

**Hyperosmolar solution effects in guinea-pig airways. IV.**

**Lipopolysaccharide-induced alterations in airway reactivity and epithelial  
bioelectric responses to methacholine and hyperosmolarity<sup>1</sup>**

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**Abbreviations:** C.I., confidence interval; D-M, D-mannitol; EpDRF, epithelium-derived

relaxing factor; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; LPS,

lipopolysaccharide; MCh, methacholine; MKHS, modified Krebs-Henseleit

solution; NO, nitric oxide; PC<sub>200</sub>, the provocative MCh concentration

producing a 2-fold increase in SRaw above the value following saline

administration; SRaw, specific airway resistance; V<sub>t</sub>, transepithelial potential

difference

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## 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). Utilizing 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, since 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 ( $V_t$ ) was increased after LPS-treatment. The increase in  $V_t$  was inhibited by amiloride and indomethacin. Concentration-response curves for changes in  $V_t$  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* following 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 which 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 sec 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 et al., 1998). One such inhibitory substance which has been shown to modulate airway reactivity is the non-nitric oxide, non-prostanoid epithelium-derived relaxing factor (EpDRF), which is released in response to hyperosmolarity<sup>2</sup> at the mucosal or serosal surface of airway epithelial cells (Munakata et al., 1988, 1989, 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 which 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 ( $V_e$ ) preceded EpDRF-induced smooth muscle relaxation elicited by elevating serosal or mucosal osmolarity with either ionic or nonionic osmolytes. Fedan et al. (1999) demonstrated that amiloride-sensitive  $\text{Na}^+$  channels and 4-4' diisothiocyanatostilbene-2-2' disulfonic acid (DIDS)-sensitive  $\text{Cl}^-$  channels are involved in EpDRF-induced smooth muscle relaxation, and Tamaoki et al. (1997) published evidence for involvement of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  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.

## 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, ip) 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 sec intervals.

The animal was acclimated in the plethysmograph while breathing air for 2 h on the day before the experiment. Eighteen hours following 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<sub>200</sub>, the provocative MCh concentration producing a 2-fold increase in SRaw above the value following saline administration. The PC<sub>200</sub> was calculated by linear interpolation of graphed data.

***In vivo* administration of N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME).** Guinea pigs were exposed to L-NAME aerosol (1.2 mM) for 5 min prior to 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.** Following anesthesia and exsanguination, trachea and lungs from saline- and LPS-treated animals were inflated with 15-20 ml of 10% buffered formalin phosphate and removed *en bloc*. Sections of the trachea as well as the azygous, left, right apical, right cardiac, and right diaphragmatic lobes of the lung were then embedded with paraplast-plus 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" fashion 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 (Fedan and Frazer., 1992; Fedan et al., 2003a), along with modifications (Dortch-Carnes et al., 1999) for measuring  $V_t$  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.

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 ( $3 \times 10^{-7}$  M), as described in Results. The extraluminal and intraluminal concentration-response curves were obtained from each trachea, the intraluminal curve first, followed 90 min later by the extraluminal curve.

**Tracheal epithelium removal.** In experiments which 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.**  $N^G$ -nitro-L-arginine methyl ester [L-NAME, nitric oxide (NO) synthase

inhibitor;  $10^{-4}$  M] and indomethacin (cyclooxygenase inhibitor;  $3 \times 10^{-6}$  M) were used to determine whether hyperosmolarity-induced smooth muscle relaxation involved an NO or prostanoid component. The inhibitors were added to the extraluminal and intraluminal baths 30 min prior to inducing tone with extraluminally-added  $3 \times 10^{-7}$  M MCh. The relaxation responses in the presence of the inhibitors were compared to relaxation responses in preparations where the inhibitor was absent.

**Effect of agents on the basal  $V_p$ .** The following agents were evaluated for their effects on epithelial bioelectric properties: indomethacin ( $3 \times 10^{-6}$  M), L-NAME ( $10^{-4}$  M), and the epithelial  $\text{Na}^+$  channel blocker, amiloride ( $3 \times 10^{-5}$  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 (mM): NaCl (113.0), KCl (4.8),  $\text{CaCl}_2$  (2.5),  $\text{KH}_2\text{PO}_4$  (1.2),  $\text{MgSO}_4$  (1.2),  $\text{NaHCO}_3$  (25.0), and glucose (5.7). The MKH solution was continuously gassed with 95%  $\text{O}_2$ -5%  $\text{CO}_2$  and maintained at  $37^\circ\text{C}$  and a pH of 7.4. The osmolarity of the MKH solution was  $281 \pm 5$  mosM.

All drugs, chemicals and agents were from Sigma Chemical Co. (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 vivo* airway reactivity studies, SRaw and MCh  $\text{PC}_{200}$  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  $\text{EC}_{50}$  values were derived from least squares analysis of a sigmoidal curve fit. When comparing the  $\text{EC}_{50}$  values, normally-distributed  $-\log\text{EC}_{50}$  values

were used. The  $EC_{50}$ s, 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 (ANOVA), as appropriate. When examining the effect of agents on the basal  $V_r$ , 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 $\pm$ 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 PC<sub>200</sub> (mg MCh/ml) values [95% confidence interval (C.I.) in parentheses] were: saline, 0.08 (0.05-0.14); 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 following LPS-treatment. Thus, the effect of an aerosol of L-NAME, a 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 prior to MCh had no effect upon airway reactivity in either the saline- or LPS-treated animals (Table 1).

**Histological examination of guinea-pig airways following 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 various

regions of the guinea-pig respiratory tract. This was done to determine if 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, etc.). 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 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 which 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 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 EpDRF-mediated relaxation responses to hyperosmolar challenge would be potentiated. Intraluminal osmolarity was increased using NaCl, D-mannitol (D-M), urea or KCl as osmolytes following contraction of the smooth muscle with extraluminally-applied MCh ( $3 \times 10^{-7}$  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 and Table 4). However, LPS-treatment had no effect on responses to KCl ( $n = 4$ ; not shown), and it reduced significantly the potency of urea ( $n = 6$ ; 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 following LPS-treatment since both are capable of relaxing smooth muscle (Folkerts and Nijkamp, 1998). Neither L-NAME nor indomethacin affected responses to hyperosmolarity (NaCl 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 tracheas removed from either control or LPS-treated guinea pigs.

**Effect of LPS-treatment on basal  $V_t$ .** In tracheas from LPS-treated animals, the basal  $V_t$  was increased by 80% compared to tracheas from saline-treated animals. The basal  $V_t$  values were  $-14.6 \pm 1.2$  mV and  $-28.3 \pm 1.9$  mV for saline- and LPS-treated animals, respectively ( $p < 0.05$ ).

**Effect of LPS *in vitro* on basal  $V_t$  in tracheas from naive animals.** 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  $V_t$  across tracheas from naive animals. Using a concentration ( $10 \mu\text{g/ml}$ ), which 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 non-treated 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.

**Effect of indomethacin, L-NAME, atropine and SNP on basal  $V_t$ .** In order to determine if the increase in the basal  $V_t$  following LPS-treatment could involve prostanoids or NO, the effects of indomethacin ( $3 \times 10^{-6}$  M) and L-NAME ( $10^{-4}$  M) were examined. Prostaglandins and NO have been shown to stimulate  $\text{Cl}^-$  secretion (Tamaoki et al, 1992; Tamai and Gaginella, 1993). Indomethacin decreased  $V_t$  in both treatment groups (Fig. 8), but the effect was significantly greater in the LPS-treated group:  $+5.5 \pm 0.7$  mV for the LPS-treated group compared to  $+3.1 \pm 0.5$  mV for the saline-treated group ( $p < 0.05$ ).

L-NAME slightly hyperpolarized the epithelium in both treatment groups; 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  $0.9 \pm 0.5$  mV in tracheas from saline-treated animals and decreased  $V_t$  by  $0.3 \pm 0.6$  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 animals, extraluminally-applied MCh produced a biphasic concentration-response curve (Fig. 9A and Table 5). At MCh concentrations less than  $10^{-6}$  M,  $V_t$  increased, while at MCh concentrations greater than  $10^{-6}$  M,  $V_t$  decreased. LPS-treatment decreased the MCh  $EC_{50}$  for hyperpolarization and increased the maximum hyperpolarization. In contrast, the MCh  $EC_{50}$  and maximum response for depolarization were not affected. The intraluminal application of MCh also generated biphasic concentration-response curves in both treatment groups (hyperpolarizing at MCh concentrations less than  $10^{-3}$  M and depolarizing at MCh concentrations greater than  $10^{-3}$  M; Fig. 9B and Table 5). In the case of intraluminally-applied MCh, the maximum hyperpolarization response was greater in the LPS-treated group with respect to the saline-treated controls, while there was no difference in the depolarization responses among the two treatment groups.

**Effect of LPS-treatment on the  $V_t$  responses to hyperosmolarity.** Smooth muscle relaxation elicited by hyperosmolarity has been reported to be preceded by depolarization of

the epithelium (Dortch-Carnes et al., 1999), indicating that the two events may be functionally linked. Since 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  $3 \times 10^{-7}$  M 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 to saline-treated controls; however, the epithelium eventually depolarized to the same level in each treatment group (Fig. 10 and 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  $EC_{50}$  for depolarization was significantly greater than the  $EC_{50}$  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 ( $3 \times 10^{-5}$  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, non-inhalational administration of LPS induces *in vivo* airway hyporeactivity to inhaled MCh in the absence of an inflammatory response. Secondly, 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 hyporeactivity to inhaled MCh while 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.

Since it is well known that LPS up-regulates inducible NO synthase, it was plausible that NO may have contributed to *in vivo* airway hyporeactivity to inhaled MCh; however, there was no effect of L-NAME on responses to MCh in saline- or LPS-treated, conscious animals. Thus, NO is not responsible for hyporeactivity to inhaled MCh after LPS administration. In contrast, Nijkamp et al. (1993) found in intubated, anesthetized animals, that L-NAME increased airway resistance.

Folkerts et al. (1988) reported the development of airway hyperreactivity to histamine and inflammation of the airways 24 h after inhalation exposure to LPS. In the report of *in vivo* airway hyporeactivity to histamine following LPS-treatment (Folkerts et al., 1988), the airways of the guinea pigs became infiltrated with large numbers of neutrophils and monocytes. In contrast, there were no signs of pulmonary inflammation or changes in the physical structure of the airways 18 h after LPS-treatment in the present study. The lack of pulmonary inflammation in our LPS-treated guinea pigs suggests that the route of LPS administration is important to the development of inflammation, and that inflammation is not a prerequisite for LPS-induced changes in the EpDRF system.

Paradoxically, airway hyporeactivity to MCh *in vivo* was not manifest *in vitro* in isolated, perfused tracheas from LPS-treated animals. There could be several reasons for this. First, the mechanism underlying the *in vivo* airway hyporeactivity may not involve the trachea, but could involve intrapulmonary bronchi and bronchioles. Second, the other mechanisms responsible for the development of *in vivo* airway hyporeactivity could be lost *in vitro*, such as

neural pathways involved in the control of reactivity.

Although there was no effect of LPS on airway smooth muscle reactivity to MCh in the perfused trachea, LPS did alter the basal bioelectric properties of the epithelium as well as its reactivity to MCh, i.e., LPS significantly hyperpolarized the trachea. LPS increased the conductance of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels in vascular smooth muscle cells (Hoang et al., 1997). In our system, LPS-treatment could have initiated epithelial cell  $\text{Ca}^{2+}$  influx and activation of  $\text{K}^+$  channels leading to hyperpolarization, especially since  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels have been shown to be involved in the synthesis and/or release of EpDRF in response to MCh in bronchial strips (Tamaoki et al., 1997). However, we observed no effect of iberiotoxin or glibenclamide on basal  $V_t$  or hyperosmolarity-induced relaxation responses (Fedan et al., 2003b; Wu et al., 2003).

Biphasic concentration-response curves were obtained in response to extraluminal and intraluminal MCh in both treatment groups. The greater potency of extraluminal MCh compared to intraluminal MCh suggests the existence of multiple muscarinic receptors on the basolateral epithelial membrane. Our results are consistent with the electrophysiological effects produced by MCh in guinea-pig nasal epithelium (Suzuki et al., 1999), in which MCh produced triphasic short-circuit current responses: a transient increase followed by a small decrease and then, finally, a sustained increase. The authors concluded that these changes were brought about by alterations in  $\text{Cl}^-$  secretion and amiloride-sensitive  $\text{Na}^+$  transport (Suzuki et al., 1999). MCh is capable of activating and/or inhibiting ion transport following its interaction with more than one muscarinic receptor subtype (Janssen et al., 1998; Somlyo and Somlyo, 1994; 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.,  $M_1$ ,  $M_2$ , and  $M_3$  muscarinic receptors have been localized in the guinea-pig lung (Mak and Barnes, 1990). A high-affinity receptor appears to be associated with hyperpolarization, while a low affinity receptor may be linked to depolarization. The effect of LPS-treatment, i.e., potentiates hyperpolarization responses, appears 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 following LPS-treatment, and observed that relaxation was potentiated. The observation that EpDRF-induced relaxation was potentiated following LPS-treatment at the time that the airways were hyporeactive 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 hyperreactivity 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 hyperosmolarity-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, non-electrogenic ion transport mechanism that is more sensitive to extracellular osmolarity changes. For example, exposure of cells to hyperosmolar solution causes cell alkalization (Miyata et al., 2000). The Na<sup>+</sup> channel blocker, amiloride, and the Cl<sup>-</sup> channel blockers, DIDS and NPPB, both inhibit relaxation responses to hyperosmolarity, and both agents are capable of inhibiting the Na<sup>+</sup>,H<sup>+</sup> exchanger and the Cl<sup>-</sup>,HCO<sub>3</sub><sup>-</sup> 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 following LPS-treatment. Because LPS is known to induce

NO synthase and cyclooxygenase (Okamoto et al., 1998), the effect of inhibitors of these enzymes on  $V_t$  was examined. Although NO has been shown to influence epithelial ion transport (Tamai and Gaginella, 1993), the small electrophysiological effects of L-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  $V_t$  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  $V_t$  to a greater extent in the LPS-treated group suggests that the role of prostanoids is heightened after LPS-treatment. The mechanism whereby this occurs cannot be determined from our experiments, but it could involve up-regulation of cyclooxygenase, changes in prostaglandin receptors or post-receptor signaling pathways. Nevertheless, the results suggest that prostanoid-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.

Intraluminal amiloride to the intraluminal surface of the trachea abolished the effect of LPS-treatment on basal  $V_t$ . This suggests that the hyperpolarization of the epithelium following 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  $V_t$ . 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  $V_t$ , and the potentiated  $V_t$  response to hyperosmolarity.

In conclusion, LPS-treatment induces *in vivo* airway hyporeactivity to MCh which 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  $V_i$  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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## Footnotes

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<sup>1</sup>Mention of brand name does not constitute product endorsement. This paper is the fourth one of a series of four companion papers which report the effects of hyperosmolar solutions in guinea-pig airways (Fedan et al., 2003a,b; Wu et al., 2003).

<sup>2</sup>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 will not draw distinctions between the two terms when describing general phenomena.

## Legends for Figures

**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,  $n = 6$ .

**Fig. 2.** Lack of effect of L-NAME on *in vivo* airway reactivity to inhaled, MCh aerosol in (A) saline- and (B) LPS-treated animals. Each animal served as its own control, and reactivity to MCh was measured 18 h following saline- or LPS-treatment, before and after delivery of aerosolized L-NAME (1.2 mM). Saline- and LPS-treated,  $n = 4$ .

**Fig. 3.** Representative Harris' hematoxylin-eosin stained histological sections obtained 18 h after saline-treatment (A, C, and E) and LPS-treatment (B, D, and F). A and B are 40 × sections of distal trachea, C and D are 10 × sections of bronchi, and E and F are 20 × sections of the alveolar regions from saline- and LPS-treated animals, respectively. Sections of the bronchi and alveolar region were taken from the left lobe of the lung. The bars (50, 200, and 100 μm) found in B, D, and F also apply to A, C, and E, respectively. These results are representative of eight different experiments from each treatment group, using animals treated specifically for histological analysis.

**Fig. 4.** Lack of effect of LPS-treatment on tracheal smooth muscle reactivity to extraluminally- and intraluminally-applied MCh in (A) epithelium-intact and (B) -denuded, isolated, perfused trachea. Extraluminal and intraluminal MCh concentration-response curves were obtained from tracheas removed from saline- and LPS-treated animals 18 h post-treatment. Saline- and LPS-treated,  $n = 6$ , for epithelium-intact, and  $n = 4$  for epithelium-denuded.

**Fig. 5.** Effect of LPS-treatment on EpDRF-induced smooth muscle relaxation elicited with intraluminal MKHS made hyperosmolar with added (A) NaCl or (B) D-M. 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 ( $3 \times 10^{-7}$  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 to saline.

**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 (A) saline- and (B) LPS-treated animals 18 h post-treatment. The tracheal smooth muscle was contracted with extraluminally-applied MCh ( $3 \times 10^{-7}$  M) before NaCl was added to the intraluminal bath in stepwise-increasing, cumulative concentrations. Saline- and LPS-treated,  $n = 4$ .

**Fig. 7.** Lack of effect of extraluminally- and intraluminally-applied indomethacin ( $3 \times 10^{-6}$  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 (A) saline- and (B) LPS-treated animals 18 h post-treatment. The tracheal smooth muscle was contracted with extraluminally-applied MCh ( $3 \times 10^{-7}$  M) before NaCl was added to the intraluminal bath in stepwise-increasing, cumulative concentrations. Saline- and LPS-treated,  $n = 4$ .

**Fig. 8.** Effect of indomethacin on the basal  $V_t$  of isolated, perfused trachea from saline- and LPS-treated animals. (A) The mV change in the basal  $V_t$  evoked by the simultaneous addition of indomethacin ( $3 \times 10^{-6}$  M) to the intraluminal and extraluminal baths. (B) The  $V_t$  values before and after the addition of indomethacin. Saline- and LPS-treated,  $n = 5$ .  $*p < 0.05$  compared to saline-treated controls.  $\dagger p < 0.05$  compared to saline-treated controls (- Indomethacin).  $\dagger\dagger p < 0.05$  compared to saline-treated controls (- Indomethacin).  $\dagger\dagger\dagger p < 0.05$  compared to LPS-treated (- Indomethacin).

**Fig. 9.** Effect of LPS-treatment on epithelial reactivity to (A) extraluminally- and (B) intraluminally-applied MCh. Positive values refer to depolarization while negative values refer to hyperpolarization. Saline-treated,  $n = 7$  and LPS-treated,  $n = 6$ .  $*p < 0.05$  compared to saline-treated controls.

**Fig. 10.** Effect of LPS-treatment on epithelial bioelectric reactivity to intraluminal hyperosmolar NaCl in the presence of extraluminally-applied MCh ( $3 \times 10^{-7}$  M). (A) The mV change in  $V_t$  evoked by the addition of intraluminal NaCl. (B) These are data from the same experiments described in (A) but the plot shows the actual  $V_t$  values following the addition of intraluminal NaCl. Saline- and LPS-treated,  $n = 4$ .  $*p < 0.05$  compared to saline-treated controls.

**Fig. 11.** Effect of LPS-treatment on the relationship between smooth muscle relaxation and epithelial depolarization elicited by hyperosmolar NaCl. The preparations were contracted with extraluminally-applied MCh before NaCl was added cumulatively to the intraluminal bath. 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) The change in the basal  $V_t$  evoked by the addition of amiloride to the intraluminal bath. (B) The  $V_t$  before and after the addition of amiloride. Saline- and LPS-treated,  $n = 5$ .  $*p < 0.05$  compared to saline-treated controls.  $\dagger p < 0.05$  compared to saline-treated controls (- Amiloride).  $\dagger\dagger p < 0.05$  compared to saline-treated controls (- Amiloride).  $\dagger\dagger\dagger p < 0.05$  compared to LPS-treated (- Amiloride).

TABLE 1

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

Treatment ( <i>n</i> )	-L-NAME	+L-NAME
	MCh PC <sub>200</sub> (mg/ml) (95% C.I.)	
Saline (4)	0.07 (0.06-0.10)	0.01 (0.00-0.94)
LPS (4)	0.78 <sup>a</sup> (0.31- .97)	0.4 (0.11-1.45)

<sup>a</sup>Significantly greater than saline (L-NAME).

TABLE 2

Effect of LPS-treatment on *in vitro* airway reactivity to MCh in epithelium-intact, perfused trachea

Treatment ( <i>n</i> )	EC <sub>50</sub> (M)		Maximum Response		IL/EL Maximum Response Ratio <sup>a</sup>
	EL	IL	EL	IL	
	(95% C.I.)		ΔP (cm H <sub>2</sub> O)		
Saline (6)	1.9×10 <sup>-7</sup> (1.4-2.5)	3.7×10 <sup>-4b</sup> (1.5-9.1)	10.7±1.5	8.4±1.1 <sup>c</sup>	0.78±0.03
LPS (6)	2.1×10 <sup>-7</sup> (1.7-2.4)	3.2×10 <sup>-4d</sup> (1.8-5.8)	6.6±0.9	4.7±0.9 <sup>e</sup>	0.70±0.12

EL, extraluminal; IL, intraluminal. The tracheas were removed from the animals 18 h post-treatment. <sup>a</sup>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 while 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). <sup>b</sup>Significantly greater than saline (EL). <sup>c</sup>Significantly less than saline (EL). <sup>d</sup>Significantly greater than LPS (EL). <sup>e</sup>Significantly less than LPS (EL).

TABLE 3

Effect of LPS-treatment on *in vitro* reactivity to MCh in epithelium-denuded, perfused trachea

Treatment ( <i>n</i> )	EL	IL	EL	IL	IL/EL Maximum Response Ratio <sup>a</sup>
	EC <sub>50</sub> (M) (95% C.I.)		Maximum Response ΔP (cm H <sub>2</sub> O)		
Saline (4)	2.1×10 <sup>-6</sup> (0.8-5.2)	7.7×10 <sup>-6</sup> (2.0-30.0)	3.8±0.7	3.5±0.6	0.97±0.12
LPS (4)	1.1×10 <sup>-6</sup> (0.5-2.2)	1.5×10 <sup>-6</sup> (0.8-2.8)	3.9±0.4	3.5±0.3	1.00±0.06

EL, extraluminal; IL, intraluminal. The tracheas were removed from the animals 18 h post-treatment. <sup>a</sup>See 2 legend.

TABLE 4

Effect of LPS-treatment on relaxation responses of perfused trachea to hyperosmolarity

Treatment ( <i>n</i> )	EC <sub>50</sub> (mosM) <sup>a</sup> (95% C.I.)	Maximum Response (% of MCh) <sup>b</sup>
NaCl		
Saline (8)	11.4 (8.2-15.8)	78.2±6.2
LPS (8)	10.4 (8.4-12.8)	113.3±9.5 <sup>c</sup>
D-Mannitol		
Saline (6)	15.2 (8.2-28.2)	86.1±4.9
LPS (6)	15.9 (11.1-22.7)	109.7±8.5 <sup>c</sup>

The tracheas were removed from the animals 18 h post-treatment. <sup>a</sup>Values refer to mosM of osmolyte added to the intraluminal MKHS. <sup>b</sup>Relaxation responses in cm H<sub>2</sub>O were normalized as a percentage of the extraluminal MCh (3×10<sup>-7</sup> M)-induced contraction. <sup>c</sup>Significantly greater than saline-treated controls.

TABLE 5

Effect of LPS-treatment on epithelial bioelectric reactivity to MCh

Treatment ( <i>n</i> )	Extraluminal	Intraluminal	Extraluminal	Intraluminal
	EC50 (M)		Maximum Response	
	(95% C.I.)		$\Delta V_t$ (mV) <sup>a</sup>	
Hyperpolarization				
<i>n</i>				
Saline (7)	6.2×10 <sup>-8</sup> (2.9-13.0)	9.8×10 <sup>-6b</sup> (2.1-45.0)	-0.9±0.3	-1.6±0.3
LPS (6)	4.2×10 <sup>-9c</sup> (1.6-11.0)	2.7×10 <sup>-6d</sup> (1.8-5.9)	-3.0±0.7 <sup>b</sup>	-5.3±1.6 <sup>e</sup>
Depolarization				
Saline (7)	2.6×10 <sup>-6</sup> (1.5-4.3)	7.6×10 <sup>-2b</sup> (2.3-23.0)	2.3±0.6	4.0±1.1
LPS (6)	1.9×10 <sup>-6</sup> (1.0-3.7)	2.8×10 <sup>-2d</sup> (0.7-10.0)	4.8±1.6	5.0±1.1

<sup>a</sup>Values refer to the change in  $V_t$ . <sup>b</sup>Significantly greater than saline (EL).

<sup>c</sup>Significantly less than saline (EL). <sup>d</sup>Significantly greater than LPS (EL).

<sup>e</sup>Significantly greater than saline (IL).

TABLE 6

Effect of LPS-treatment on mechanical and bioelectric reactivity to intraluminal hyperosmolar NaCl

Treatment ( <i>n</i> )	Relaxation (% of MCh) <sup>a</sup>	Depolarization $\Delta V_t$ (mV) <sup>b</sup>	Relaxation (% of MCh)	Depolarization $\Delta V_t$ (mV)
	EC50 (mosM) <sup>c</sup> (95% C.I.)		Maximum Response	
Saline (7)	11.4 (8.2-15.8)	121.6 <sup>d</sup> (75.0-197.2)	78.2±6.2	15.3±0.5
LPS (6)	10.4 (8.4-12.8)	183.6 <sup>e</sup> (120.2-280.4)	113.3±9.5 <sup>f</sup>	38.0±4.1 <sup>f</sup>

<sup>a</sup>Relaxation responses (cm H<sub>2</sub>O) were normalized as a percentage of the extraluminal MCh-induced contraction; saline- and LPS- treated, *n* = 8. <sup>b</sup>Values refer to the change of V<sub>t</sub> after the administration of extraluminal MCh; saline- and LPS-treated, *n* = 4. <sup>c</sup>Values refer to mosM of NaCl added to the intraluminal MKH solution. <sup>d</sup>Significantly greater than saline (relaxation). <sup>e</sup>Significantly greater than LPS (relaxation). <sup>f</sup>Significantly greater than saline-treated controls.

Fig. 1  
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Revised Combined JPET  
Ms. 051672 and 051680

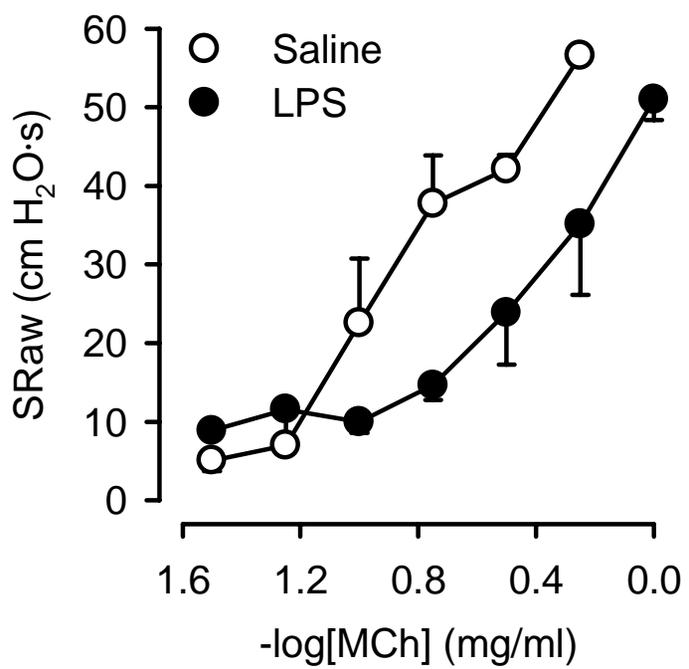


Fig. 2  
Johnston et al.  
Revised Combined JPET

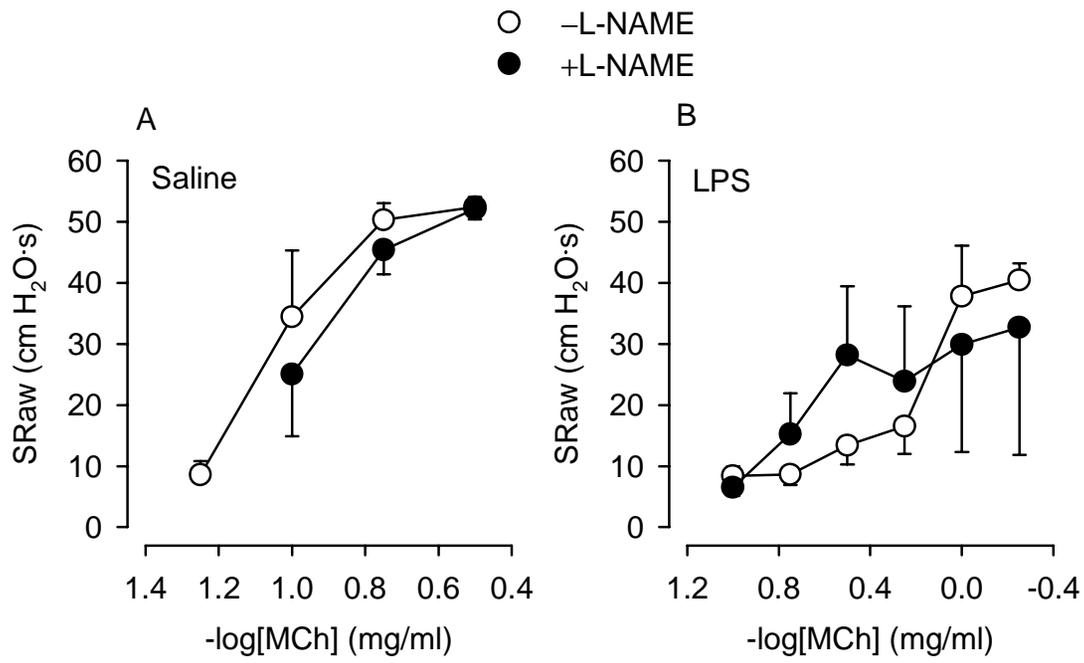


Fig. 3  
Johnston et al.  
Revised Combined JPET  
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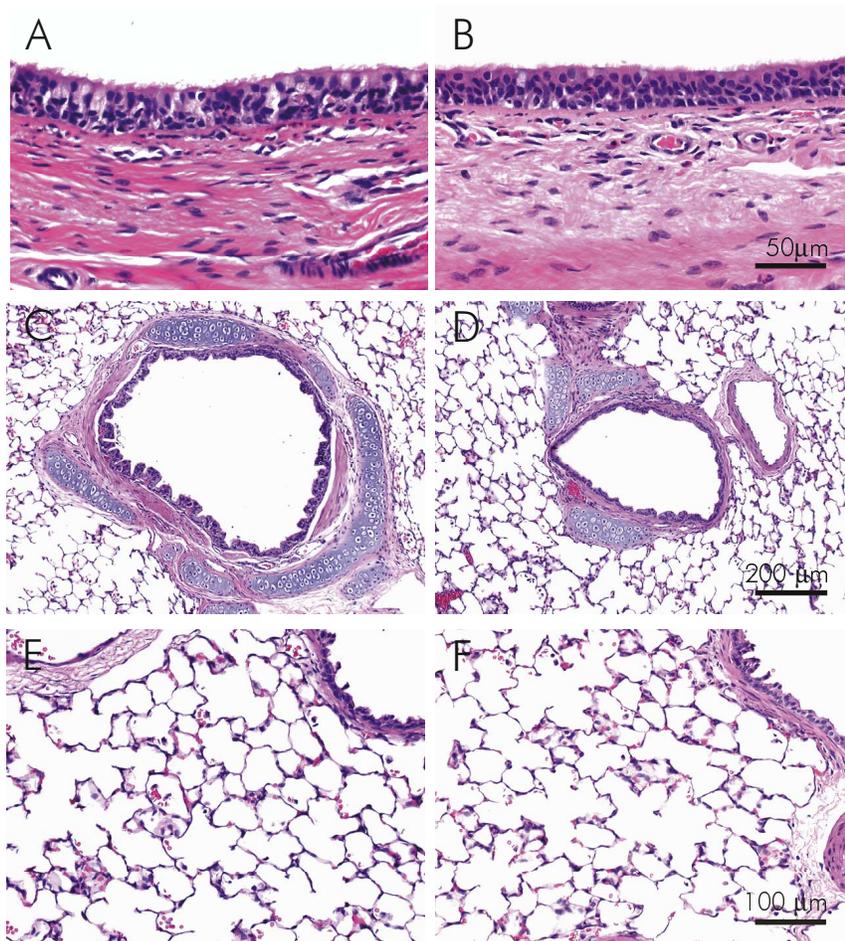


Fig. 4  
Johnston et al.  
Revised Combined JPET  
Ms. 051672 and 051680

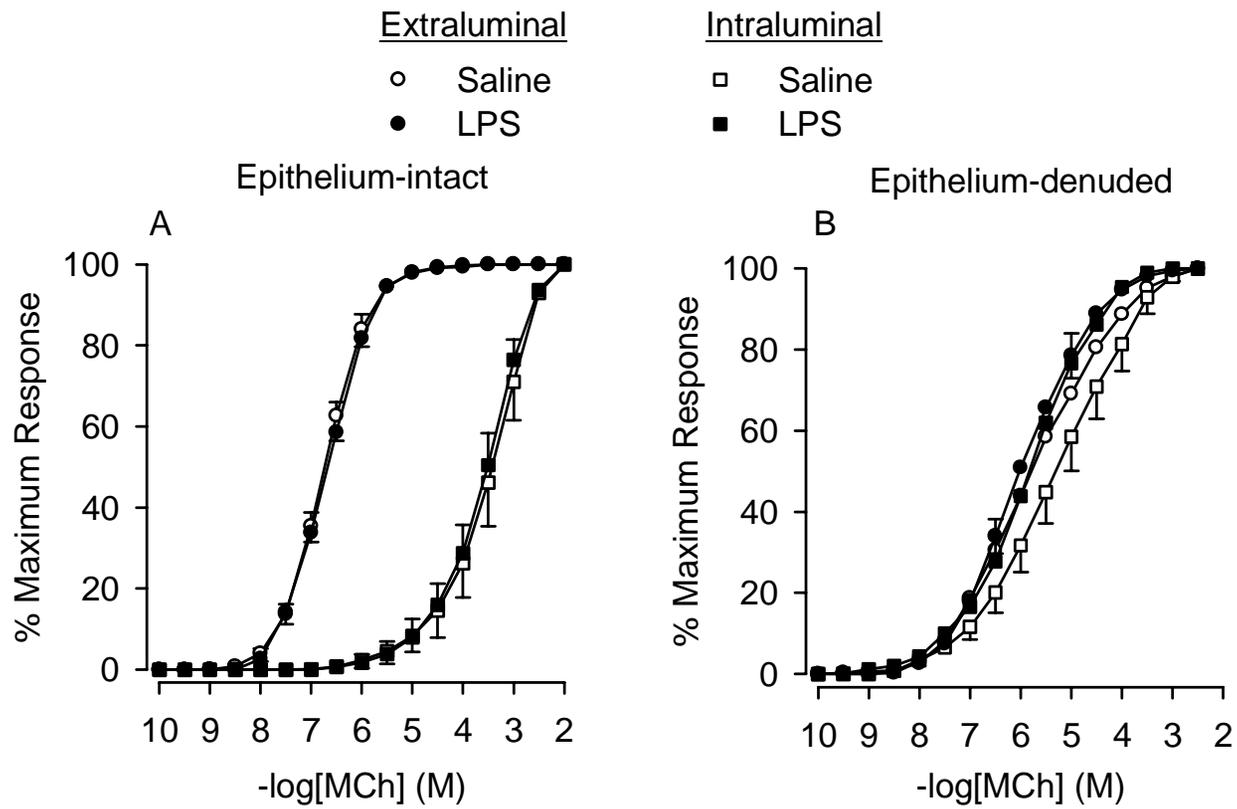


Fig. 5  
Johnston et al.  
Revised Combined JPET  
Ms. 051672 and 051680

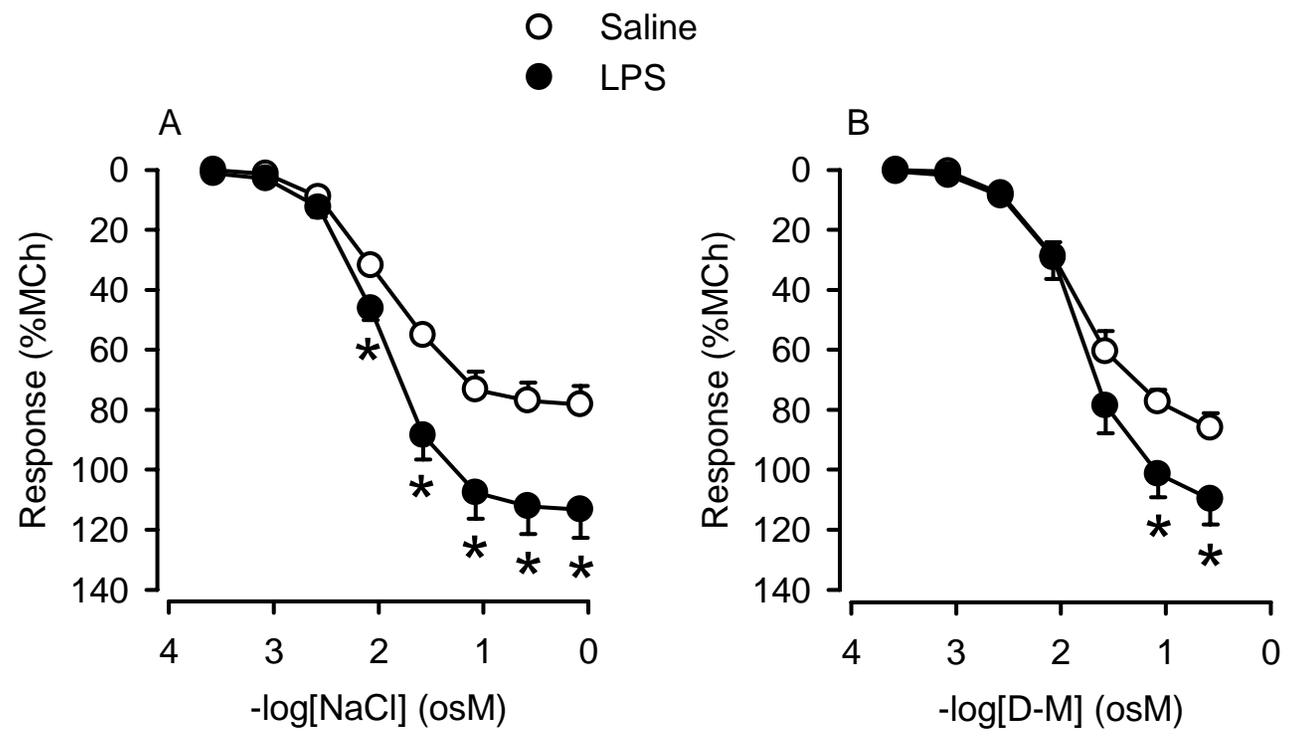


Fig. 6  
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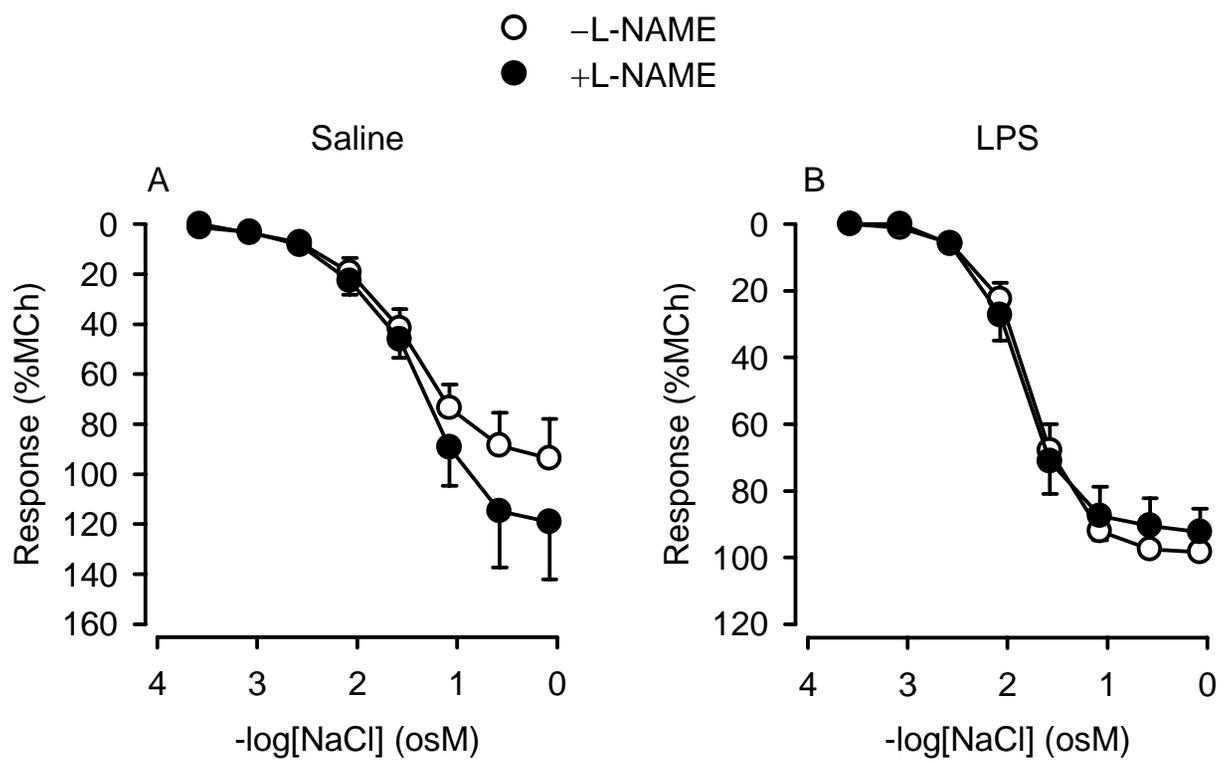


Fig. 7  
Johnston et al.  
Revised Combined JPET  
Ms. 051672 and 051680

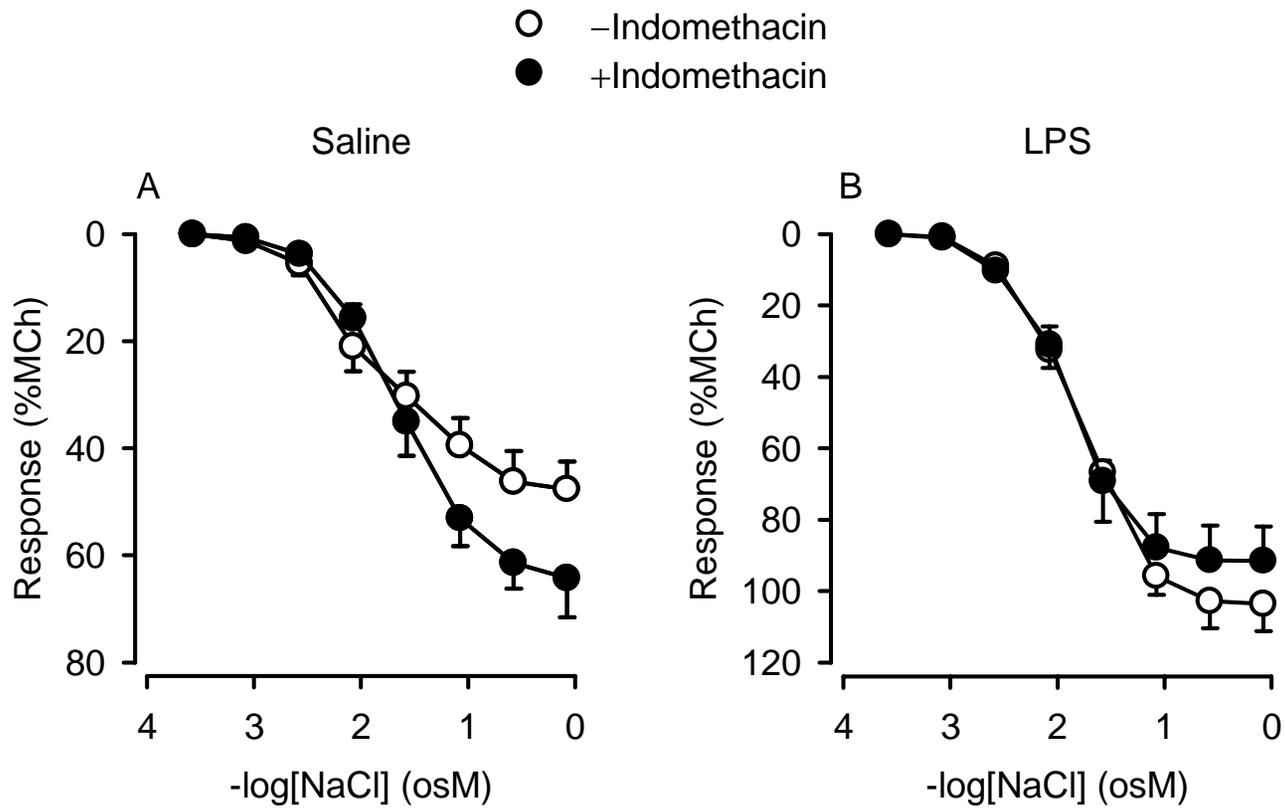


Fig. 8  
Johnston et al.  
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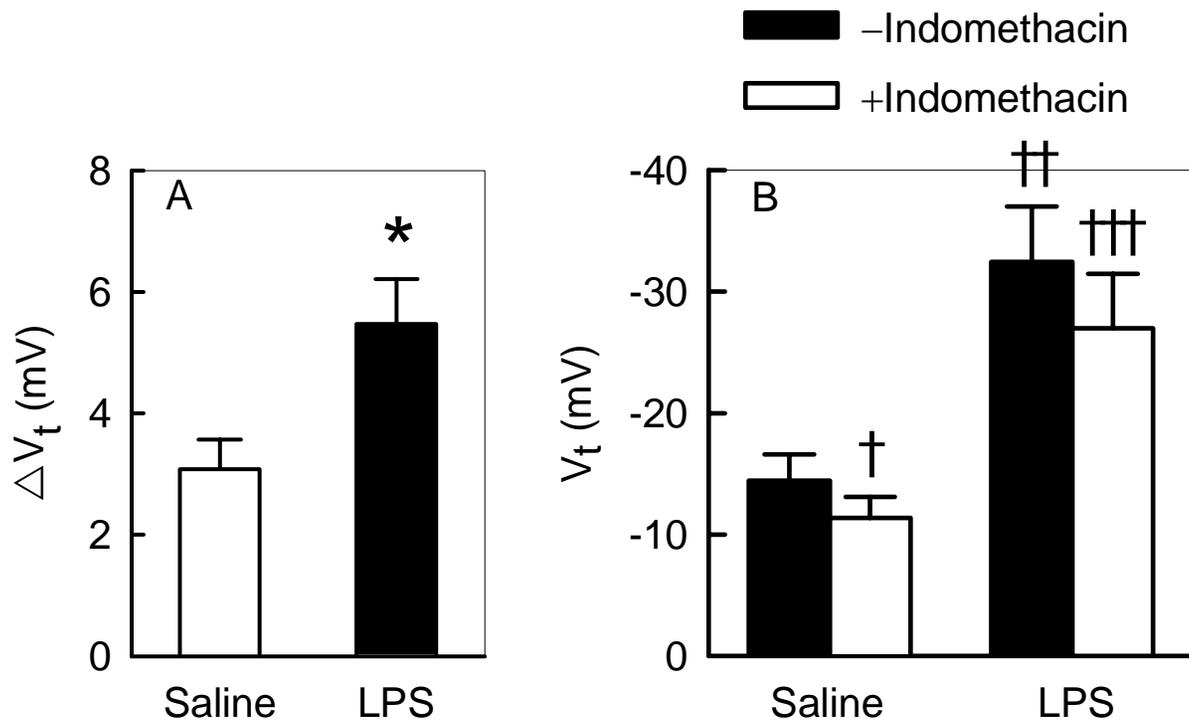


Fig. 9  
Johnston et al.  
Revised Combined JPET  
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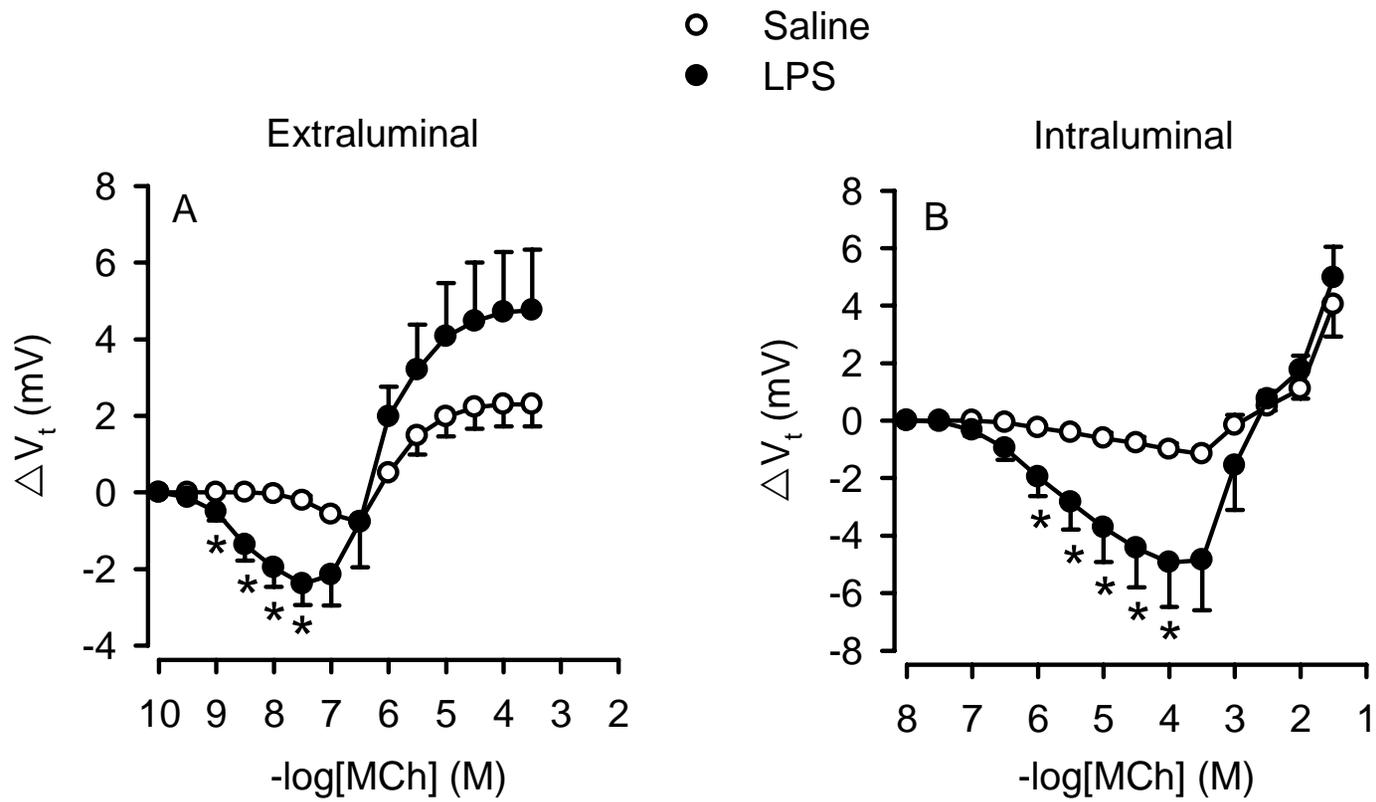


Fig. 10  
Johnston et al.  
Revised Combined JPET  
Ms. 051672 and 051680

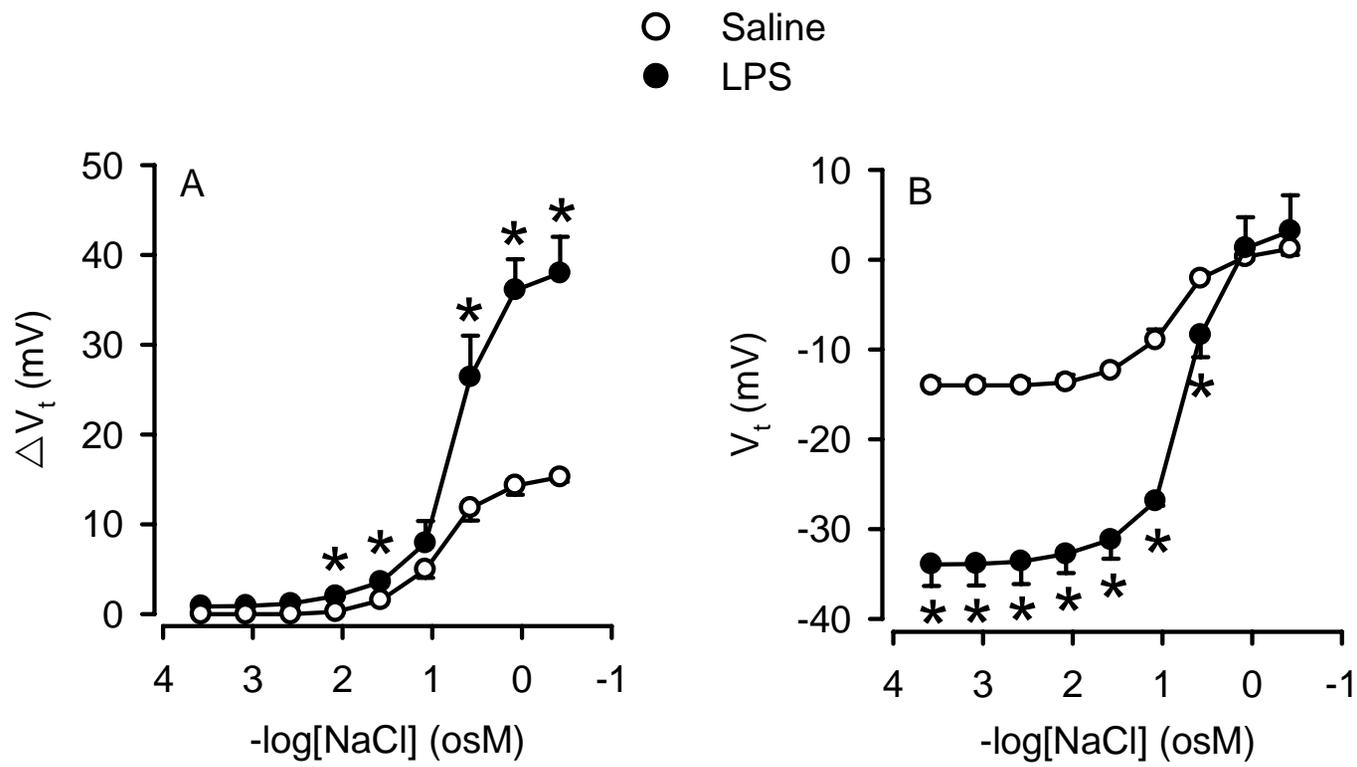


Fig. 11  
Johnston et al.  
Revised Combined JPET  
Ms. 051672 and 051680

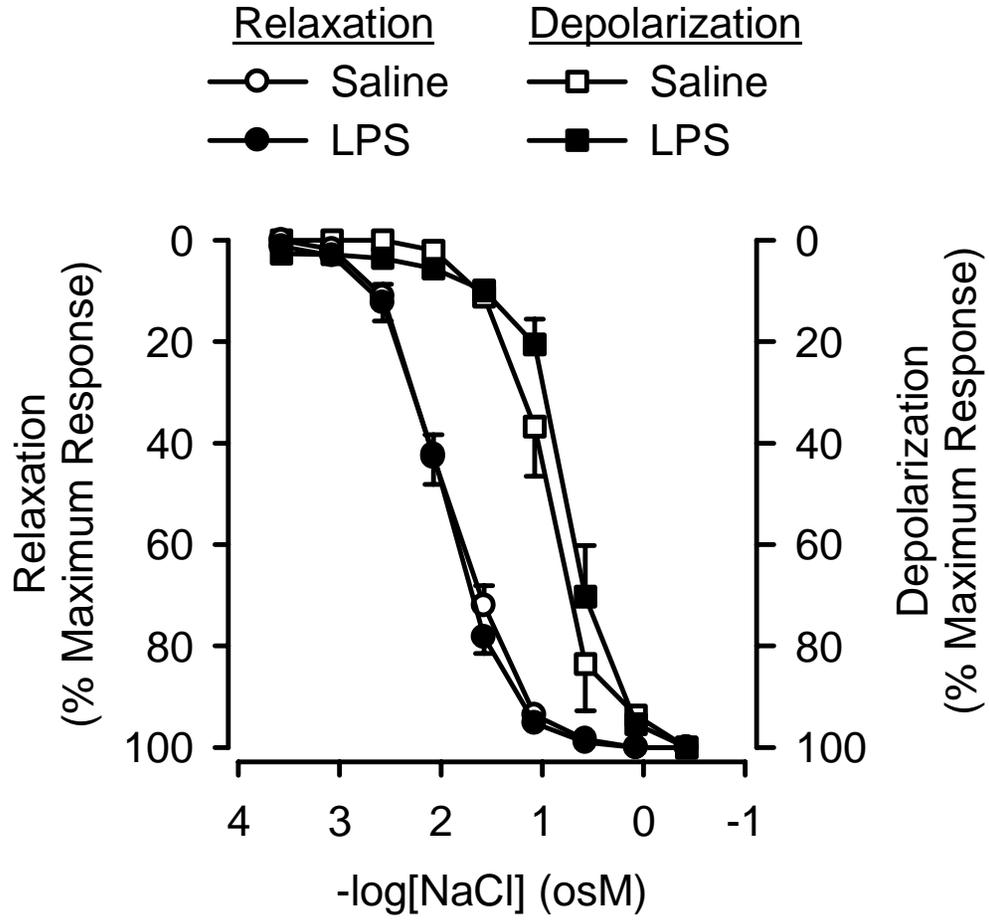


Fig. 12  
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Revised Combined JPET  
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