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Characterization of the endothelin receptor subtype mediating epithelium-derived relaxant nitric oxide release from guinea-pig trachea

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1 The endothelin (ET) receptor subtype that mediates niric oxide (NO)-dependent airway relaxation in tracheal tube preparations precontracted with carbachol and pretreated with indomethacin was investigated. The release of NO induced by ET from guinea-pig trachea using a recently developed porphyrinic microsensor was also measured.

2 ET-1 (1 pM – 100 nM) contracted tracheal tube preparations pretreated with the NO-synthase inhibitor, L-NMMA, and relaxed, in an epithelium-dependent manner, preparations pretreated with the inactive enantiomer D-NMMA. The effect of L-NMMA was reversed by L-Arg, but not by D-Arg.

3 The selective ET_B receptor agonists, IRL 1620 or sarafotoxin S6c, both (1 pM – 100 nM) contracted tracheal tube preparations in a similar manner either after treatment with D-NMMA or with L-NMMA. In the presence of the ET_A receptor antagonist, FR139317 (10 μM), ET-1 administration resulted in a contraction that was similar after either L-NMMA or D-NMMA. In the presence of the ET_B receptor antagonist, BQ788 (1 μM), ET-1 relaxed and contracted tracheas pretreated with D-NMMA and L-NMMA, respectively.

4 Exposure of tracheal segments to ET-1 (1 – 1000 nM) caused a concentration-dependent increase in NO release that was reduced by L-NMMA. IRL1620 (1 μM) did not cause any significant NO release. FR139317 (10 μM), but not, BQ788 (1 μM), inhibited the NO release induced by ET-1.

5 These results demonstrate that in the isolated guinea-pig trachea activation of ET_B receptors results in a contractile response, whereas activation of ET_A receptors cause both a contraction, and an epithelium-dependent relaxation that is mediated by NO release.

Keywords: Endothelin; ET_A and ET_B receptors; nitric oxide; trachea; airway epithelium

Introduction

Endothelin-1 (ET-1) belongs to a family of regulatory peptides originally recognized as potent vasoconstrictor agents (Yanagisawa *et al*., 1988), although, since then additional effects of ETs have been discovered in a large variety of tissues and organs (Rubanyi & Plokoff, 1994). The biological responses of ETs are mediated by, at least, two receptor subtypes, namely, the ET_A and ET_B receptors (Arai *et al*., 1990; Sakurai *et al*., 1990). ETs were first identified as products of endothelial cells (Yanagisawa *et al*., 1988). However, ETs may be produced by other cell types, including airway epithelial and submucosal gland cells (Giaid *et al*., 1991). In the airways ETs cause plasma extravasation and contraction of the smooth muscle (Barnes, 1994), stimulate mucus secretion and also are mitogenic for fibroblasts (Takuwa *et al*., 1989) and airway smooth muscle cells (Glassberg *et al*., 1994), suggesting a possible role in airway wall remodeling. The biological effects of ETs in the airways and their increased expression in asthmatic airways (Springall *et al*., 1991) has suggested a role of these peptides in asthma (Hay *et al*., 1993).

The bronchomotor action of ET-1 in the guinea-pig airways is particularly complex. ET-1 may both contract and relax isolated guinea-pig bronchi (Battistini *et al*., 1994). Both ET_A and ET_B receptors mediate the bronchoconstrictor response to ET-1 (Battistini *et al*., 1994). Indirect evidence has suggested that ET-1 dependent bronchorelaxation might be due to prostanoid and nitric oxide (NO) release (Battistini *et al*., 1994; Filep *et al*., 1993). In order to identify which receptor subtype(s) mediates the NO-dependent relaxation caused by ET-1 we used guinea-pig isolated tracheal tube preparations in which stimuli are administered into the tracheal lumen in a manner independent from the external fluid bathing the serosal surface of the trachea (Nijkamp *et al*., 1993). This preparation has been used previously to study NO-dependent relaxation caused by histamine (Nijkamp *et al*., 1993) and bradykinin (Figini *et al*., 1995). In addition, we used a recently developed method that utilizes a porphyrinic based microsensor (Malinski & Taha, 1992) in order to measure the release of NO from guinea-pig tracheal segments *in vitro*.

Methods

Tracheal tube preparation

Male guinea-pigs (350 – 500 g) were killed with sodium pentobarbital (80 mg kg^{-1}, i.p.) and the trachea isolated and perfused with a Krebs solution of the following composition

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(mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 8.3. The solution was maintained at 37°C and was aerated continuously by bubbling with a mixture of 95% O_2-5% CO_2, which maintained a pH of 7.4. Experiments were performed using apparatus and techniques similar to those reported previously (Nijkamp *et al*., 1993). Briefly, proximal ends of the trachea were mounted in an organ bath that permitted independent circulation of fluid within the lumen of the tracheal segment or around the exterior of the tracheal segment. Two hooks were passed through the tracheal wall around two adjacent cartilaginous rings as close as possible to the muscle. The lower hook was fixed and the upper hook was connected to an isometric force transducer (Basile, Italy). Resting tension was set at an optimal counterweight of 2 g. The inside of the trachea was perfused with Krebs solution at a constant flow rate of 2 ml min^{-1} with a peristaltic pump. During a 90 min equilibration period Krebs buffer was changed on both sides every 15 min. All drugs were added intraluminally. Thereafter, the intraluminal side of the trachea was perfused for 30 min with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) or N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-D-arginine methyl ester (D-NAME, 100 μM) or N^G-monomethyl-D-arginine (D-NMMA). In some experiments L-arginine (L-Arg, 1 mM) or D-arginine (D-Arg, 1 mM) were co-incubated with L-NAME. In other experiments, FR139317 (10 μM) or BQ788 (1 μM), selective antagonists to ET_A and ET_B, respectively, were added at the same time as D-NMMA or L-DMMA. Indomethacin (5 μM) was added to the perfusion solution because without indomethacin, administration of ET-1 could result in both contraction or relaxation. This finding is consistent with the observation that ET-1 may release contractile or relaxing prostanoids from the epithelium (Battistini *et al*., 1994).

Tracheal tubes were precontracted with intraluminal perfusion of carbachol (Cch, 10 μM). The contraction in response to Cch remained stable for at least 25 min. As soon as a stable contraction to Cch was obtained, ET-1 (1 pM–0.1 μM), or one of the two selective agonists of ET_B receptor we tested (IRL1620, 0.1–100 nM and sarafotoxin S6c 0.1–100 nM), were added intraluminally. In each trachea only one challenge with Cch tone was performed. Cumulative agonist concentration-response curves were constructed by adding increasing concentrations of the agonists as soon as a plateau to the previous concentration was reached.

In a separate set of experiments the epithelial layer of tracheal tube segments was removed with a cotton swab (Nijkamp *et al*., 1993). To verify that the tissues were denuded of epithelium, histologic examinations were performed. The tissues were fixed by immersion in formaldehyde (4%) and embedded in paraffin blocks. Sections measuring 5 μm were cut and stained with hematoxylin and eosin for histologic evaluation. Histologic examination showed that the epithelial layer was completely removed, whereas no damage was observed to the lamina propria (data not shown).

Determination of NO release using a porphyrinic microsensor

Tracheas were excised as reported above. We prepared tracheal segments 3–4 cartilage rings wide that were opened by a longitudinal cut of the anterior surface. Segments were placed in a Petri dish containing an oxygenated Krebs solution (10 ml) and maintained at 37°C as reported above. After a resting period of 30 min, a porphyrinic microsensor was immersed into the bathing fluid on to the mucosal surface of the tracheal segment. Vehicle or drug was then added (50 μl) with a Hamilton syringe and the NO release measurement performed. Currents proportional to NO concentration were measured by a chronoamperometric method by using a voltametric analyzer Autolab 20 electrochemical work station (ECHO-Chemie, The Netherlands) and a porphirinic microsensor (Malinski & Taha, 1992). Calibration curve was obtained with NO standard solution (2 mmol l^{-1}) prepared as described previously (Masaros *et al*., 1997). Detected NO represented a local concentration which was established on the tissue surface or at a close proximity (0.2–1 μm). The response time of the microsensor was about 1 ms. Therefore, the sensor could only detect a concentration of NO which was not consumed by the extremely fast intracellular chemical reaction of NO with superoxide anion (Kobayashi & Miki, 1994).

Drugs

L-Arg, D-Arg, D-NMMA, L-NMMA, D-NAME, L-NAME, carbachol, indomethacin and sodium nitroprusside were obtained from Sigma Chemical (U.S.A.). ET-1 and sarafotoxin S6c were purchased from NovaBiochem (Switzerland). IRL 1620 (Suc-[Glu^9,Ala^{11-15}] endothelin-1(8-21)), FR139317 (2 (R)-[2(R)-(2(S)-{[1-(hexahydro-1H-azepinyl)]carbonyl}amino-4-methylpentanoyl)-amino-3-[3-(1-methyl-1H-indolyl)]propri-onyl]-amino-3-(2-pyridyl)propionic acid) and BQ788 (N-*cis*-2,6-dimethylpiperidinocarbonyl-L-g-methylleucyl-D-1-meth-oxy-carbonyllltryptophanyl-D-norleucin) were kind gift from Dr T. Okada (Novartis, Japan, Ltd.). All ET agonists and antagonists were dissolved in dimethylsulfoxide. All the other drugs were dissolved in 0.9% saline. Stock solution of peptides were stored at −20°C. L-Arg, D-Arg, L-NAME, D-NAME, carbachol were freshly prepared for each experiment.

Statistical analysis

Values in the text and figures are the means ± s.e.mean. Statistical comparisons were performed using Student's *t*-tests for unpaired values or the one way analysis of variance and Dunnett's test. In all cases, a P value of less than 0.05 was considered significant.

Results

ET-1 induced contraction and relaxation

Pretreatment with Cch (10 μM) produced a contraction of guinea-pig tracheal tube preparations that averaged 475 ± 67 mg ($n = 7$) and 545 ± 71 mg ($n = 6$) after pretreatment with D-NMMA and L-NMMA, respectively. Contractions produced by Cch under both these conditions remained stable for at least 30 min. In tracheal tube preparations pretreated with D-NMMA (100 μM) and precontracted with Cch, administration of ET-1 (1 pM–100 nM) caused a concentration-dependent relaxation (Figure 1A). Pretreatment with L-NMMA abolished the ET-1 induced relaxation such that a concentration-dependent contraction was evident (Figure 1A). After L-NAME (100 μM), ET-1 contracted tracheal tube preparations, whereas after D-NAME (100 μM), ET-1 caused a relaxation (Figure 1B). In tracheal tube preparations pretreated with L-NMMA (100 μM), addition of L-Arg (1 mM), but not of D-Arg (1 mM), reverted the contraction into a relaxation (Figure 1C). In tracheal tube preparations in which the epithelium was removed, ET-1 caused a concentration-dependent contraction that was similar in the presence of either D-NMMA or L-NMMA (Figure 2).

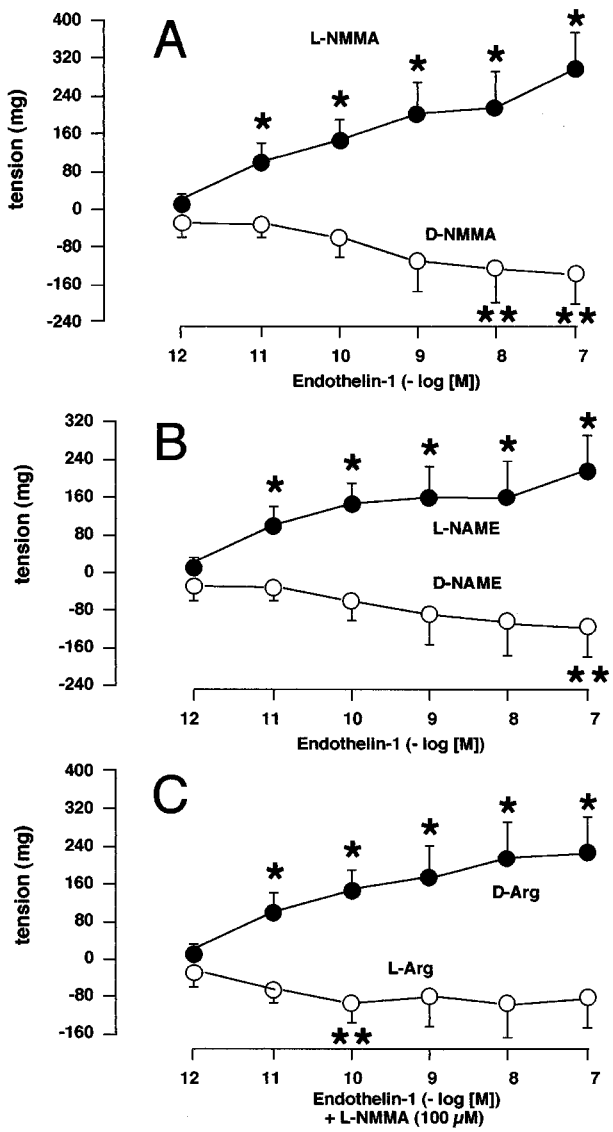

Figure 1 (A) The concentration-dependent relaxation induced by endothelin-1 after pretreatment with D-NMMA (100 μM, ○) is changed into a contraction after pretreatment with L-NMMA (100 μM, ●). (B) D-NAME (100 μM, ○) and L-NAME (100 μM, ●) were used. (C) Preparations were pretreated with L-NMMA (100 μM) and D-arginine (100 μM, ○) or L-arginine (100 μM, ●). Guinea-pig tracheal tube preparations precontracted with carbachol (10 μM) and pretreated with indomethacin (5 μM) were used. Entries are means±s.e.mean of at least five experiments. *$P<0.05$ vs ○; **$P<0.05$ vs baseline.

Selective agonists: ET_B receptor activation

In tracheal tubes preparations precontracted with Cch (10 μM) the selective ET_B receptor agonists, sarafotoxin S6c and IRL1620 caused concentration-dependent contraction (Figure 3A,B). In both cases the concentration-response curves to these agonists were similar in preparations pretreated with D-NMMA and L-NMMA (Figure 3A,B).

Selective antagonists

Pretreatment with the selective ET_B receptor antagonist, BQ788 (1 μM), did not affect the contraction produced by Cch (10 μM, data not shown). In the presence of BQ788 and after pretreatment with D-NMMA, ET-1 (1 pM–100 nM) caused a concentration-dependent relaxation. Maximum ET-1-induced relaxation in the presence of BQ788 (−355±63 mg, $n=5$) (Figure 4A) was significantly greater than that produced by ET-1 in the absence of BQ-788 (−110±37 mg, $n=6$,

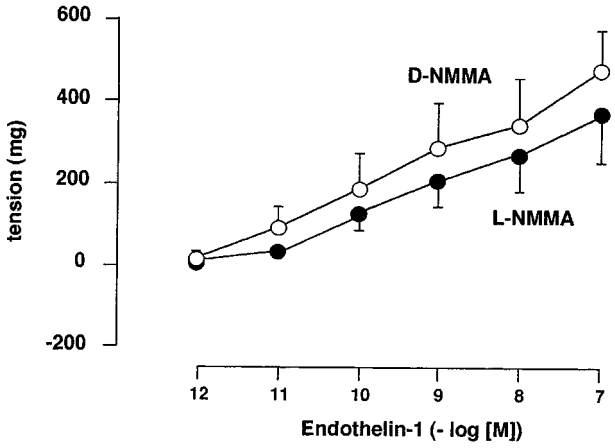

Figure 2 Concentration-dependent motor response induced by endothelin-1 in epithelium-denuded preparations after pretreatment with D-NMMA (100 μM, ○) or L-NMMA (100 μM, ●). Guinea-pig tracheal tube preparations precontracted with carbachol (1 μM) and pretreated with indomethacin (5 μM) were used. Entries are means±s.e.mean of at least five experiments. *, $P<0.05$ vs ○.

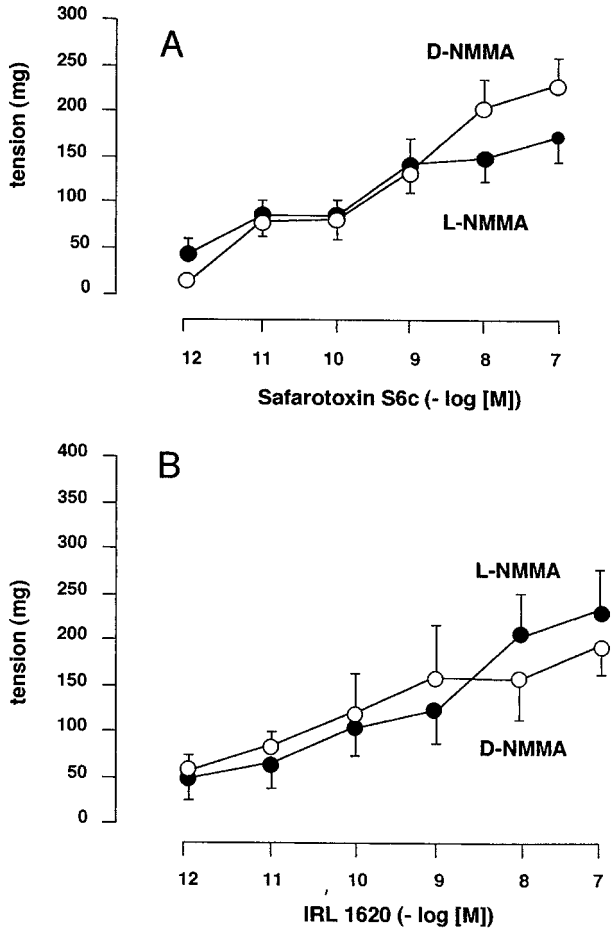

Figure 3 Concentration-dependent contraction induced by the selective ET_B receptor agonists, sarafotoxin S6c (A) and IRL 1620 (B), after pretreatment with D-NMMA (100 μM, ○) or L-NMMA (100 μM, ●). Guinea-pig tracheal tube preparations precontracted with carbachol (1 μM) and pretreated with indomethacin (5 μM) were used. Entries are means±s.e.mean of at least five experiments.

Table 1 Nitric oxide (fmol) levels in the bathing fluid over mucosal surface of guinea-pig tracheal segments measured by a porphyrinic microsensor

Vehicle	45±7			
IRL1620 (1 μM)	58±18			
ET-1		10 nM	100 nM	1 μM
ET-1+D-NMMA (100 μM)		110±43*	412±107*	672±214*
ET-1+L-NMMA (100 μM)			127±43**	216±49**
ET-1+BQ788 (1 μM)			511±92	
ET-1+FR139317 (10 μM)			158±55***	

Each value is the mean±s.e.mean of at least four experiments. *$P<0.05$ vs vehicle; **$P<0.05$ vs respective ET-1+D-NMMA; and ***$P<0.05$ vs ET-1+D-NMMA (100 nM). D- or L-NMMA were added 20 min before the stimulus. Receptor antagonists were added 10 min before the stimulus.

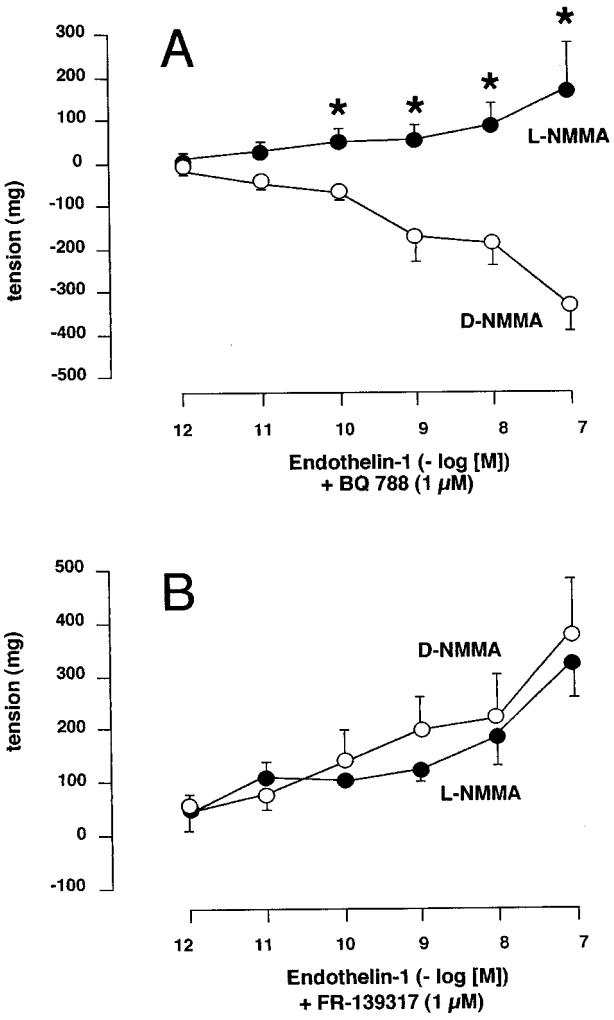

Figure 4 Concentration-dependent response induced by endothelin-1 after pretreatment with D-NMMA (100 μM, ○) or L-NMMA (100 μM, ●). (A) Preparations were pretreated with the ET_B receptor antagonist BQ788 (1 μM); (B) Preparations were pretreated with the ET_A receptor antagonists FR139317 (10 μM). Guinea-pig tracheal tube preparations precontracted with carbachol (1 μM) and pretreated with indomethacin (5 μM) were used. Entries are means ±s.e.mean of at least five experiments.

$P<0.05$). In the presence of the combination of L-NMMA and BQ788 ET-1 produced a concentration-dependent contraction. Under these conditions maximum contraction (120±35 mg, $n=5$) induced by ET-1, acting only on ET_A receptors, was significantly lower than that induced in the presence of L-NMMA and without the BQ788 (290±35 mg, $n=6$, $P<0.05$).

Pretreatment with the selective ET_A receptor antagonist FR139317 (10 μM) did not affect the contraction produced by Cch (10 μM, data not shown). In the presence of FR139317 and after pretreatment with D-NMMA, ET-1 caused a concentration-dependent contraction: maximum contraction (ET-1 100 nM, 375±51 mg, $n=5$) (Figure 4B) was not different from that produced by ET-1 in the absence of FR139317 (297±37 mg, $n=6$). In the presence of FR139317, the concentration-response curve to ET-1 after pretreatment with L-NMMA was not different from that observed after pretreatment with D-NMMA (Figure 4B).

NO release studies

Addition of ET-1 vehicle (0.9% saline, 50 μl) to the tracheal segments did not cause any detectable change of the baseline signal. Addition of ET-1 caused a upward shift of the baseline, that reached a maximum within 5–15 s. The amplitude of response to ET-1 in NO levels was concentration-dependent between the range 0.1 nM–1 μM (Table 1). The ability of ET-1 (1 μM) to increase NO release from intact tracheal segments (590±177 fmol, $n=5$) was significantly reduced in epithelium-denuded preparations (346±82 fmol, $n=4$, $P<0.05$). After pretreatment with L-NMMA (100 μM), but not with D-LMMA, the ability of ET-1 to increase NO level was reduced by about 70% (Table 1). In the presence of the ET_B receptor antagonist, BQ788 (1 μM), the increase in NO level induced by ET-1 was not significantly changed, whereas in the presence of ET_A receptor antagonist, FR139317 (10 μM), it was significantly reduced (Table 1).

Discussion

The results reported in the present paper show that ET-1 causes an epithelium-dependent relaxation of guinea-pig tracheal tube preparations precontracted with Cch. The relaxation caused by ET-1 was apparently due to NO release because it was abolished following inhibition of NO synthase (NOs) activity with L-NMMA or L-NAME. Further support for a role of NO in this effect is given by the observation that L-Arg, but not D-Arg, reverted the effect of NOs inhibition. A previous report has shown that in guinea-pig bronchi that ET-1 causes a relaxant response that is reduced by NOs inhibitors (Filep *et al*., 1993). Present data reinforce the hypothesis that NO of epithelial origin relaxes guinea-pig airways. The role of epithelium to release NO was underlined by experiments showing that after its removal, ET-1 was no longer able to cause any detectable relaxation of tracheal tube preparations, and contractions induced by ET-1 in the presence of L-NMMA, were similar to those obtained in the presence of D-NMMA. The present paper provides direct biochemical evidence of NO release from the airway tissue after challenge with ET-1. The ability of ET-1 to increase NO levels as detected by the porphyrinic based microsensor was dependent

on ET-1 concentration and markedly reduced by pretreatment with L-NMMA. The reason and the physiological significance of why a concentration of L-NMMA (100 μM) that blocked relaxation to ET-1, did not completely abolished NO release induced by ET-1 remains to be determined.

The other piece of evidence obtained in the present work is the demonstration that the NO- and epithelium-dependent relaxation induced by ET-1 in guinea-pig tracheal tube preparations is mediated by ET_A receptors. This demonstration is derived from various findings obtained with ET_A and ET_B receptor agonists and antagonists. Firstly, the two selective agonists of ET_B receptors safarotoxin S6c and IRL1620 were unable to relax tracheal tube preparations. In addition, pretreatment with L-NMMA or D-NMMA did not affect concentration-response curves to safarotoxin S6c and IRL1620. Secondly, in the presence of the selective ET_B receptor antagonist BQ788, the relaxation produced by ET-1 was significantly greater than that observed in the absence of the ET_B receptor antagonist. Thus, after blockade of ET_B receptors with BQ788, the epithelium-dependent relaxation mediated by ET_A receptors seems to predominate over the direct ET_A-mediated contraction. Thirdly, the selective ET_A receptor antagonist, FR139317, abolished the ET-1 induced relaxation, and concentration-response curves to ET-1 in the presence of either L-NMMA or D-NMMA were similar. Measurement of NO from tracheal segments after different pharmacological manipulations or epithelium removal confirmed the role of ET_A receptors to increase NO levels in the medium. In fact, the ET_A receptor antagonist, FR139317, inhibited the increase in NO produced by ET-1, whereas the ET_B receptor antagonist BQ788 was without effect.

The effect to ET-1 on bronchomotor tone in the guinea-pig airways *in vitro* is particularly complex since both ET_A and ET_B receptors are involved and direct smooth muscle action and indirect epithelium-dependent components have a role. Previous studies in isolated strips or rings of guinea-pig trachea or main bronchi have shown that ET-1 may contract guinea-pig airways by stimulating ET_B receptors (Battistini *et al*., 1994), although a smaller contractile component mediated by activation of ET_A receptors is also present (Battistini *et al*., 1994). ET-1 was also found to cause an epithelium-dependent relaxant response that is, in part, mediated by NO release (Filep *et al*., 1993). By using the tracheal tube preparation and the porphyrinic based microsensor we confirmed that ET-1 stimulates the release of NO from the guinea-pig trachea and demonstrated that epithelium- and NO-dependent relaxation to ET-1 is mediated by ET_A receptor activation. In the present experimental conditions (tracheal tube preparations precontracted with Cch) the relaxation mediated by ET_A receptors *via* NO release predominates over the direct smooth muscle contraction due to ET_B and ET_A receptor activation. In addition, we confirmed previous observations (Battistini *et al*., 1994) that ET_A receptors may contribute to increase bronchial tone. However, the major component of the contractile response to ET-1 is due to ET_B receptors (Battistine *et al*., 1994), because after ET_B receptor blockade and NOs inhibition, the contraction caused by ET-1 was smaller than that observed after ET_A receptor blockade.

Various relaxing factors may be released from the airway epithelium, including prostanoids, NO, and hyperpolarizing factor. Recently, a large variety of mediators and agents have been found to cause relaxation due to NO release from the epithelium.

These mediators include histamine, bradykinin and high K^+. NO release by these agents seems to have an important role in pathophysiological models of disease, such as asthma. Thus, viral infection caused increased bronchial responsiveness to histamine in guinea-pigs, that was similar to the increased bronchial responsiveness to this autacoid caused by inhibition of NOs activity in the airway epithelium (Folkerts *et al*., 1995). This finding suggests that viral infection may damage a NO-dependent protective mechanism activated by inflammatory mediators such as histamine: downregulation of this protective mechanism may, thus, contribute to hyperresponsiveness.

These findings obtained in experimental animals are important for the interpretation of recent results obtained in asthma. In mild asthmatic patients $PD_{20}FEV_1$ to inhaled bradykinin was decreased by 3.2 doubling doses after L-NMMA pretreatment (Ricciardolo *et al*., 1996). In contrast, in severe asthmatic patients who showed in baseline conditions remarkable hyperresponsiveness to bradykinin, $PD_{20}FEV_1$ to aerosolized bradykinin was not further decreased after NOs inhibition (Ricciardolo *et al*., 1997). Thus, in severe asthma, as in virus-infected guinea-pigs, the bronchoprotective function of NO seems to be lost. It has been proposed that ETs may have a role in airway and lung disease (Barnes, 1994; Hay *et al*., 1993) because ETs cause long lasting contractions of human airway. The present data suggest that a precise localization of ET receptor subtypes in the human airways and of their biological role in airway motility both *in vitro* and *in vivo* condition can be useful to identify possible bronchodilator activity of endothelin and its role in asthma.

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References

ARAI, H., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature,* **348,** 730–732.

BARNES, P.J. (1994). Endothelins and pulmonary diseases. *J. Appl. Physiol.,* **77,** 1051–1059.

BATTISTINI, B., WARNER, T.D., FOURNIER, A. & VANE, J.R. (1994). Characterization of ET_B receptors mediating contractions induced by endothelin-1 or IRL 1620 in guinea-pig isolated airways: effect of BQ-123, FR139317 or PD 145065. *Br. J. Pharmacol.,* **111,** 1009–1016.

FIGINI, M., RICCIARDOLO, F.L.M., JAVDAN, P., NIJKAMP, F.P., EMANUELI, C., PRADELLES, P., FOLKERTS, G. & GEPPETTI, P. (1995). Evidence that epithelium-derived relaxing factor released by bradykinin in guinea pig trachea is nitric oxide. *Am. J. Respir. Crit. Care Med.,* **153,** 918–923.

FILEP, J., BATTISTINI, B. & SIROIS, P. (1993). Induction by endothelin-1 of epithelium-dependent relaxation of guinea-pig trachea in vitro: role of nitric oxide. *Br. J. Pharmacol.,* **109,** 637–644.

FOLKERTS, G., VAN DER LINDE, H.J. & NIJKAMP, F.P. (1995). Virus-induced hyperresponsiveness in guinea pigs is related to a deficiency in nitric oxide. *J. Clin. Invest.,* **95,** 26–30.

GIAID, A., POLAK, J.M., GAITONDE, V., HAMID, S., MOSCOS, G., LOGON, S., UWANGHO, D., RONCALLI, M., SHIMI, O., KIMURA, S., YANAGISAWA, M., MASAKI, T. & SRINGALL, D.R. (1991). Distribution of endothelin-like immunoreactivity and mRNA in the developing and adult human lung. *Am. Rev. Respir. Dis.,* **4,** 50–58.

GLASSBERG, M.K., ERGUL, A., WANNER, A. & PUETT, D. (1994). Endothelin-1 promotes mitogenesis in airway smooth muscle cells. *Am. J. Respir. Cell. Mol. Biol.,* **10,** 316–321.

HAY, D.W., HENRY, P.J. & GOLDIE, R.G. (1993). Endothelin and the respiratory system. *Trends Pharmacol. Sci.,* **14,** 29–32.

KOBAYASHI, K. & MIKI, M. (1994). A direct demonstration of reaction of nitric oxide with superoxide anion by the use of pulse radiolysis. In Asada, K. & Toshikawa, T. (eds). *Frontiers of reactive oxygen species in biology and medicine*. Elsevier Science: Oxford, pp. 223–224.

MALINSKI, T. & TAHA, Z. (1992). Nitric oxide release from a single cell in situ by a porphyrinic-based microsensor. *Nature,* **358,** 676–678.

MASAROS, S., GRUNFELD, S., MESAROVA, A., BUSTIN, D. & MALINSKI, T. (1997). Determination of nitric oxide saturated (stock) solution by chronoamperometry on a porphyrine microelectrode. *Analyt. Chim. Acta,* **339,** 265–270.

NIJKAMP, F.P., VAN DER LINDE, H.J. & FOLKERTS, G. (1993). Nitric oxide synthesis inhibitors induce airway hyperresponsiveness in the guinea pig in vivo and in vitro. Role of the epithelium. *Am. Rev. Respir. Dis.,* **148,** 727–734.

RICCIARDOLO, F.L.M., DI MARIA, G.U., SAPIENZA, M.A., MISTRETTA, A. & GEPPETTI, P. (1997). Impairment of bronchoprotection by NO in severe asthma. *Lancet,* **350,** 1297–1298.

RICCIARDOLO, F.L.M., GEPPETTI, P., MISTRETTA, A., NADEL, J.A., SAPIENZA, M.A., BELLOFIORE, S. & DI MARIA, G.U. (1996). Randomised double-blind placebo-controlled study of the effect of inhibition of nitric-oxide synthesis in bradykinin-induced asthma. *Lancet,* **348,** 374–377.

RUBANYI, G.M. & PLOKOFF, M.A. (1994). Endothelins: molecular biology biochemistry, pharmacology, physiology and pathophysiology. *Pharmacol. Rev.,* **46,** 325–415.

SAKURAI, T., YANAGISAWA, M.Y, MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non isopeptide selective subtype of the endothelin receptor. *Nature,* **348,** 732–735.

SPRINGALL, D.R., HOWARTH, P.H., COUNIHAN, H., DJUKANOVIC, R., HOLGATE, S.T. & POLAK, J.M. (1991). Endothelin immunoreactivity of airway epithelium in asthmatic patients. *Lancet,* **337,** 697–701.

TAKUWA, N., TAKUWA, Y., YANAGISAWA, M., YAMASHITA, K. & MASAKI. (1989). A novel vasoactive peptide endothelin stimulates mitogenesis through inositol lipid turnover in Swiss 3T3 fibroblasts. *J. Biol. Chem.,* **264,** 7856–7861.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KABAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature,* **332,** 411–415.

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