ABSTRACT
We investigated the in vivo and in vitro effects of lipopolysaccharide (LPS) treatment (4 mg/kg i.p.) on guinea pig airway smooth muscle reactivity and epithelial bioelectric responses to methacholine (MCh) and hyperosmolarity. Hyperosmolar challenge of the epithelium releases epithelium-derived relaxing factor (EpDRF). Using a two-chamber, whole body plethysmograph 18 h post-treatment, animals treated with LPS were hyporeactive to inhaled MCh aerosol. This could involve an increase in the release and/or actions of EpDRF, because LPS treatment enhanced EpDRF-induced smooth muscle relaxation in vitro in the isolated perfused trachea apparatus. In isolated perfused tracheas the basal transepithelial potential difference (Vt) was increased after LPS treatment. The increase in Vt was inhibited by amiloride and indomethacin. Concentration-response curves for changes in Vt in response to serosally and mucosally applied MCh were biphasic (hyperpolarization, \( <3 \times 10^{-7} \) M; depolarization, \( >3 \times 10^{-7} \) M); MCh was more potent when applied serosally. The hyperpolarization response to MCh, but not the depolarization response, was potentiated after LPS treatment. In both treatment groups, mucosally applied hyperosmolar solution (using added NaCl) depolarized the epithelium; this response was greater in tracheas from LPS-treated animals. The results of this study indicate that airway hyporeactivity in vivo after LPS treatment is accompanied by an increase in the release and/or actions of EpDRF in vitro. These changes may involve LPS-induced bioelectric alterations in the epithelium.

Asthma is a chronic disease that is characterized by reversible airway obstruction, airway inflammation, and airway hyperresponsiveness. Lipopolysaccharide (LPS) or endotoxin, the major component of the outer membrane of gram negative bacteria, poses problems for asthmatic patients. For example, inhaled endotoxin causes a slight decrease in the forced expiratory volume in 1 s and an increase in histamine responsiveness in asthmatic patients, but not in normal subjects (Michel et al., 1989, 1996). Several reports have demonstrated that LPS administration leads to the development of airway hyperreactivity and inflammation in mice (Held and Uhlig, 2000), rats (Pauwels et al., 1990), and guinea pigs (Toward and Broadley, 2000).

Airway reactivity is under the control of numerous physiological mechanisms, including epithelial-derived excitatory and inhibitory substances (Fedan et al., 1988; Goldie and Hay, 1997; Folkerts and Nijkamp, 1998). One such inhibitory substance that has been shown to modulate airway reactivity is the non-nitric oxide nonprostanoid epithelium-derived relaxing factor (EpDRF), which is released in response to hy-
perosmolarity\(^1\) at the mucosal or serosal surface of airway epithelial cells (Munakata et al., 1988, 1990; Fedan et al., 1999, 2003a). EpDRF release during hyperosmolar challenge occurs in response to the incremental increase in osmolarity (Fedan et al., 2003a) and is associated with epithelial bioelectric events (Dortch-Carnes et al., 1999; Wu et al., 2003). Evidence has been obtained to suggest that hyperosmolar challenge of the epithelium releases carbon monoxide (Fedan et al., 2003b). Once released, EpDRF initiates airway smooth muscle relaxation via an unknown mechanism.

There have been several reports indicating the importance of EpDRF in modulating airway reactivity. For example, in the guinea pig isolated perfused trachea, reactivity to methacholine (MCh) is decreased substantially when the release of EpDRF is stimulated by challenging the epithelium with hyperosmolar solution (Fedan et al., 1999). In addition, EpDRF-induced smooth muscle relaxation is significantly attenuated in isolated perfused tracheas from guinea pigs exposed to ozone, which is accompanied by in vitro and in vivo hyperreactivity to MCh (Fedan et al., 2000). Finally, preliminary evidence indicates that sensitization and challenge of guinea pigs with ovalbumin enhances the release of EpDRF and causes in vitro airway hyporeactivity to MCh, at a time when the animals exhibit hyperreactivity to inhaled MCh (Warner et al., 1996).

In addition, there is evidence that indicates that the synthesis, release, and/or effects of EpDRF are functionally linked to the electrical activity of epithelial cells. Dortch-Carnes et al. (1999) demonstrated that a decrease in the transepithelial potential difference (Vt) preceded EpDRF-in-duced smooth muscle relaxation elicited by elevating aerosol or mucosal osmolarity with either ionic or nonionic osmolytes. Fedan et al. (1999) demonstrated that amiloride-sensitive Na\(^+\) channels and 4,4'-diisothiocyanato-2,2'-stilbene disulfonate-sensitive Cl\(^-\) channels are involved in EpDRF-induced smooth muscle relaxation, and Tamaoki et al. (1997) published evidence for involvement of Ca\(^{2+}\)-activated K\(^+\) channels in the synthesis and/or release of EpDRF in human bronchial strips.

LPS has been shown to alter airway reactivity in human and animal subjects. Therefore, the purpose of this study was to examine the possible relationship between alterations in airway reactivity induced by LPS and alterations in the smooth muscle effects of EpDRF and the epithelial bioelectric events associated with its release.

**Materials and Methods**

**Animals.** These studies were conducted in facilities accredited fully by the Association for the Assessment and Accreditation of Laboratory Animal Care International and were approved by the institutional Animal Care and Use Committee. The animals were anesthetized with sodium pentobarbital (65 mg/kg i.p.) and sacrificed by thoracotomy and bleeding before removing the trachea. Other details of animal use have been given (Fedan et al., 2003a).

**In Vivo Saline and LPS Treatment.** Guinea pigs were injected i.p. with 4 mg/kg LPS from *Salmonella typhimurium* or a volume equivalent of saline (control). Eighteen hours post-treatment, in vivo or in vitro experiments were conducted.

**Two-Chamber, Whole-Body Plethysmograph.** This method has been described in detail previously (Lawrence et al., 1997). Briefly, a two-chamber, whole-body plethysmograph (Buxco Electronics, Inc., Sharon, CT) was used to measure specific airway resistance (SRaw) before and after delivery of MCh aerosol to measure airway reactivity in saline- and LPS-treated animals. Each chamber of the plethysmograph was connected to a differential pressure transducer and then to a noninvasive LS-20 airway mechanics analyzer (Buxco Electronics, Inc.) connected to a computer for logging acquired data at 6-s intervals.

The animal was acclimated in the plethysmograph while breathing air for 2 h on the day before the experiment. Eighteen hours after saline- or LPS treatment, the animal was placed in the plethysmograph and SRaw measurements were recorded for 4 min after an initial 16-min acclimation period in air. Dose-response curves for responses to aerosolized MCh were then generated.

**In Vivo MCh Dose-Response Curves.** The animal was exposed to aerosolized saline as a vehicle control. The saline was delivered for 3 min, followed by a 10-min recording period. Dose-response curves were generated by administering MCh aerosol (Ultra Neb 99 nebulizer; Devilbiss Co., Somerset, PA) in half-log concentrations ranging from 0.03 to 3.16 mg/ml. Each MCh aerosol was delivered for 3 min, followed by a 10-min recording period. The peak SRaw value was taken as the response to that particular concentration of MCh. Before the next higher dose of MCh was administered, SRaw was allowed to return to baseline. Airway reactivity to MCh was determined by calculating the MCh PC\(_{200}\), the provocative MCh concentration producing a 2-fold increase in SRaw above the value after saline administration. The PC\(_{200}\) was calculated by linear interpolation of graphed data.

**In Vivo Administration of N\(^{\text{N}}\)-Nitro-L-arginine Methyl Ester (L-NAME).** Guinea pigs were exposed to L-NAME aerosol (1.2 mM) for 5 min before the delivery of each MCh concentration. Two MCh dose-response curves were generated in both saline- and LPS-treated animals, the first in the absence and the second after treatment with L-NAME. This protocol is similar to that used by Nijkamp et al. (1993).

**Histological Analysis of Guinea Pig Airways.** After anesthesia and exsanguination, trachea and lungs from saline- and LPS-treated animals were inflated with 15 to 20 ml of 10% buffered formalin phosphate and removed en bloc. Sections of the trachea as well as the tracheal, left, right apical, right cardiac, and right diafragmatic lobes of the lung were then embedded with paraffin before being cut into 5-μm slices with a microtome. Individual sections were stained according to Harris' hematoxylin and eosin procedure and prepared for reading. The slides were examined in a “blind” manner under the light microscope by a veterinary pathologist.

**Modified Isolated Perfused Trachea Apparatus for Simultaneous Measurement of Transepithelial Potential Difference and Mechanical Responses.** The isolated perfused trachea preparation was used to measure responses of the smooth muscle elicited by challenge of the epithelium with hyperosmolar solution, and other drug effects. This preparation permits separate application of agents to the mucosal (intraluminal) or serosal (extraluminal) surfaces of the trachea. The method has been described previously (Fedan and Frazer, 1992; Fedan et al., 2003a), along with modifications (Dortch-Carnes et al., 1999) for measuring Vt simultaneously with smooth muscle contractile and relaxant responses.

**MCh and Elevated Osmolarity Concentration-Response Curves.** MCh concentration-response curves were generated by adding MCh in stepwise-increasing, cumulative concentrations to the extraluminal and intraluminal baths. Both extraluminal and intraluminal MCh concentration-response curves were obtained from each trachea, the extraluminal curve first, followed 90 min later by the intraluminal curve, with washes every 15 min.

\(^1\) Hypertonic solutions are those that cause cell shrinkage. Hyperosmolar solutions have osmolarity greater than that of the physiological extracellular solution. For simplicity, in this report we do not draw distinctions between the two terms when describing general phenomena.
Concentration-response curves for the effects of hyperosmolarity were generated by adding NaCl, D-mannitol (D-M), urea, or KCl in stepwise-increasing cumulative concentrations to the extraluminal and/or intraluminal baths in the presence or absence of extraluminal MCh (3 × 10⁻⁷ M), as described under Results.

Tracheal Epithelial Removal. In experiments that required removal of the tracheal epithelium, a trimmed 6-cm segment of pipe cleaner was slowly inserted into the tracheal lumen and then withdrawn while rotating slowly (Fedan and Frazer, 1992).

Inhibitors. L-NAME [nitric-oxide (NO) synthase inhibitor; 10⁻⁴ M] and indomethacin (cyclooxygenase inhibitor; 3 × 10⁻⁶ M) were used to determine whether hyperosmolarity-induced smooth muscle relaxation involved an NO or prostacyclin component. The inhibitors were added to the extraluminal and intraluminal baths 30 min before inducing tone with extraluminally added 3 × 10⁻⁷ M MCh. The relaxation responses in the presence of the inhibitors were compared with relaxation responses in preparations where the inhibitor was absent.

Effect of Agents on the Basal V₀. The following agents were evaluated for their effects on epithelial bioelectric properties: indomethacin (3 × 10⁻⁶ M), L-NAME (10⁻⁴ M), and the epithelial Na⁺ channel blocker amiloride (3 × 10⁻⁵ M). Indomethacin and L-NAME were added simultaneously to the extraluminal and intraluminal baths, whereas amiloride was only added to the intraluminal bath.

Solutions and Reagents. The modified Krebs-Henseleit solution (MKHS) contained 113.0 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25.0 mM NaHCO₃, and 5.7 mM glucose. The MKHS was continuously gassed with 95% O₂, 5% CO₂ and maintained at 37ºC and pH 7.4. The osmolality of the MKHS was 281 ± 5 mosM.

All drugs, chemicals, and agents were from Sigma-Aldrich (St. Louis, MO) and dissolved in saline unless otherwise noted. Amiloride was dissolved in distilled water; indomethacin was dissolved in 100 mM sodium carbonate.

Statistical Analysis of Results. For in vitro airway reactivity studies, SRaw and MCh PC200 values among saline- and LPS-treated animals were compared using the paired or nonpaired Student’s t test or the Mann-Whitney rank sum test, as appropriate. For in vitro airway reactivity studies, geometric EC₅₀ values were derived from least-squares analysis of a sigmoidal curve fit. When comparing the EC₅₀ values, normally distributed logEC₅₀ values were used. The EC₅₀ values, maximum responses, and the intraluminal/extraluminal maximum response ratios were analyzed using the paired or nonpaired Student’s t test or one-way repeated measures analysis of variance, as appropriate. When examining the effect of agents on the basal V₀, the data were analyzed statistically using either the paired or nonpaired Student’s t test or the Mann-Whitney rank sum test, as appropriate. The results, except where noted, were expressed as the mean ± S.E; n is the number of separate experiments. p < 0.05 was considered significant.

Results

Effect of Saline and LPS Treatments on Basal Pulmonary Function and in Vivo Airway Reactivity to MCh. Basal respiratory rate was slightly, but significantly, decreased by saline treatment (data not shown). However, there were no other differences between the pre- or post-treatment breathing frequency and SRaw values within each treatment group. LPS treatment induced a rightward shift in the MCh dose-response curve (Fig. 1). The MCh PC₂₀₀ (milligrams of MCh per milliliter) values [95% confidence interval (CI) in parentheses] were saline, 0.08 (0.05–0.14); and LPS-treated, 0.26 (0.08–0.79) (p < 0.05).

Effect of L-NAME on in Vivo Airway Reactivity to MCh in Saline- and LPS-Treated Animals. Because LPS is known to induce NO synthase activity in the lung (Salter et al., 1991) and because NO is capable of modulating airway smooth muscle reactivity to agonists (Fedan et al., 1995; Folkerts and Nijkamp, 1998), it was hypothesized that NO could account for the in vivo airway hyporeactivity to MCh after LPS treatment. Thus, the effect of an aerosol of L-NAME, an NO synthase inhibitor, on in vivo airway reactivity to MCh was determined (Fig. 2). MCh dose-response curves obtained in the absence of L-NAME indicated that the LPS-treated animals were hyporeactive to MCh (Table 1). Administration of L-NAME before MCh had no effect upon airway reactivity in either the saline- or LPS-treated animals (Table 1).

Histological Examination of Guinea Pig Airways after LPS Treatment. Because L-NAME failed to provide any clues to the mechanisms underlying LPS-induced in vivo airway hyporeactivity to MCh, we examined the effects of LPS treatment on the structure of the guinea pig respiratory tract. This was done to determine whether LPS treatment altered the structure of the airways in such a way as to limit access of MCh to the airway smooth muscle (i.e., excessive mucus secretion, epithelial and basement membrane hyperplasia). Histological sections of the distal trachea as well as the azygous, left, right, apical, right cardiac, and right diaphragmatic lobes of the lung from saline- and LPS-treated animals were examined. The sections

![Fig. 1. Effect of LPS treatment on in vivo airway reactivity to inhaled MCh aerosol. Reactivity to MCh was measured 18 h after saline or LPS treatment. Saline- and LPS-treated, n = 6.](image1)

![Fig. 2. Lack of effect of L-NAME on in vivo airway reactivity to inhaled MCh aerosol in saline- (A) and LPS-treated (B) animals. Each animal served as its own control, and reactivity to MCh was measured 18 h after saline or LPS treatment, before and after delivery of aerosolized L-NAME (1.2 mM). Saline- and LPS-treated, n = 4.](image2)
from both treatment groups were indistinguishable from one another, at least at the level of the light microscope. Representative sections of the distal trachea and left lobe of the lung from saline- and LPS-treated animals are shown in Fig. 3. The bronchial and tracheal epithelium as well as the alveolar sacs remained structurally intact within each treatment group. Finally, in both treatment groups, a few inflammatory cells, namely, eosinophils and neutrophils, could be seen infiltrating the airways in some regions of the respiratory tract that were examined (data not shown).

Effect of LPS Treatment on in Vitro Reactivity to MCh in Epithelium-Intact and -Denuded Isolated Perfused Trachea. Because LPS treatment caused in vivo airway hyporeactivity to inhaled MCh, we investigated whether this effect manifested itself in vitro in epithelium-intact and -denuded isolated perfused trachea.

In epithelium-intact tracheas, there was no difference in reactivity to extraluminally and intraluminally applied MCh between the two treatment groups. The tracheas from both treatment groups were more sensitive to extraluminally applied MCh (Fig. 4A; Table 2). In addition, there was no difference in the intraluminal/extraluminal maximum response ratio (Fedan and Frazer, 1992) between the saline- and LPS-treated groups.

In epithelium-denuded tracheas, the differences between extraluminal and intraluminal reactivity to MCh were abolished, and the intraluminal/extraluminal maximum response ratio approached unity (Fig. 4B; Table 3). In addition, there was no difference in reactivity to extraluminally and intraluminally applied MCh in epithelium-denuded tracheas from saline- and LPS-treated animals.

Effect of Hyperosmolar Solution-Induced Smooth Muscle Relaxation in Isolated Perfused Trachea. Because LPS treatment caused in vivo airway hyporeactivity to MCh, it was hypothesized that EpDRF-mediated relaxation responses to hyperosmolar challenge would be potentiated. Intraluminal osmolarity was increased using NaCl, d-M, urea, or KCl as osmolytes after contraction of the smooth muscle with extraluminally applied MCh (3 × 10⁻⁷ M). When NaCl and d-M were used to increase osmolarity, relaxation responses of LPS-treated animals were potentiated in tracheas from LPS-treated animals (Fig. 5; Table 4). However, LPS treatment had no effect on responses to KCl (n = 4; data not shown), and it reduced significantly the potency of urea (n = 6; data not shown).

Effect of l-NAME and Indomethacin on Hyperosmolar Solution-Induced Smooth Muscle Relaxation in Isolated Perfused Trachea from Saline and LPS-Treated Guinea Pigs. Because LPS is known to up-regulate mRNA of both inducible NO synthase and cyclooxygenase (Okamoto et al., 1998), the effect of l-NAME and indomethacin on relaxant responses to hyperosmolar challenge was examined. This was done to rule out the possible contribution of NO and prostaglandins to the potentiation of EpDRF-induced smooth muscle relaxation after LPS treatment because both are capable of relaxing smooth muscle (Folkerts and Nijkamp, 1998). Neither l-NAME nor indomethacin affected responses to hyperosmolarity (NaCl

### Table 1

Effect of l-NAME on airway reactivity to inhaled MCh in saline- and LPS-treated guinea pigs

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>MCh PC₂₀₀ (mg/ml) (95% CI)</th>
<th>L-NAME</th>
<th>+L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (4)</td>
<td>0.07 (0.06–0.10)</td>
<td>0.01</td>
<td>0.00–0.94</td>
</tr>
<tr>
<td>LPS (4)</td>
<td>0.78 (0.31–0.97)</td>
<td>0.40</td>
<td>0.11–1.45</td>
</tr>
</tbody>
</table>

* Significantly greater than saline (–L-NAME).
added) in isolated perfused tracheas from saline- and LPS-treated guinea pigs (Figs. 6 and 7). These findings buttress the view that EpDRF is neither a prostanoid nor NO in the view that EpDRF is neither a prostanoid nor NO in the presence of the epithelium. As the modulatory effect of the epithelium becomes more inhibitory, the ratio becomes smaller (Fedan and Frazer, 1992; Fedan et al., 2000).

In view of the hyperpolarization observed in tracheas from LPS-treated animals, it was of interest to investigate whether LPS applied in vitro could affect Vt across tracheas from naive animals. Using a concentration (10 µg/ml) that was shown previously to inhibit MCh-induced airway smooth muscle contraction in vitro (Fedan et al., 1995), LPS was added simultaneously to the solutions bathing the extraluminal and intraluminal surfaces of tracheas isolated from nontreated animals. Within minutes, LPS began to hyperpolarize the epithelium (data not shown). The time required for stabilization to occur varied from trachea to trachea but ranged between 1 to 3 h. Over similar intervals, control tracheas run in parallel exhibited a small hyperpolarization, which did not achieve statistical significance.

**Table 2**

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>EC50 (M) (95% CI)</th>
<th>Maximum Response (cm H2O)</th>
<th>IL/EL Maximum Response Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EL</td>
<td>IL</td>
<td>EL</td>
</tr>
<tr>
<td>Saline (6)</td>
<td>1.9 × 10^-7 (1.4–2.5)</td>
<td>3.7 × 10^-4 (1.5–9.1)</td>
<td>10.7 ± 1.5</td>
</tr>
<tr>
<td>LPS (6)</td>
<td>2.1 × 10^-7 (1.7–2.4)</td>
<td>3.2 × 10^-4 (1.8–5.8)</td>
<td>6.6 ± 0.9</td>
</tr>
</tbody>
</table>

EL, extraluminal; IL, intraluminal.

* The IL/EL maximum response ratio is used to gauge the modulatory effect of the epithelium on the IL maximum contractile response. In the absence of the epithelium, the ratio is unity, whereas it becomes less than unity in the presence of the epithelium. As the modulatory effect of the epithelium becomes more inhibitory, the ratio becomes smaller (Fedan and Frazer, 1992; Fedan et al., 2000).

+ Significantly greater than saline (EL).

+ Significantly less than saline (EL).

+ Significantly greater than LPS (EL).

+ Significantly less than LPS (EL).

**Table 3**

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>EC50 (M) (95% CI)</th>
<th>Maximum Response (cm H2O)</th>
<th>IL/EL Maximum Response Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EL</td>
<td>IL</td>
<td>EL</td>
</tr>
<tr>
<td>Saline (4)</td>
<td>2.1 × 10^-6 (0.8–5.2)</td>
<td>7.7 × 10^-3 (2.0–30.0)</td>
<td>3.8 ± 0.7</td>
</tr>
<tr>
<td>LPS (4)</td>
<td>1.1 × 10^-6 (0.5–2.2)</td>
<td>1.5 × 10^-3 (0.8–2.8)</td>
<td>3.9 ± 0.4</td>
</tr>
</tbody>
</table>

EL, extraluminal; IL, intraluminal.

* See Fig. 2 legend.

**Table 4**

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>EC50 (mosM) (95% CI)</th>
<th>Maximum Response (Percentage of MCh)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EL</td>
<td>IL</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (8)</td>
<td>11.4 (8.2–15.8)</td>
<td>78.2 ± 6.2</td>
</tr>
<tr>
<td>LPS (8)</td>
<td>10.4 (8.4–12.8)</td>
<td>113.3 ± 9.5</td>
</tr>
<tr>
<td>n-Mannitol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (6)</td>
<td>15.2 (8.2–28.2)</td>
<td>86.1 ± 4.9</td>
</tr>
<tr>
<td>LPS (6)</td>
<td>15.9 (11.1–22.7)</td>
<td>109.7 ± 8.5</td>
</tr>
</tbody>
</table>

* Values refer to milliosmoles of osmolyte added to the intraluminal MKHS.

+ Relaxation responses in centimeters of H2O were normalized as a percentage of the extraluminal MCh (3 × 10^-7 M)-induced contraction.

+ Significantly greater than saline-treated controls.

**Figure 5.** Effect of LPS treatment on EpDRF-induced smooth muscle relaxation elicited with intraluminal MKHS made hyperosmolar with added NaCl (A) or D-M (B). The concentration-response curves were obtained from tracheas removed from saline- and LPS-treated animals 18 h post-treatment. The tracheal smooth muscle was contracted with extraluminally applied MCh (3 × 10^-7 M) before NaCl or D-M were added to the intraluminal bath in stepwise-increasing, cumulative concentrations. Saline- and LPS-treated, n = 8 for NaCl and n = 6 for D-M. *p < 0.05 compared with saline.
tracheal smooth muscle was contracted with extraluminally applied MCh from saline- (A) and LPS-treated (B) animals 18 h post-treatment. The concentration-response curves were obtained from tracheas removed with intraluminal MKHS made hyperosmolar with added NaCl. The results suggest that basal $V_t$ is under a modest cholinergic influence.

Intraluminal and extraluminally applied indomethacin (3 × 10^{-6} M) on EpDRF-induced smooth relaxation elicited with intraluminal MKHS made hyperosmolar with added NaCl. The concentration-response curves were obtained from tracheas removed from saline- (A) and LPS-treated (B) animals 18 h post-treatment. The tracheal smooth muscle was contracted with extraluminally applied MCh (3 × 10^{-7} M) before NaCl was added to the intraluminal bath in stepwise-increasing, cumulative concentrations. Saline- and LPS-treated, $n = 4$.

Saline- and LPS-treated, $n = 4$.

![Fig. 6. Lack of effect of extraluminally and intraluminally applied l-NAME (10^{-4} M) on EpDRF-induced smooth relaxation elicited with intraluminal MKHS made hyperosmolar with added NaCl. The concentration-response curves were obtained from tracheas removed from saline- (A) and LPS-treated (B) animals 18 h post-treatment. The tracheal smooth muscle was contracted with extraluminally applied MCh from saline- and LPS-treated animals.](image)

Intraluminal and extraluminally applied indomethacin. Saline- and LPS-treated, $n = 4$.

![Fig. 7. Lack of effect of extraluminally and intraluminally applied l-NAME (3 × 10^{-6} M) on EpDRF-induced smooth relaxation elicited with intraluminal MKHS made hyperosmolar with added NaCl. The concentration-response curves were obtained from tracheas removed from saline- (A) and LPS-treated (B) animals 18 h post-treatment. The tracheal smooth muscle was contracted with extraluminally applied MCh (3 × 10^{-7} M) before NaCl was added to the intraluminal bath in stepwise-increasing, cumulative concentrations. Saline- and LPS-treated, $n = 4$.](image)

There was no effect of LPS on the magnitude of the hyperpolarization ($n = 4$; data not shown). SNP (10^{-6} M) caused a very small depolarization ($n = 4$; data not shown). These results suggest that $V_t$ is under a slight influence of NO. However, the effect of l-NAME could have resulted from muscarinic receptor blockade (Buxton et al., 1993). Therefore, the effect of atropine (10^{-6} M) was examined to compare the effect of muscarinic receptor blockade with that of l-NAME. Atropine caused depolarization or hyperpolarization responses in different preparations. On average atropine increased $V_t$ by 0.9 ± 0.5 mV in tracheas from saline-treated animals and decreased $V_t$ by 0.3 ± 0.6 mV in tracheas from LPS-treated animals ($p > 0.05$; data not shown). Therefore, atropine did not mimic l-NAME, but the results suggest that basal $V_t$ is under a modest cholinergic influence.

Effect of LPS Treatment on Bioelectric Reactivity to MCh. In tracheas from both saline- and LPS-treated anim-
that the two events may be functionally linked. Because LPS treatment potentiated smooth muscle relaxation in response to hyperosmolar challenge of the epithelium, we sought to determine whether LPS treatment also affected epithelial bioelectric responses to hyperosmolar solution.

NaCl concentration-response curves for bioelectric responses were generated in the presence of extraluminally applied MCh (Fig. 10), to mimic the conditions utilized for studying relaxation responses. In tracheas from LPS-treated animals, the intraluminal addition of NaCl led to a significantly greater depolarization response compared with saline-treated controls; however, the epithelium eventually depolarized to the same level in each treatment group (Fig. 10; Table 6).

The concentration-response relationships for hyperosmolarity-induced epithelial depolarization and smooth muscle relaxation are compared in Fig. 11. In both treatment groups, the EC50 value for depolarization was significantly greater than the EC50 value for EpDRF-induced smooth muscle relaxation (Table 6).

**Effect of Amiloride on Basal V_t.** It has been demonstrated previously that relaxation and bioelectric responses of the perfused trachea to hyperosmolar solutions involve epithelial Na⁺ transport (Fedan et al., 1999; Wu et al., 2003). We, therefore, considered the possibility that the bioelectric changes resulting from LPS treatment could involve a change in Na⁺ transport (Fig. 12). In tracheas from both saline- and LPS-treated animals amiloride (3 × 10⁻⁵ M) applied to the intraluminal bath decreased basal V_t. The depolarization was larger in the LPS-treated group. In the presence of amiloride, there was no longer any difference in the V_t values between the saline- and LPS-treated groups.

**Discussion**

Several general conclusions can be drawn from this study. First, systemic, noninhalational administration of LPS induces in vivo airway hyperreactivity to inhaled MCh in the absence of an inflammatory response. Second, LPS treatment potentiates EpDRF-induced airway smooth muscle relaxation in response to hyperosmolar solution in an osmolyte-specific manner and is associated with potentiated epithelial depolarization responses. Finally, epithelial depolarization does not always accompany EpDRF-induced smooth muscle relaxation, as judged by the disparity in the potency of NaCl as a relaxant and depolarizing osmolyte. This could indicate, at least in the case of NaCl, either that multiple mechanisms underlie epithelium-dependent relaxation, and only one of these is linked to depolarization of the epithelium, or that EpDRF release and epithelial depolarization are coincident but unrelated phenomena.

The LPS treatment protocol (4 mg/kg i.p.; 18 h post-treatment) used in this study caused in vivo airway hyperreactivity to inhaled MCh, whereas no differences in in vitro reactivity to MCh existed in the isolated perfused tracheas taken from saline- and LPS-treated animals. There have been several reports concerning the effect of LPS treatment on in vitro and in vivo guinea pig airway reactivity to contractile agents. LPS has been reported to induce hyperreactivity (Folkerts et al., 1989; Yamawaki et al., 1990; Van Oosterhout et al., 1991), hyporeactivity (Folkerts et al., 1988), and no change in reactivity of airway smooth muscle (Fedan et al., 1995). In the present study, there were no differences in airway reactivity to MCh in epithelium-intact and -denuded isolated perfused tracheas. This is consistent with a previous study from our laboratory (Fedan et al., 1995), even though the 18 h post-treatment time point was not examined. In vivo, LPS-treated guinea pigs were hyporeactive to the effects of inhaled MCh. This is not consistent with the earlier published report from our laboratory (Fedan et al., 1995), but again, the 18 h post-treatment time point may account for the differences.

Because it is well known that LPS up-regulates inducible NO synthase, it was plausible that NO may have contributed

**Table 5**

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>EC50 (M) (95% CI)</th>
<th>Maximum Response ΔV_t (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EL</td>
<td>IL</td>
</tr>
<tr>
<td>Hyperpolarization (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (7)</td>
<td>6.2×10⁻⁸ (2.9–13.0)</td>
<td>9.6×10⁻⁸ (2.1–45.0)</td>
</tr>
<tr>
<td>LPS (6)</td>
<td>4.2×10⁻⁹ (1.6–11.0)</td>
<td>2.7×10⁻⁹ (1.8–5.9)</td>
</tr>
<tr>
<td>Depolarization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (7)</td>
<td>2.6×10⁻⁶ (1.5–4.3)</td>
<td>7.6×10⁻⁶ (2.3–23.0)</td>
</tr>
<tr>
<td>LPS (6)</td>
<td>1.9×10⁻⁶ (0.5–3.7)</td>
<td>2.8×10⁻⁶ (0.7–10.0)</td>
</tr>
</tbody>
</table>

EL, extraluminal; IL, intraluminal.

* Values refer to the change in V_t.

† Significantly greater than saline (EL).

‡ Significantly less than saline (EL).

§ Significantly greater than LPS (EL).

||
| FIG. 10. Effect of LPS treatment on epithelial bioelectric reactivity to intraluminal hyperosmolar NaCl in the presence of extraluminally applied MCh (3 × 10⁻⁷ M). A, millivolt change in V_t evoked by the addition of intraluminal NaCl. B, data from the same experiments described in A, but the plot shows the actual V_t values after the addition of intraluminal NaCl. Saline- and LPS-treated, n = 4. *, p < 0.05 compared with saline-treated controls.
Table 6

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>EC_{50} (mM) (95% CI)</th>
<th>Depolarization ( \Delta V_t ) (mV)</th>
<th>Maximum Response</th>
<th>Relaxation (Percentage of MCh)</th>
<th>Depolarization ( \Delta V_t ) (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (7)</td>
<td>11.4 (8.2–15.8)</td>
<td>121.6 (75.0–197.2)</td>
<td>183.6 (120.2–280.4)</td>
<td>78.2 ± 6.2</td>
<td>15.3 ± 0.5</td>
</tr>
<tr>
<td>LPS (6)</td>
<td>10.4 (8.4–12.8)</td>
<td></td>
<td></td>
<td>113.3 ± 9.5</td>
<td>38.0 ± 4.1</td>
</tr>
</tbody>
</table>

* Values refer to the change of \( V_t \) after the administration of extraluminal MCh; saline- and LPS-treated, \( n = 8 \).
* Values refer to the percentage of MCh-induced contraction; saline- and LPS-treated, \( n = 8 \).
* Significantly greater than saline (relaxation); signification difference.
* Significantly greater than LPS (relaxation).

Fig. 11. Effect of LPS treatment on the relationship between smooth muscle relaxation and epithelial depolarization elicited by hyperosmolar NaCl. Saline- and LPS-treated for relaxation, \( n = 8 \) and saline- and LPS-treated for depolarization, \( n = 4 \).

Fig. 12. Effect of amiloride on the basal \( V_t \) of isolated perfused trachea from saline- and LPS-treated animals. A, change in the basal \( V_t \) evoked by the addition of amiloride to the intraluminal bath. B, \( V_t \) before and after the addition of amiloride. Saline- and LPS-treated, \( n = 5 \). * \( p < 0.05 \) compared with saline-treated controls (–amiloride). ††, \( p < 0.05 \) compared with saline-treated controls (–amiloride). †††, \( p < 0.05 \) compared with LPS-treated (–amiloride).

The authors concluded that these changes were brought about by alterations in Cl– secretion and amiloride-sensitive Na+ transport (Suzuki et al., 1999). MCh is capable of acti-
vating and/or inhibiting ion transport after its interaction with more than one muscarinic receptor subtype (Somlyo and Somlyo, 1994; Janssen et al., 1998; Roux et al., 1998). The activation of more than one channel was most likely responsible for the biphasic MCh concentration-response curve, but it is not known at present which muscarinic receptor subtypes were initiating these responses, i.e., M1, M2, and M3 muscarinic receptors have been localized in the guinea pig lung (Mak and Barnes, 1990). A high-affinity receptor seems to be associated with hyperpolarization, whereas a low-affinity receptor may be linked to depolarization. The effect of LPS treatment, i.e., potentiation of hyperpolarization responses, seems to be mediated by the high-affinity hyperpolarizing receptor.

To explore retained changes in the airway wall in vitro that might have affected responses to MCh in vivo, we examined whether hyperosmolar solution-induced relaxation mechanism is altered after LPS treatment and observed that relaxation was potentiated. The observation that EpDRF-induced relaxation was potentiated after LPS treatment at the time that the airways were hypereactive to inhaled MCh supports the general view that EpDRF modulates airway reactivity. This is the third instance in which alterations in the actions of EpDRF have been observed to accompany changes in in vivo and/or in vitro airway reactivity. For example, exposure to ozone causes a decrease in the actions of EpDRF, which is associated with in vivo and in vitro airway hypereactivity to MCh (Fedan et al., 2000). In addition, sensitization and challenge of guinea pigs with ovalbumin potentiated EpDRF-induced relaxation, which is accompanied by in vitro airway hyporeactivity to MCh (Warner et al., 1996).

We observed that LPS treatment potentiated hyperosmolality-induced smooth muscle relaxation and depolarization of the epithelium. These results support the hypothesis that the relaxation and bioelectric events are functionally linked. This hypothesis originated in the observation that depolarization of the epithelium in response to elevation of the intraluminal or extraluminal osmolarity preceded the relaxation response (Dortch-Carnes et al., 1999). However, in the present study, in both treatment groups, the smooth muscle relaxed at lower hyperosmolar concentrations than were required to elicit depolarization. Relaxation occurred when as little as 0.8 mosM of NaCl was added to the intraluminal bath; however, depolarization did not occur until the osmolarity was raised by 8.4 mosM. If epithelial depolarization in response to hyperosmolarity signaled the release of EpDRF (Dortch-Carnes et al., 1999), the epithelium would have depolarized with the same osmolar concentration dependence as that for relaxation. It is necessary to reformulate the hypothesis linking bioelectric and mechanical events as cause and effect phenomena, at least insofar as NaCl is concerned. At low hyperosmolar concentrations, EpDRF release occurs independently of epithelial depolarization, whereas at higher elevations of osmolarity the epithelium becomes depolarized, which may signal the release of greater quantities of EpDRF. The meaning of these two pathways in terms of ion transport is not currently understood. The effects of low level hyperosmolarity could be mediated by electrically neutral ion transport, whereas at the higher osmotic particle concentrations permeation through ion channels also would be engaged. Transepithelial depolarization might be a coincident phenomenon associated with EpDRF release stimulated by a different, nonelectrogenic ion transport mechanism that is more sensitive to extracellular osmolarity changes. For example, exposure of cells to hyperosmolar solution causes cell alkalinization (Miyata et al., 2000). The Na–Cl channel blocker amiloride and the Cl– channel blockers 4,4′-disothiocyanato-2,2′-stilbene disulfonate and 5-nitro-2-(3-phenylpropylamino)benzoic acid both inhibit relaxation responses to hyperosmolarity, and both agents are capable of inhibiting the Na+,H+ exchanger and the Cl–,HCO3– exchanger (Kreydiyyeh, 2000; Lamb et al., 2000), respectively. These pharmacological inhibitors could be predicted to influence intracellular pH.

Finally, we investigated mechanism(s) that could have participated in the hyperpolarization of the epithelium after LPS treatment. Because LPS is known to induce NO synthase and cyclooxygenase (Okamoto et al., 1998), the effect of inhibitors of these enzymes on Vt was examined. Although NO has been shown to influence epithelial ion transport (Tanai and Gaggin, 1993), the small electrophysiological effects of 1-NAME and SNP suggest that NO plays little, if any, role in regulating ion transport in guinea pig tracheal epithelium. On the other hand, indomethacin decreased markedly Vt in both treatment groups, but significantly more so in the LPS-treated group. This depolarizing effect is consistent with an inhibition of prostanoid-stimulated Cl– secretion (Tamaoki et al., 1992). The fact that indomethacin decreased Vt to a greater extent in the LPS-treated group suggests that the role of prostanooids is heightened after LPS treatment. The mechanism whereby this occurs cannot be determined from our experiments, but it could involve up-regulation of cyclooxygenase, or changes in prostaglandin receptors or postreceptor signaling pathways. Nevertheless, the results suggest that prostanooid-stimulated Cl– secretion may contribute to the hyperpolarization of the epithelium after LPS treatment. Inasmuch as the hyperpolarization caused by LPS was abolished by amiloride and inhibited by indomethacin, it is tempting to speculate that a functional relationship between prostanoids and Na+ channels exists, which is altered by LPS.

Amiloride applied to the intraluminal surface of the trachea abolished the effect of LPS treatment on basal Vt. This suggests that the hyperpolarization of the epithelium after LPS treatment was due primarily to an increase in Na+ transport. An increase in Na+ removal from the lumen, via these channels, would generate a more negative Vt. Amiloride has been shown to inhibit EpDRF-mediated relaxation responses to hyperosmolar solution (Fedan et al., 1999). An increase in the activity of amiloride-sensitive Na+ channels may therefore be linked to potentiated responses to hyperosmolar challenge, the increase in basal Vt, and the potentiated Vt response to hyperosmolarity.

In conclusion, LPS treatment induces in vivo airway hyperreactivity to MCh that may be due, at least in part, to an increase in the actions of EpDRF. In addition, our results demonstrate that LPS treatment induces appreciable alterations in basal Vt of tracheal epithelium as well as its reactivity to MCh. These changes affect the airway smooth muscle indirectly in that the EpDRF-mediated relaxation response to hyperosmolarity is also potentiated. It is conceivable that such alterations in the airways could exist during gram negative bacterial infections of the airways.
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References


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