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Effects of lipopolysaccharide on epithelium-dependent relaxation in coaxial bioassay

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Abstract

This study investigated the effects of airway inflammation elicited by intraperitoneal and intratracheal lipopolysaccharide administration to guinea pigs on the activity of tracheal epithelium-derived relaxant factor (EpDRF). Acetylcholine induced epithelium-dependent relaxation in precontracted rat anococygeus muscle placed in guinea pig trachea (coaxial bioassay). Indomethacin, *N*-nitro-L-arginine methyl ester, aminoguanidine and L-canavanine did not alter this relaxation excluding the role of prostaglandins and nitric oxide (NO). Intraperitoneal lipopolysaccharide potentiated the acetylcholine response, which was reversed by aminoguanidine and L-canavanine while intratracheal lipopolysaccharide inhibited acetylcholine-induced relaxation. Lipopolysaccharide pretreatments did not cause epithelial damage but induced inflammatory cell infiltration. These results suggested that systemic lipopolysaccharide administration did not alter the EpDRF response but resulted in NO synthase induction, thus NO participated in relaxation to acetylcholine in coaxial bioassay system. On the other hand, airway inflammation induced by intratracheal lipopolysaccharide attenuated the synthesis/release of EpDRF without altering the epithelium morphology.

Keywords: Trachea, guinea-pig; Anococcygeus muscle, rate

1. Introduction

Inflammation of the airways involves influx of inflammatory cells, increased mucus secretion, edema formation and bronchial hyperreactivity with epithelial damage (Folkerts et al., 1988). Allergens and respiratory viruses induce inflammatory changes in the airways of experimental animals (Chung, 1986; Folkerts et al., 1993). Endotoxin of gram-negative bacteria is also implicated in inflammatory airway diseases (Harlar et al., 1983; Hutchinson et al., 1983). Lipopolysaccharide induces the release of cytokines, which triggers the inflammatory cascade in the airways, and plays an important role in sepsis-associated lung injury and acute respiratory distress syndrome (Brigham and Meyrick, 1986).

Airway epithelium is involved in the regulation of bronchial reactivity by releasing relaxing substances, such as cylooxygenase products, i.e. prostaglandin E_2, and nitric oxide (NO) (Butler et al., 1987; Munakata et al., 1990). Impaired synthesis of prostaglandin E_2 in epithelial cells has been suggested to participate in endotoxin-induced airway hyperreactivity (Folkerts et al., 1989). Increased thromboxane synthesis in the airways may also contribute to the bronchoconstriction seen in gram-negative sepsis (Uhlig et al., 1996). NO has been suggested as an inflammatory mediator that modifies airway responses (Barnes and Belvisi, 1993; Gaston et al., 1994; Barnes and Liew, 1995). Increased synthesis of NO by inducible NO synthase in human bronchial epithelial cells was demonstrated after exposure to cytokines (Asano et al., 1994).

Tracheal epithelium releases an epithelium-derived relaxing factor (EpDRF), besides cyclooxygenase products and NO. The nature of EpDRF has not been identified yet but its release has been demonstrated in a coaxial bioassay system consisting of guinea pig trachea as the donor organ, and rat

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aorta or anococcygeus muscle as the assay tissue (Ilhan and Sahin, 1986; Güc et al., 1988; Spina and Page, 1991). We have previously shown that EpDRF release from guinea pig tracheal epithelium was impaired by reactive oxygen species (Burcin et al., 1999). Increased production of reactive oxygen species with the infiltration of inflammatory cells is a common feature of the airway inflammation. Therefore, in the present study, we have investigated the effects of airway inflammation elicited by intraperitoneal and intratracheal lipopolysaccharide administration to guinea pigs, on tracheal EpDRF-mediated relaxation in the coaxial bioassay system.

2. Materials and methods

The present study was approved by Hacettepe University Animal Ethics Committee.

2.1. Coaxial bioassay system

In the first group of experiments the guinea pigs (350–450 g) were treated by an intraperitoneal (i.p.) injection of lipopolysaccharide (4 mg/kg). The controls of this group were injected equal volume of saline by the same route. After 16 h, the tracheas were isolated for coaxial bioassay experiments.

In the second group of experiments, guinea pigs were treated by an intratracheal injection of lipopolysaccharide (0.02 mg/kg) under urethane anaesthesia (1.5 g/kg, i.p). Guinea pigs receiving equivalent volume of saline (0.5 ml) by intratracheal injection were used as their controls. The tracheas were isolated 6 h after intratracheal injection.

In the coaxial bioassay model, the guinea pig trachea was used, as the donor organ and rat anococcygeus muscle was the assay tissue (Güc et al., 1988). The animals, used in the coaxial bioassay studies, were killed by a sharp blow to the head and bleeding. The rat anococcygeus muscle was always isolated from untreated control group of rats and prepared as described by Gillespie (1972). The guinea pig trachea isolated from controls or lipopolysaccharide-treated groups was used in its original tubular shape. The anococcygeus muscle was placed in the lumen of the trachea and mounted under a resting tension of 1 g in a 20-ml organ bath filled with Krebs–Henseleit solution at 37 °C and gassed with 95% O_2–5% CO_2. The composition of the Krebs–Henseleit solution was (in mM): NaCl, 118.0; KCl, 4.7; $MgS0_4$, 1.2; $CaCl_2$, 2.5; KH_2P0_4, 1.2; $NaHC0_3$, 25.0; glucose, 11.6.

The tissues were allowed to equilibrate 45 min before each experimental procedure. Isometric changes in tension were recorded with an isometric force transducer and "MAY 95-transducer data acquisition system" (Commat, Ankara, Turkey) on an IBM-compatible personal computer.

The rat anococcygeus muscle that was mounted within the trachea was precontracted by 3×10^{-7}–3×10^{-6} M phenylephrine (60–80% of the maximum contraction).

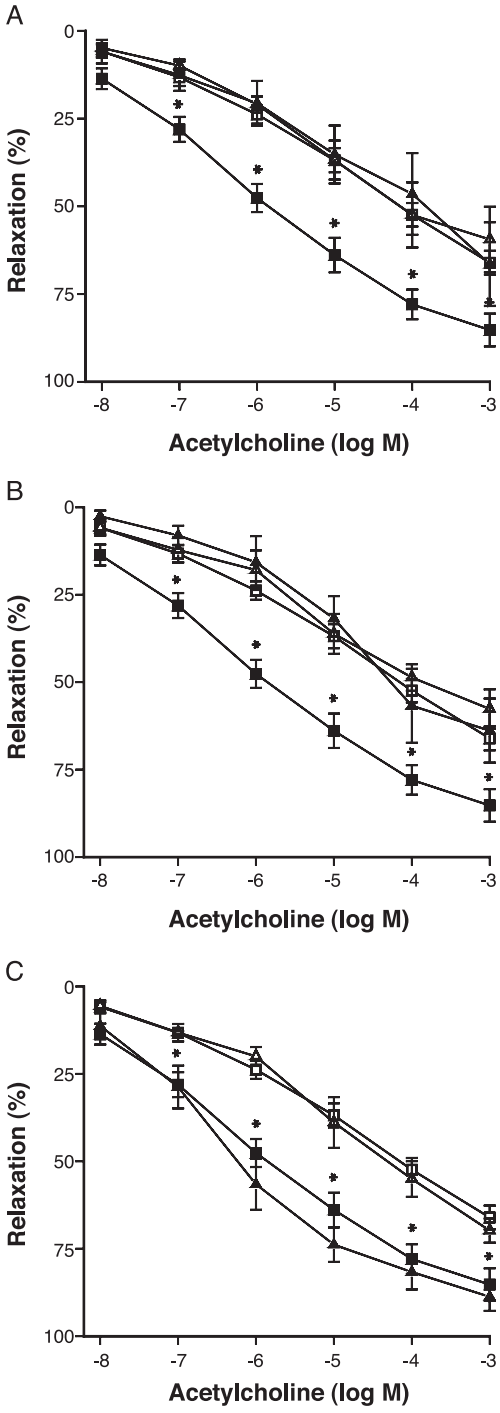

Fig. 1. Acetylcholine-induced relaxation response in rat anococcygeus muscle mounted within trachea isolated from intraperitoneal lipopolysaccharide (4 mg/kg ■) and saline (□)-pretreated guinea pigs and the effects of incubation with (A) aminoguanidine (B) L-canavanine, and (C) indomethacin and L-NAME (intraperitoneal lipopolysaccharide ▲, saline △). The data are expressed as percentages of papaverine-induced relaxation and shown as mean ± S.E.M. *Significantly different from saline group, $P < 0.05$ ($n = 5$–11).

Then the relaxation responses to increasing concentrations of acetylcholine (10^{-8}–10^{-3} M) were obtained. Some experiments were conducted after the removal of the tracheal epithelium by rubbing the mucosal surface with a probe coated with cotton wool. Removal of the epithelium was confirmed by histological examination. Acetylcholine-induced relaxation response was also elicited after pre-incubation for 30 min with aminoguanidine (10^{-4} M), L-canavanine (10^{-4} M), indomethacin (10^{-5} M) and *N*-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M), as described above.

2.2. Cell count in bronchoalveolar lavage

The sequestration of inflammatory cells was assessed in the bronchoalveolar lavage fluid of the guinea pigs from either intraperitoneal or intratracheal lipopolysaccharide-pretreated guinea pigs that were not used in the coaxial bioassay experiments. Under urethane anaesthesia (1.5 g/kg, i.p.), the trachea was trimmed free of connective tissue and a small incision at the proximal end was made for insertion of a cannula. The lumen was lavaged with 5 ml saline twice and then once more with 1 ml saline; fluid recovery was approximately 90%. Bronchoalveolar lavage fluid was centrifuged at 1000 rpm for 10 min at 4 °C, and the supernatant was separated. The cell pellet was resuspended in 1 ml of saline and the cells were counted using a haemocytometer (Coulter max M). For differential counts, the cell monolayers from bronchoalveolar lavage fluid were prepared by cytocentrifugation (1500 rpm, 5 min) (Shandon Cytospin-3). Differential counts were performed on 100 cells from smears stained with May–Grünwald Giemsa.

2.3. Serum and bronchoalveolar lavage nitrite assay

The concentration of nitrite, an indicator of NO synthesis in the serum and bronchoalveolar lavage fluid was measured by a spectrophotometric method based on the Griess reaction (Green et al., 1982). Blood samples were collected from carotid arteries of intraperitoneal and intratracheal lipopolysaccharide-pretreated guinea pigs and centrifuged at 3000 rpm for 15 min to obtain the serum. The supernatants from the bronchoalveolar lavage samples were isolated as described above. All samples were stored at −20 °C until use. 100 ml sample was pipetted into microplates, and 100 ml of Griess reagent (50 ml 1% sulphanilamide and 50 ml 0.1% *N*-(1-naphthyl) ethylenediamide dissolved in phosphoric acid 2.5%) was added. After incubation for 15 min at room temperature, absorbance was measured at 550 nm with an automated microplate reader (Automated Bio-Tek Instruments®, Microplate Reader). Nitrite concentrations of the samples were calculated from a curve using sodium nitrite as the standard.

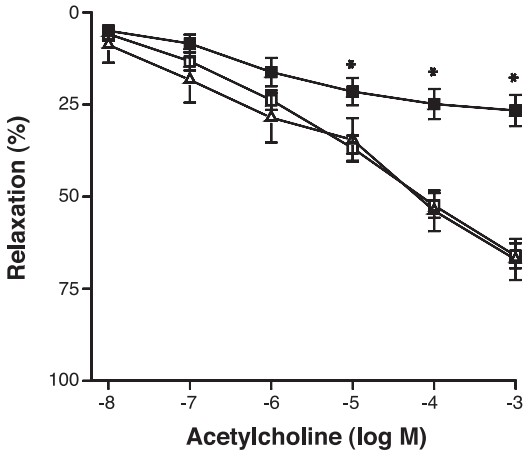

Fig. 2. Acetylcholine-induced relaxation response in rat anococcygeus muscle mounted within trachea isolated from intratracheal lipopolysaccharide (0.02 mg/kg, ■)-intratracheal saline (△)- and intraperitoneal saline (□)-pretreated guinea pigs. The data are expressed as percentages of papaverine-induced relaxation and shown as mean ± S.E.M. *Significantly different from saline-pretreated groups, $P<0.05$ ($n=5$–11).

2.4. Histological examination

Specimens of the trachea isolated from control and lipopolysaccharide-pretreated (intraperitoneal and intratracheal) guinea pigs were stained with haematoxylin and eosin for histological examination.

2.5. Statistical analysis

In the coaxial bioassay system, relaxation responses to acetylcholine are expressed as the percentage of the papaverine (10^{-4} M)-induced relaxation response. All data

Table 1
The amount of total inflammatory cells, macrophages, neutrophils and lymphocytes (cell × 10^6/ml) in bronchoalveolar lavage fluids obtained from intraperitoneal and intratracheal lipopolysaccharide-pretreated guinea pigs

	Total inflammatory cells	Macrophages	Neutrophils	Lymphocytes
Control	1.48 ± 0.26	0.31 ± 0.05	0.50 ± 0.06	0.60 ± 0.21
Lipopolysaccharide (intraperitoneal)	4.16 ± 0.81[a]	1.73 ± 0.35[a]	2.04 ± 0.47[a]	0.38 ± 0.11
Lipopolysaccharide (intratracheal)	4.25 ± 1.32[a]	1.64 ± 0.55[a]	1.95 ± 0.77[a]	0.65 ± 0.16

The data are shown as mean ± S.E.M.
[a] Significantly different from control, $P<0.05$ (n = 4–5).

are expressed as means ± standard error of the mean (S.E.M.). Statistical analysis performed by using analysis of variance (ANOVA) with repeated measurements and Bonferroni test; paired samples were compared by Student's *t*-test. A *P* value of less than 0.05 was considered significant.

2.6. Drugs used

Phenylephrine hydrochloride, acetylcholine hydrochloride, papaverine hydrochloride, lipopolysaccharide (*E. coli* lipopolysaccharide, 0.55:B5), aminoguanidine hemisulphate, L-canavanine, indomethacin, *N*-nitro-L-arginine methyl ester (L-NAME), sulphanilamide, *N*-(1-naphthyl) ethylenediamide, phosphoric acid and sodium nitrite were purchased from SIGMA (USA).

3. Results

3.1. Effects of i.p. lipopolysaccharide-pretreatment

After precontraction by phenylephrine (3×10^{-7}–3×10^{-6} M), acetylcholine (10^{-8}–10^{-3} M) induced a concentration-dependent relaxation response in rat anococcygeus muscle mounted within the guinea pig trachea isolated from control group (Fig. 1). This relaxation response was epithelium-dependent since it was inhibited by mechanical denudation of the trachea epithelium (data not shown). On the other hand, incubation with the cyclooxygenase inhibitor indomethacin (10^{-5} M) and NO synthase inhibitors L-NAME (10^{-4} M), aminoguanidine (10^{-4} M) and L-canavanine (10^{-4} M) did not alter the concentration response curve for acetylcholine in the coaxial bioassay system (Fig. 1).

The acetylcholine-induced relaxation response was significantly augmented when tracheas from intraperitoneal lipopolysaccharide (4 mg/kg)-pretreated guinea pigs were mounted within rat anococcygeus muscle in the coaxial bioassay system (Fig. 1). Incubation with either aminoguanidine or L-canavanine significantly inhibited this potentiation of the relaxation response while indomethacin and L-NAME were ineffective in the lipopolysaccharide-pretreated group (Fig. 1).

Table 2
The nitrite levels (μM) in serum and bronchoalveolar lavage fluids obtained from intraperitoneal and intratracheal lipopolysaccharide-pretreated guinea pigs

	Serum	Bronchoalveolar lavage
Control	18.32 ± 1.44	9.56 ± 0.70
Lipopolysaccharide (intraperitoneal)	41.29 ± 2.92[a]	27.79 ± 4.51[a]
Lipopolysaccharide (intratracheal)	20.25 ± 3.70	6.56 ± 1.58

The data are shown as mean ± S.E.M.
[a] Significantly different from control, $P < 0.05$ ($n = 4$–10).

3.2. Effects of intratracheal lipopolysaccharide-pretreatment

Acetylcholine-induced relaxation in the coaxial bioassay system consisting of tracheas from intratracheal saline injected guinea pigs was not significantly different from intraperitoneal saline injected control group (Fig. 2). On the other hand, acetylcholine-induced relaxation response was significantly attenuated in the coaxial bioassay system when tracheas were isolated from intratracheal lipopolysaccharide (0.02 mg/kg)-pretreated guinea pigs (Fig. 2).

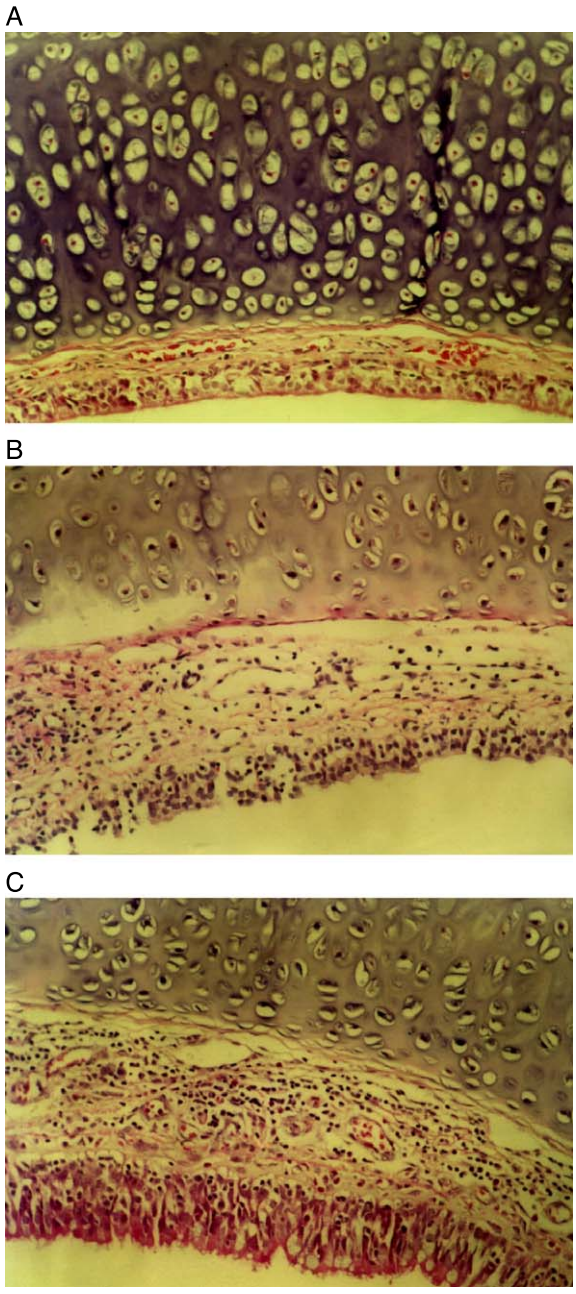

Fig. 3. Histological sections of the tracheas isolated from (A) control, (B) intraperitoneal lipopolysaccharide (4 mg/kg)- and (C) intratracheal lipopolysaccharide (0.02 mg/kg)-pretreated group of guinea pigs (H + E × 460).

3.3. Cell count in bronchoalveolar lavage

The total number of the inflammatory cells recovered in bronchoalveolar lavage fluids was significantly increased in both intraperitoneal and intratracheal lipopolysaccharide-pretreated group of guinea pigs. The amount of macrophages and neutrophils in bronchoalveolar lavage fluids were also increased after lipopolysaccharide (i.p. and intratracheal)-pretreatments while the number of lymphocytes was not different from control values (Table 1).

3.4. Serum and bronchoalveolar lavage nitrite levels

Intraperitoneal lipopolysaccharide-pretreatment to guinea pigs resulted in a significant increase in the serum and bronchoalveolar lavage nitrite concentrations when compared to control group. However, lipopolysaccharide injection by intratracheal route did not cause any increase in the serum and bronchoalveolar lavage nitrite concentrations (Table 2).

3.5. Histological examination

The histological evaluation revealed that lipopolysaccharide-pretreatment of guinea pigs by intraperitoneal route did not cause any morphological change in the tracheal epithelium. Only a few inflammatory cells were seen in the cross-sections of trachea specimens from lipopolysaccharide (i.p.)-pretreated guinea pigs.

Intratracheal exposure to lipopolysaccharide also did not cause any morphological change in the epithelial layer of the trachea, but infiltration of inflammatory cells was seen in all cross-sections of specimens (Fig. 3).

4. Discussion

In the present experiments, we have investigated the effects of airway inflammation on guinea-pig tracheal EpDRF-mediated relaxation in the coaxial bioassay system. For this purpose, two models of experimental inflammation-systemic and local administration of lipopolysaccharide were used. Lipopolysaccharide is involved in the pathogenesis of acute and chronic airway diseases (Harlar et al., 1983; Hutchinson et al., 1983). Thus, lipopolysaccharide was administered intraperitoneally for the systemic effects, and by intratracheal route for the local effects, and EpDRF response in the coaxial system was evaluated 16 and 6 h after injections, respectively. In these models, lipopolysaccharide doses and different time courses were chosen by taking the similar increases in the inflammatory cells in bronchoalveolar lavage fluid as an indicator of inflammation in the airways, and in accordance with the previous studies (Tasaka et al., 1996; Jarreau et al., 1994; Parker and Adams, 1993).

Systemic lipopolysaccharide, endotoxin or live bacteria administration to experimental animals (i.e. guinea pigs) is considered an experimental model of sepsis or acute respiratory distress syndrome-like lung injury (Parker and Adams, 1979; Esbenshade et al., 1982; Matsuo, 1999). Sepsis induced by gram-negative bacteria is one of the most common causes of acute respiratory disease syndrome in clinics (Newman, 1985). Inflammatory cytokines and mediators induce the inflammatory cascade in airway epithelium leading to lung injury mediated by acute respiratory distress syndrome and sepsis (Brigham and Meyrick, 1986; Windsor et al., 1993). EpDRF that is neither a cylooxygenase product nor NO, mediates the relaxation to acetylcholine in the precontracted assay tissue (i.e. rat anococcygeus muscle) placed in guinea pig trachea (coaxial bioassay system) (Ilhan and Sahin, 1986; Spina and Page, 1991). In the present study, we have shown that acetylcholine-induced relaxation in the coaxial bioassay system was potentiated when tracheas were removed from intraperitoneal lipopolysaccharide injected guinea pigs. The potentiation of the acetylcholine response was elicited in the presence of indomethacin excluding the role of cylooxygenase products after lipopolysaccharide treatment in this experimental model. Although, previous studies in our laboratory support the proposition that EpDRF demonstrated by the coaxial bioassay assembly is not NO, in the present experiments the augmented relaxation response to acetylcholine after intraperitoneal lipopolysaccharide injection to guinea pigs was inhibited by aminoguanidine and L-canavanine; the selective inhibitors of inducible NO synthase (Takano et al., 1997; Liaudet et al., 1998; Vona-Davis et al., 2002; Mansart et al., 2003). Increased NO synthesis by the induction of inducible NO synthase in various tissues and increased nitrite levels in the serum has been demonstrated in systemic lipopolysaccharide-treated experimental animals (Liu et al., 1993; Arkovitz et al., 1996; Vona-Davis et al., 2002). It has been shown that pre-treatment with the inhibitors of inducible NO synthase, aminoguanidine and L-canavanine reduced the serum nitrite levels in experimental sepsis models (Takano et al., 1997; Liaudet et al., 1998; Vona-Davis et al., 2002; Mansart et al., 2003). NO synthesized by inducible NO synthase has also been suggested as an important mediator of the inflammatory response in the airways (Barnes and Belvisi, 1993; Gaston et al., 1994; Barnes and Liew, 1995). Inducible NO synthase is expressed in human bronchial epithelial cells after exposure to inflammatory cytokines and is also expressed to a greater extent in the airway epithelium of patients with asthma than in normal subjects (Asano et al., 1994). In lung injury due to sepsis NO in expired air as well as serum and bronchoalveolar lavage nitrite/nitrate; the products of NO neutralisation in vivo, are increased (Matsuo, 1999). Thus, our finding that the serum and bronchoalveolar lavage nitrite levels are increased after systemic (i.p.) lipopolysaccharide administration could also be due to the excessive NO production through inducible NO synthase in the present experimental model. Furthermore, in the present study, NO, which could not be detected in the control conditions in the coaxial bioassay system, likely causes

the potentiation of the acetylcholine-induced relaxation response when tracheas were isolated from intraperitoneal lipopolysaccharide-treated group of guinea pigs. On the other hand, EpDRF release seems to be unaltered by systemic lipopolysaccharide administration as relaxation to acetylcholine in the presence of inducible NO synthase inhibitors was not different to control. The possible interaction of NO with other mediators of inflammation including epithelium-derived contractile factors or reactive oxygen species might be the reason of the preserved EpDRF response in the present model.

L-NAME, despite being described as an inhibitor of NO synthesis did not alter the potentiation of the acetylcholine response in the coaxial bioassay system in the intraperitoneal lipopolysaccharide-treated group. This finding may suggest that L-NAME did not inhibit the responses due to excessive NO production by inducible NO synthase induction at the concentration used in the present experiments. In fact, the selectivity of L-NAME towards the constitutive and inducible isoforms of NO synthase is not absolutely clear as it is sometimes used as the selective inhibitor of the constitutive isoform or the nonselective inhibitor in different studies. A recent radioligand binding study has reported that L-NAME has affinity towards only constitutive NO synthase but not to inducible NO synthase, and thus considered it as a selective inhibitor of the constitutive isoform (Boer et al., 2000). Thus our data confirms the studies, which found L-NAME ineffective in inhibiting responses due to inducible NO synthase induction in experimental animals (Fischer et al., 1993; Kankuri et al., 2001).

In the second part of the study, lipopolysaccharide was administered by intratracheal route to guinea pigs. We have demonstrated that airway inflammation elicited by intratracheal lipopolysaccharide injection to guinea pigs in vivo resulted in the impairment of the EpDRF response in the coaxial bioassay system. Furthermore, we found that unlike systemic lipopolysaccharide administration, bronchoalveolar lavage nitrite level was not increased, and this could be taken as an evidence against inducible NO synthase induction and increased NO synthesis in the airways. It has been shown that lipopolysaccharide administration into airways by inhalation or intratracheal instillation causes airway inflammation with infiltration of inflammatory cells and increased production of the reactive oxygen species (Brigham, 1986; Vincent et al., 1993; Toward and Broadley, 2000). In our previous study, we have shown that reactive oxygen species are capable of impairing the guinea pig tracheal EpDRF-mediated relaxation in a coaxial bioassay system (Burcin et al., 1999). Thus, airway inflammation with the involvement of inflammatory mediators and/or reactive oxygen species in the absence of NO synthesized through inducible NO synthase, could be suggested for the impairment of EpDRF response after intratracheal lipopolysaccharide administration in the present study. Other possibility might be the influence of epithelium-derived contractile factors that are upregulated during airway inflammation. Thus, these factors could mask the demonstration of EpDRF in the coaxial bioassay system after intratracheal lipopolysaccharide administration.

In spite of the loss of EpDRF response in coaxial system, we could not demonstrate any histological change in the trachea epithelium after intratracheal lipopolysaccharide injection. On the other hand, edema and inflammatory cell infiltration under the epithelial layer was observed. Furthermore, the increases in inflammatory cell counts, neutrophils and macrophages in bronchoalveolar lavage fluid are the other indicators of airway inflammation in guinea pigs after intratracheal lipopolysaccharide injection. Controversial data have been reported in the literature, regarding the histological changes in epithelial cell morphology after lipopolysaccharide administration. Some previous studies reported the absence of any epithelial alteration after intratracheal injection or inhalation of lipopolysaccharide to experimental animals (Lantz et al., 1985; Vincent et al., 1993). On the other hand Folkerts et al. (1988) reported epithelial damage accompanied by bronchial hyporeactivity and inflammatory cell influx to the airways after lipopolysaccharide administration. Our data seems to in agreement with the former studies and suggest an epithelial dysfunction, in terms of EpDRF synthesis/release rather than morphological damage after local exposure to lipopolysaccharide. Likewise, in our previous study, reactive oxygen species did not cause any alteration in the guinea-pig tracheal epithelium morphology but resulted in impairment of the EpDRF synthesis/release (Burcin et al., 1999).

Inflammatory mediators have been suggested to play a role in the bronchial hyperreactivity associated with airway inflammation (Folkerts et al., 1993). The present finding that EpDRF response was impaired after intratracheal lipopolysaccharide injection could be taken as an evidence for its contribution on the airway hyperreactivity in lipopolysaccharide-induced airway inflammation. However, the nature of EpDRF and its participation in the regulation of tracheal contractility are still obscure to make such an assumption.

In conclusion, the present results suggested that systemic exposure to lipopolysaccharide caused the induction of NO synthase probably without altering the activity of EpDRF, and NO that was produced in high amounts by inducible NO synthase, participated in acetylcholine-induced relaxation in the coaxial bioassay system. However, local airway inflammation elicited by intratracheal injection of lipopolysaccharide resulted in attenuated synthesis and/or release of EpDRF in the coaxial bioassay system without altering the morphology of tracheal epithelium.

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