 and Table 2 show that LPS, in concentrations of 0.001, 1.0, and 100 $\mu\text{g}/\text{mL}$ increased significantly the maximum response of the tissues to histamine. The remaining LPS concentrations tested (0.01, 0.1, and 10.0 $\mu\text{g}/\text{mL}$) appeared to exert a similar effect on the maximum response, but for these concentrations the potentiations were not significant (e.g., compare panels B and D). The EC_{50} values for histamine were unaffected by LPS. Concentration–response curves for methacholine and isoproterenol were not altered significantly by LPS (Figs. 3 and 4; Table 2). These findings suggested that LPS might *in vivo* potentiate the effects of endogenous histamine, and contribute to bronchoconstriction.

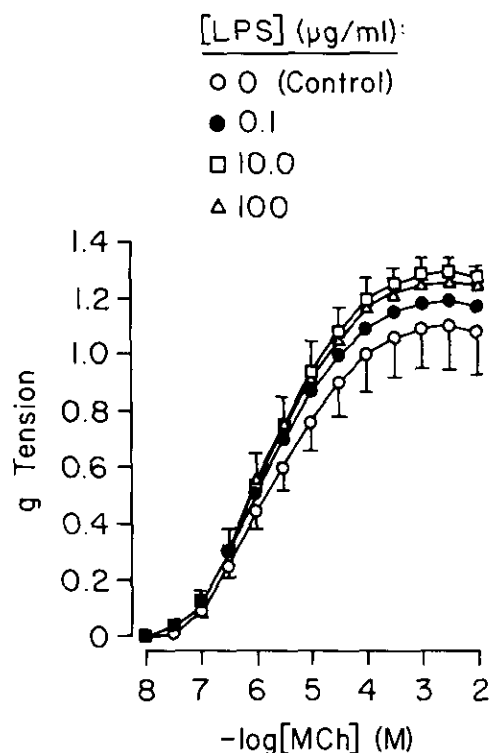


FIGURE 3. Effect of *E. coli* LPS on the methacholine (MCh) concentration–response relationship of the GPT. $n = 4-8$ (see Table 2).

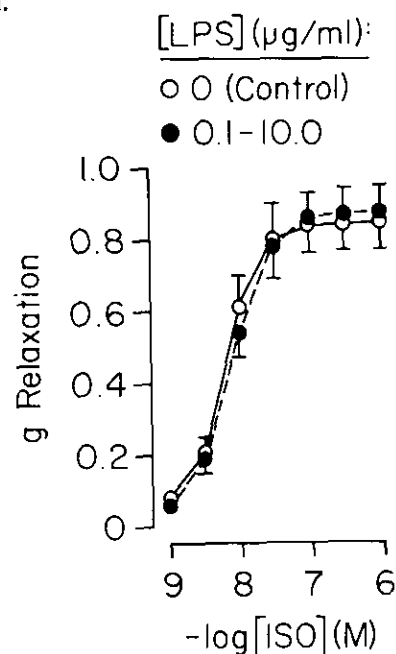


FIGURE 4. Effect of *E. coli* LPS on the isoproterenol (ISO) concentration–response curve of the GPT. The concentrations of LPS which were used are shown in Table 2. However, only the results obtained with 10 $\mu\text{g}/\text{mL}$ LPS are plotted, as the other curves (not shown) were nearly superimposable on the ones depicted. The curve for 10 $\mu\text{g}/\text{mL}$ LPS is therefore representative of the lack of effect of LPS in any of the concentrations used ($n = 4$ for each concentration of LPS).

Table 2. Effect of various concentrations of LPS on agonist-induced responses of the guinea pig isolated trachealis.

Agonist	[LPS] $\mu\text{g/mL}$	$-\log EC_{50}$ (M)	Maximum response, g^a	<i>n</i>
Histamine	0.0	5.36 ± 0.11	0.60 ± 0.11	8
	0.001	5.23 ± 0.11	$0.86 \pm 0.12^*$	4
	0.01	5.18 ± 0.08	0.98 ± 0.20	5
	0.1	5.31 ± 0.13	0.73 ± 0.10	7
	1.0	5.39 ± 0.07	$0.92 \pm 0.10^*$	8
	10.0	5.39 ± 0.12	0.87 ± 0.08	4
	100.0	5.48 ± 0.16	$0.92 \pm 0.13^*$	4
Methacholine	0.0	5.59 ± 0.12	1.10 ± 0.15	8
	0.001	5.70 ± 0.10	1.11 ± 0.18	4
	0.01	5.47 ± 0.15	1.08 ± 0.15	4
	0.1	5.62 ± 0.10	1.19 ± 0.13	8
	1.0	5.68 ± 0.09	1.25 ± 0.16	8
	10.0	5.56 ± 0.16	1.29 ± 0.05	4
	100.0	5.47 ± 0.20	1.25 ± 0.22	4
Isoproterenol	0.0	8.26 ± 0.03	0.84 ± 0.10	4
	0.1	8.18 ± 0.04	0.75 ± 0.19	4
	1.0	8.16 ± 0.12	0.88 ± 0.11	4
	10.0	8.42 ± 0.10	0.86 ± 0.14	4

^a Contraction for histamine and methacholine; relaxation for isoproterenol.

* $p \leq 0.5$ (paired *t*-test).

suggested that LPS might *in vivo* potentiate the effects of endogenous histamine, and contribute to bronchoconstriction.

The LPS stock solution used in these experiments had been made up using distilled water and the container kept at 4°C. After the experiments were concluded, we noted that the LPS stock had become cloudy. Even though the above effect of LPS was specific for histamine, we suspected that bacterial(?) contamination in the stock, and not the LPS *per se*, was responsible for the observed potentiation. We, therefore, repeated these experiments using a fresh batch of LPS. This time the LPS was dissolved in sterile, nonpyrogenic water, and aliquots were frozen for use. The results obtained with selected concentrations of LPS are presented in Table 3. In this series of experiments there were no significant effects of LPS on the EC_{50} or maximum responses of the tissues to histamine and methacholine. At this point, preliminary findings indicate that responses to KCl and isoproterenol also were unaffected by LPS.

Since the second series of experiments was performed using the appropriate precautions to prevent contamination of the LPS, we conclude that LPS probably is pharmacologically innocuous in the GPT preparation, at

least with respect to the reactivity of the tissues to histamine, methacholine, KCl, and isoproterenol. We have not yet performed experiments to determine whether LPS can interfere with the development of neurogenic responses. That is, while LPS may not modify postjunctionally the actions of these agonist, it remains possible that the release of neurotransmitters could be modified by LPS. Since endotoxin increases the spontaneous release of acetylcholine at the neuromuscular junction, and also affects postjunctional calcium conductance (26), it would seem appropriate because of our previous findings to conduct evaluations of LPS on neurogenic responses of the DT and GPT.

The possibly artifactual results obtained initially with LPS may have some merit, because they suggest that cruder bacterial products could have pharmacological effects not seen with the LPS fraction. It is, therefore, our intention to repeat these experiments using crude endotoxin. An additional reason for this line of pursuit is the report (27) that α -adrenoceptor-mediated contractile responses of human bronchial smooth muscle were potentiated markedly by endotoxin from *E. coli*. In our laboratory, we have been unable to confirm this interaction using the GPT and LPS. A species difference in this

Table 3. Second evaluation of the effect of various concentrations of LPS on histamine- and methacholine-induced responses of guinea pig trachealis.

Agonist	[LPS], $\mu\text{g/mL}$	$-\log EC_{50}$ (M)	Maximum response, g^a	<i>n</i>
Histamine	0.0	5.24 ± 0.18	0.71 ± 0.15	5
	0.1	5.02 ± 0.06	0.81 ± 0.17	5
	1.0	5.20 ± 0.13	0.64 ± 0.14	5
	10.0	5.20 ± 0.09	0.61 ± 0.13	5
Methacholine	0.0	5.53 ± 0.12	0.93 ± 0.11	5
	0.1	5.56 ± 0.18	0.98 ± 0.14	5
	1.0	5.55 ± 0.11	1.01 ± 0.13	5
	10.0	5.59 ± 0.18	1.03 ± 0.09	5

^a Contraction. There were no significant effects of LPS in this series of experiments.

effect of endotoxin seems possible.

Last, recent evidence indicates that *E. coli* LPS, following its acute administration to dogs, resulted in a decrease in the number of hepatic β -adrenergic receptors (28–30). Moreover, *E. coli* LPS caused, 4 days after IP injection of the animals, a reduction in β -adrenoceptor number in guinea pig lung (31). These reports could indicate that there is species-dependent diversity in the effects of LPS in the β -adrenoceptor coupling system, or that time is required to induce the alterations in lungs which correlate to the role of endotoxins in the development of byssinosis. If the latter is true, then it could explain why, in our laboratory, LPS had no effect on the responses of the GPT to isoproterenol.

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