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Isoproterenol Increases Cl Diffusion Potential Difference of Rabbit Trachea through Nitric Oxide Generation[1]

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ABSTRACT

To determine whether nitric oxide (NO) formation is involved in Cl secretion across airway mucosa in response to *beta* adrenergic agonists, we studied the effect of isoproterenol (ISO) on the Cl diffusion potential difference of rabbit tracheal mucosa and measured NO formation by a highly specific electrode for this molecule *in vivo*. Perfusion of ISO on the tracheal mucosal surface increased the Cl diffusion potential difference, as determined in the presence of amiloride, in a dose-dependent fashion, the maximal increase from the base-line value being 12.1 ± 1.7 mV ($P < .001$). Application of N^G-nitro-L-arginine methylester (10^{-3} M) decreased the Cl diffusion potential difference by itself and attenuated the subsequent response to ISO, causing a rightward displacement of ISO concentration-response curves, whereas N^G-nitro-D-arginine methylester had no effect. This inhibitory effect of N^G-nitro-L-arginine methylester was reversed by L-arginine but not by D-arginine. Addition of ISO dose-dependently increased polarographic current and, hence, NO concentration in the perfusate, the maximal increase from the base-line levels being 178 ± 10 nM. Histochemistry for NADPH diaphorase activity showed a strong staining within epithelial cells. These results suggest that NO formation may play a role in the *beta* adrenoceptor-mediated Cl secretion by tracheal mucosa.

NO, a multipurpose messenger molecule generated from the amino acid L-arginine by NOS, may be functioning in the regulation of airway and vascular smooth muscle tone, host defense and pulmonary neurotransmission (Gaston *et al.*, 1994; Persson *et al.*, 1990; Belvisi *et al.*, 1992). Recently, the localization of NOS-like activity has been found in airway epithelial cells, which suggests that this cell type may generate NO (Kobzik *et al.*, 1993; Philip *et al.*, 1994).

Inhaled *beta* adrenergic agonists are widely used for the treatment of bronchial asthma. Stimulation of *beta* adrenergic receptors not only relaxes airway smooth muscle but also stimulates mucus secretion and epithelial ciliary motility (Nadel *et al.*, 1985; Sanderson and Dirksen, 1989). Previous experiments on isolated tracheal epithelial sheets (Al-Bazzaz and Cheng, 1979) and cultured epithelial cells (Welsh, 1986) have demonstrated that the *beta* adrenergic agonist ISO stimulates Cl secretion by epithelium from the submucosal side toward the respiratory lumen, thereby increasing water secretion. However, these *in vitro* findings may not necessarily reflect ion transport *in vivo* because of the lack of innervation and blood supply. Therefore, to determine whether *beta* adrenergic agonists likewise stimulate Cl secretion *in vivo*, we studied the effect of ISO on Cl-PD across tracheal mucosa in anesthetized rabbits. Recent reports have shown that formation of NO contributes to *beta* adrenoceptor-mediated relaxation of vascular smooth muscle (Parent *et al.*, 1993) and ciliary stimulation of airway epithelium (Jain *et al.*, 1993). Therefore, we investigated the possible involvement of NO generation in the action of ISO on Cl-PD by using an amperometric sensor highly specific for this molecule.

Materials and Methods

Measurement of PD of tracheal mucosa. Male Japanese white rabbits (2.2–2.5 kg) were anesthetized with intraperitoneal α-chloralose (50 mg/kg) and Urethane (500 mg/kg), the trachea was exposed and artificial ventilation (tidal volume 10 ml/kg, respiratory rate 60/min) was performed (model SN-480–7, Shinano Co., Tokyo, Japan) through the polyethylene tube inserted 5 mm above the tracheal carina. Then cartilage rings of upper trachea were incised transaxially, and the surface of the membranous portion was fully exposed. The exploring bridge for *in vivo* measurement of airway epithelial PD, constructed of polyethylene tube (2.5 mm in diameter), was placed on the surface of the tracheal mucosa (Boucher *et al.*, 1980). Contact with the tracheal surface was ensured by continuous

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ABBREVIATIONS: NO, nitric oxide; ISO, isoproterenol; PD, potential difference; Cl-PD, Cl diffusion potential difference; L-NAME, N^G-nitro-L-arginine methylester; D-NAME, N^G-nitro-D-arginine methylester; NOS, nitric oxide synthase; K-H solution, Krebs-Henseleit solution; SNAP, S-nitroso-*N*-acetyl-*dl*-penicillamine; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazole-3-oxide-1-oxyl; PBS, phosphate-buffered saline; L-NMMA, N^G-monomethyl-L-arginine; D-NMMA, N^G-monomethyl-D-arginine.

perfusion (0.3 ml/min) through the bridge with warmed (37°C), gassed (95% O_2/5% CO_2) K-H solution containing 118 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 1.2 mM $NaHCO_3$ and 25.5 mM glucose. The perfusion reservoir was connected to the calomel half-cell *via* a polyethylene tube (1.5 mm in diameter) filled with K-H solution in 3% agar. The reference bridge, a 21-gauge needle that contained K-H solution in 3% agar, was inserted into the subcutaneous space of the right anterior chest wall. Each bridge was connected by a calomel half-cell to a high-impedance voltmeter (CEZ-9100, Nihon Kohden, Tokyo). The electrical signal was filtered to remove 60-cycle interference and was continuously recorded (model SR 6335, Graphtec, Tokyo). Because preliminary experiments showed that the subcutaneous space was isoelectric with the adventitial surface of the trachea, electrical potential difference between the tracheal mucosal surface and the subcutaneous space was recorded as transepithelial PD.

Effect of ISO on Cl-PD. To measure Cl-PD of the tracheal mucosa, the K-H solution superfusing the mucosal surface was switched to one that contained amiloride (10^{-4} M), a Na channel blocker (Al-Bazzaz and Zevin, 1984). This maneuver decreases PD, and the remaining PD (Cl-PD) is an index of epithelial cellular and paracellular paths available for Cl diffusion (Stutts *et al.*, 1990). When Cl-PD became stable, amiloride-containing solution was changed to a similar solution that contained ISO (10^{-6} M).

To assess the role of NO formation in the ISO-induced increase in Cl-PD, L-NAME (10^{-3} M), an inhibitor of NO synthesis from L-arginine (Rees *et al.*, 1990), was added to the superfusing solution in the presence of amiloride, and when the response of Cl-PD reached a plateau, ISO (10^{-6} M) was subsequently added. Then, after the response to ISO became stable, either D-arginine or L-arginine at 10^{-3} M was added to the solution. Furthermore, to assess whether NO actually affects Cl-PD, SNAP (10^{-3} M), a stable standard NO generator (Boughton-Smith *et al.*, 1990), was added to the superfusate in the presence of amiloride.

In constructing the concentration-response curve for ISO, we cumulatively added ISO (10^{-8}–10^{-5} M) in half-molar increments to the amiloride-containing superfusate in the absence and presence of L-NAME or of its inactive enantiomer D-NAME (10^{-3} M) and measured Cl-PD for 10 min after the application of each concentration onto the tracheal surface. This interval was chosen as a convenient period, because preliminary experiments had shown that the ISO-induced maximal change in Cl-PD occurred within 10 min of its addition.

Measurement of NO release. Measurement of NO formation was based on the measurement of pA-order redox current between the working electrode and the counterelectrode (Ichimori *et al.*, 1994; Malinski and Taha, 1992). The NO-selective working electrode and the counterelectrode (NOE-55 and NOR-20, respectively, Inter Medical Co., Tokyo) were placed 5 mm apart in the solution perfusing the surface of the tracheal mucosa. The redox current between electrodes was recorded by a NO monitor (model NO-501, Inter Medical Co.). The working electrode was made of Pt/I alloy wire (0.2 mm in diameter) coated with three membranes consisting of KCl, NO-selective resin, and normal silicone membranes. The KCl membrane was electrochemically deposited on the Pt/I wire to suppress overvoltage in the NO discharge. The NO-selective resin was made of puroxyline lacquer, coated by immersion of the wire in 0.8% nitrocellulose solution and dried under air for 12 hr. The silicone membrane was affixed as the outermost membrane to avoid a nonspecific ionic effect and electrochemical reactions. The counterelectrode was made of carbon fiber. The concentration of NO was measured in terms of the current the basis of the following electrochemical reaction:

$$NO + 4OH \rightarrow NO^- + 2H_2O + 3e^- \quad (1)$$

Calibration of the electrode was performed using SNAP. SNAP generates NO as follows:

$$SNAP\ (R\text{-}SNO) \rightarrow \tfrac{1}{2}\text{-}R\text{-}SS\text{-}R + NO \quad (2)$$

where R represents $^-OCOCH(NHCOCH_3)C(CH_3)_2$, which is the main body of SNAP excluding the S-nitrosothiyl group. A recording of the electrode currents generated by various concentrations of SNAP resolved in K-H solution (10^{-6}–10^{-3} M) was used as a calibration line (fig. 1). Because this calibration line showed linear regression between SNAP concentration and electrode current, the measured current was converted to NO concentration, [NO]. The signal-to-noise ratio of the current was approximately 1 when 5×10^{-6} M SNAP was applied. To test the selectivity of the electrode, we studied effects of NO_2^- and NO_3^-, which are reaction products of NO and O_2, and found that these molecules did not affect the electrode current. Furthermore, to test whether SNAP itself alters the current, we examined the effect of heat-decomposed SNAP, which contained less than 0.1% of SNAP as determined by spectrophotometry. This substance exhibited only 2% of the current produced by the original SNAP solution. In addition, NO scavengers including oxyhemoglobin (4×10^{-6} M) and PTIO (3×10^{-5} M) reduced SNAP (5×10^{-5} M)-induced current by more than 90%. Thus the current detected in the SNAP solution was due to the NO liberated from SNAP.

Effect of ISO on NO release. In our separate studies, addition of L-NAME (10^{-3} M) or ISO (10^{-6} M) to K-H solution that did not perfuse the tracheal mucosa had no effect on the electrode current, which suggests that there were no direct actions on the electrode. To assess whether the release of NO is stimulated by ISO, after establishing the base-line current, we added ISO (10^{-6} M) to K-H solution superfusing the tracheal mucosal surface and monitored the response of electrode current. In addition, to assess whether the detected NO was derived from neural tissues, we examined the effects of tetrodotoxin (10^{-6} M) and atropine (10^{-5} M) on ISO (10^{-6} M)-induced current. For determination of the concentration-dependent effect of ISO on NO generation, ISO (10^{-8}–10^{-5} M) was cumulatively added to K-H solution, and the response of the current was measured and expressed as [NO].

Histochemical staining of NOS. Because NOS is active in histochemical assay for NADPH diaphorase (Schmidt *et al.*, 1992), to identify the source of NO generated from the tracheal surface, we performed histochemical staining of NADPH diaphorase activity according to the method of Kobzik and associates (Kobzik *et al.*, 1993). Cryostat sections (8 μm) were prepared from rabbit trachea on gelatin-coated slides. Sections were fixed in fresh 2% buffered paraformaldehyde for 10 min and rinsed with PBS. Slides were then covered with a reaction mixture consisting of 0.25 mg/ml nitroblue

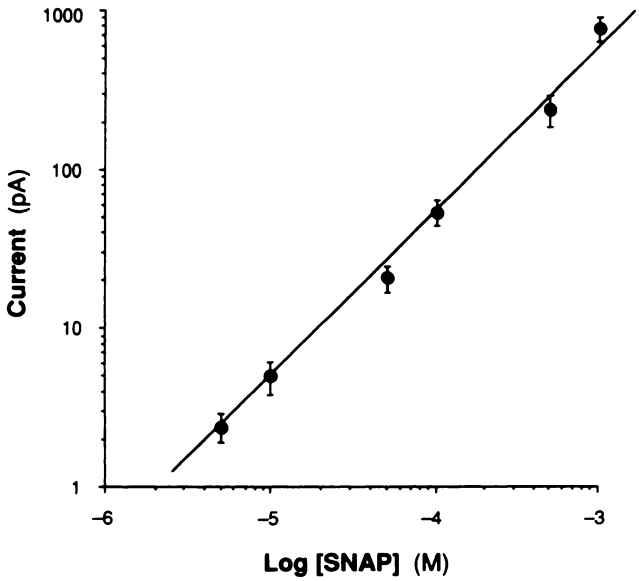

Fig. 1. Relationship between the concentration of SNAP in K-H solution at 37°C and the electrical current detected by a NO-selective electrode. Data are means ± S.E.; n = 18 to 24 for each point.

tetrazolium, 1 mg/ml NADPH and 0.5% Triton X-100 in 0.1 M Tris buffer, pH 7.6. After 1 hr, sections were washed with PBS and covered with glycerol mounting medium, and coverslips were applied. In control experiment, the substrate was omitted and 15 mM *p*-nitrophenylphosphate was incubated in the reaction medium to inhibit endogenous phosphatases that can convert NADPH to NADH and cause false-positive staining by NADH dehydrogenases (Leeflang-dePijper and Hulsmann, 1974).

Drugs. The following drugs were used: SNAP (Inter Medical Co.), DL-isoproterenol hydrochloride (Nacarai Tesque, Kyoto, Japan), amiloride, L-NAME, D-NAME, L-arginine, D-arginine, tetrodotoxin, atropine sulfate, PTIO, nitroblue tetrazolium, NADPH and *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO).

Statistics. All data were expressed as means ± S.E. Statistical analysis was performed by Student's *t* test, and a P value of less than .05 was considered statistically significant.

Results

Tracheal Cl-PD. As demonstrated in figure 2, the base-line value of *in vivo* PD of rabbit tracheal mucosa was 19.7 ± 1.8 mV (n = 22), lumen negative. Application of amiloride (10^{-4} M) to the perfusing solution reduced PD to 13.1 ± 2.9 mV (n = 9), which referred as Cl-PD. The addition of ISO (10^{-6} M) increased Cl-PD by 11.3 ± 2.4 mV ($P < .001$, n = 9). L-NAME (10^{-3} M) by itself decreased Cl-PD from 11.8 ± 1.2 to 6.9 ± 1.1 mV ($P < .01$, n = 8), and the subsequent addition of ISO (10^{-6} M) increased Cl-PD only by 4.7 ± 0.5 mV (n = 8), which was significantly less than the response in the absence of L-NAME ($P < .001$). On the other hand, D-NAME (10^{-3} M) had no such effects. This inhibitory effect of L-NAME was not affected by D-arginine but was completely reversed by L-arginine: in the presence of L-NAME (10^{-3} M) and ISO (10^{-6} M), consecutive application of D-arginine (10^{-3} M) and L-arginine (10^{-3} M) increased Cl-PD by 0.3 ± 0.2 and 12.1 ± 2.6 mV, respectively (n = 8). Addition of SNAP (10^{-3} M) increased Cl-PD from 13.2 ± 1.5 to 19.8 ± 2.0 mV ($P < .001$, n = 9).

The addition of ISO increased Cl-PD in a concentration-dependent fashion, the maximal increase from the base-line values being 12.2 ± 1.8 mV ($P < .001$, n = 7, fig. 3). Pretreatment of tracheal mucosa with L-NAME (10^{-3} M) attenuated Cl-PD in response to ISO. Pretreatment with D-NAME (10^{-3} M) was without effect on the concentration-response curves for ISO.

NO release. The output current of the NO-selective electrode in K-H solution superfusing rabbit tracheal mucosa is shown in fig. 4. When the electrode was immersed in the superfusate, the base-line current was detected with a wide variation of 9 to 36 pA, which referred to [NO] at 17 ± 4 nM (n = 23). Application of ISO (10^{-6} M) to the superfusate caused a rapid increase in the current by 93 ± 17 pA ($P < .001$, n = 8). The addition of L-NAME (10^{-6} M) decreased the current from 19 ± 5 to 4 ± 2 pA ($P < .001$, n = 7) and inhibited the stimulatory effect of 10^{-6} M ISO by approximately 80% ($P < .001$). The addition of tetrodotoxin (10^{-6} M) and atropine (10^{-5} M) did not alter the response to ISO: the ISO (10^{-6} M)-induced increases in the current were 85 ± 11 (n = 7) and 91 ± 16 pA (n = 6), respectively. The addition of ISO increased [NO] in a concentration-dependent manner, the maximal increase in [NO] from the base-line value being 178 ± 10 nM ($P < .001$, n = 8; fig. 5).

Histochemistry for NADPH diaphorase. Histochemical staining of NADPH diaphorase activity of rabbit trachea showed a positive staining within airway epithelium, whereas the staining in vascular endothelium was generally weak and variable between samples. No staining was observed in the control experiment in which the substrate was omitted from the reaction medium (fig. 6).

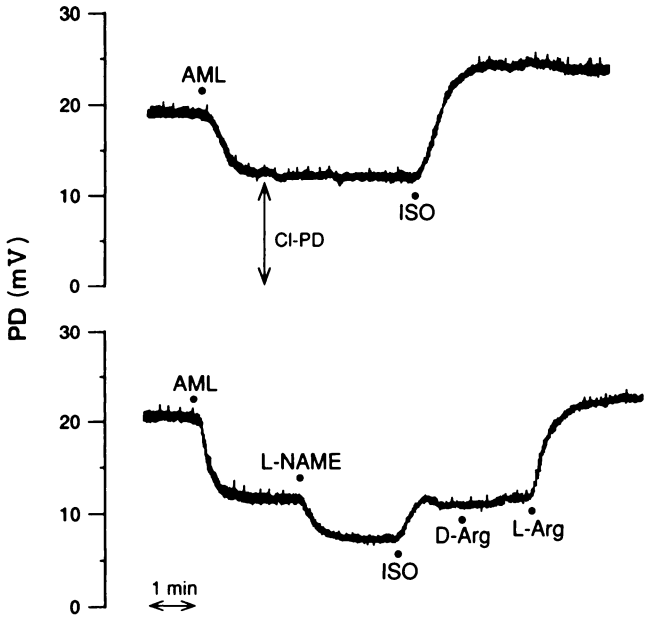

Fig. 2. Representative tracing of rabbit tracheal potential difference (PD) *in vivo*. Upper panel: Addition of amiloride (AML, 10^{-4} M) to K-H solution superfusing the tracheal mucosa decreased PD, which is referred to as Cl-PD. When Cl-PD became stable, ISO (10^{-6} M) was added. Lower panel: Response of Cl-PD to ISO (10^{-6} M) in the presence of L-NAME (10^{-3} M). After ISO, D-arginine (D-Arg, 10^{-3} M) and L-arginine (L-Arg, 10^{-3} M) were consecutively added to the superfusate.

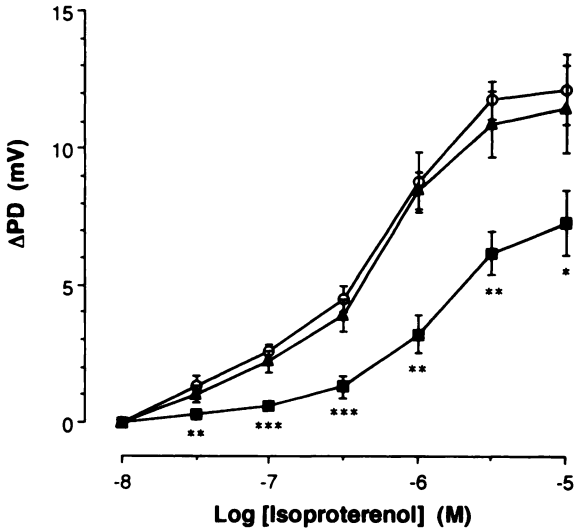

Fig. 3. Concentration-dependent effect of ISO on Cl-PD of rabbit tracheal mucosa *in vivo*. ISO was cumulatively added to the superfusate in the absence (control, open circles) and presence of D-NAME (10^{-3} M, closed triangles) or L-NAME (10^{-3} M, closed square). Values are expressed as the increase in Cl-PD from the base-line values (ΔPD). Data are means ± S.E.; n = 7 for each point. * $P < .05$, ** $P < .01$, *** $P < .001$, significantly different from corresponding control values.

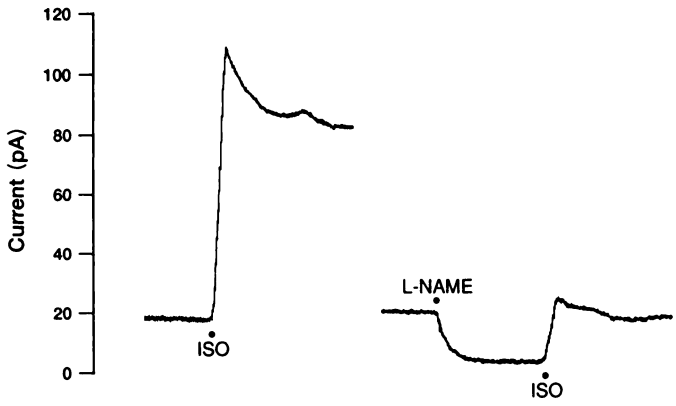

Fig. 4. Representative tracing of the current detected by NO-selective electrode in K-H solution superfusing rabbit tracheal mucosa. After equilibration, ISO (10^{-6} M) was added to the superfusate in the absence or presence of L-NAME (10^{-3} M).

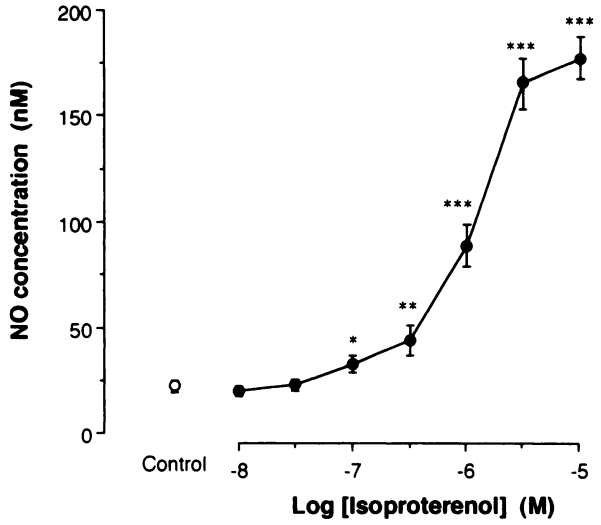

Fig. 5. Concentration-dependent effect of ISO on the release of NO from rabbit tracheal mucosa. ISO was cumulatively added to the superfusate (closed circles). In the control experiment, no drug was added (open circle). Responses are expressed as means ± S.E.; n = 8 for each point. * $P < .05$, ** $P < .01$, *** $P < .001$, significantly different from control values.

Discussion

Our present studies demonstrate that NO is spontaneously released from rabbit tracheal mucosa and that ISO increases Cl-PD *in vivo*, an effect that is accompanied by the enhanced release of NO. Thus NO derived through activation of NOS may play a role in the *beta* adrenoceptor-mediated Cl secretion across the tracheal mucosa.

Airway epithelial cells regulate electrolyte and fluid transport across airway mucosa and, hence, the output and composition of the respiratory tract secretions (Welsh, 1987). Active ion transport mechanisms generate lumen negative PD, and PD appears to result largely from secretion of Cl into the lumen and reabsorption of Na toward the interstitium (Boucher *et al.*, 1980). Mean PD of rabbit trachea in the present study was 2-fold greater than that previously reported in excised trachea *in vitro* (Jarnigan *et al.*, 1983). The reason for this difference is uncertain, but one possibility is that autonomic neurotransmitters and/or endogenous bioactive substances in the circulating blood contribute to the regulation of PD *in vivo*. Addition of amiloride to eliminate the component of Na transport (Al-Bazzaz and Zevin, 1984) decreased PD, and the remaining PD has been proposed to be dependent entirely on Cl secretion (Stutts *et al.*, 1990). This Cl-PD was increased by ISO in a concentration-dependent fashion, which indicates that stimulation of *beta* adrenoceptor enhances Cl secretion toward the respiratory lumen. This result was in accordance with the previous observations *in vitro* (Al-Bazzaz and Cheng, 1979), but the response to ISO was much less sensitive. This difference could be due to the difference in experimental conditions and/or to the species difference.

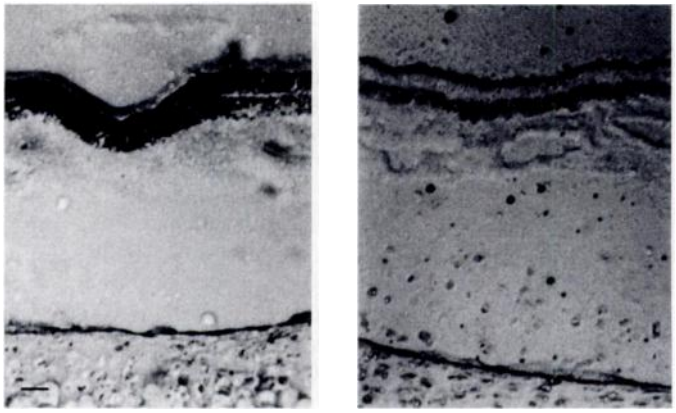

Fig. 6. Histochemical staining with NADPH diaphorase activity in rabbit tracheal mucosa showing strong blue reaction products within the epithelial cells (left panel). In the control experiment, NADPH was omitted and *p*-nitrophenylphosphate was included in the reaction medium (right panel). (Calibration bar = 30 μm.)

It is known that NO, a gaseous molecule that freely permeates cell membranes, is formed by NOS, which catalyzes the 5-electron oxidation of the guanidino nitrogen moiety of L-arginine to citrulline *via* N^G-hydroxyl-L-arginine. The existence of NOS in the respiratory tract has recently been shown in various cell types, including vascular endothelial cells, macrophages, mast cells, smooth muscle cells, nerve cells and airway epithelial cells (Kobzik *et al.*, 1993). We found that pretreatment of tracheal mucosa with L-NAME, an inhibitor of NOS (Rees *et al.*, 1990), by itself decreased Cl-PD, which indicates that NO may be spontaneously released and involved in the generation of Cl-PD under unstimulated conditions. Jain and co-workers (1993) recently reported that the NOS inhibitor L-NMMA inhibited the ISO-induced increase in ciliary beat frequency of bronchial epithelial cells. In accordance with this study, we found that L-NAME greatly reduced the ISO-induced increase in Cl-PD, whereas D-NAME did not, and that this inhibitory effect of L-NAME was reversed by the subsequent addition of L-arginine but not of D-arginine. These results suggest ISO enhances *in vivo* tracheal Cl secretion at least in part by stimulating NO synthesis. This notion is supported by the finding that the addition of SNAP, a NO donor (Boughton-Smith *et al.*, 1990), also increased Cl-PD.

However, the conclusion that NO is functioning in certain biological responses has generally been derived from the enantiomer-specific effect of NOS inhibitors, *e.g.*, effective inhibition of the responses by L-NAME or L-NMMA and the lack of the effect of D-NAME or D-NMMA, which is based on the premise that L-NAME, L-NMMA and other such arginine

analogs are specific inhibitors of NOS. On the other hand, amperometric sensors specific for NO have recently been developed to measure NO in aqueous media (Ichimori *et al.*, 1994; Malinski and Taha, 1992). Therefore, to determine whether NO was actually released from tracheal mucosa, we measured [NO] in the solution superfusing rabbit tracheal mucosa by an NO-selective electrode. Immersion of the electrode in the superfusate detected the electrical current and it was reduced by L-NAME, which suggests that NO is spontaneously released from the tracheal mucosa. Addition of ISO increased [NO] in a concentration-dependent manner, and the threshold concentration was similar to that observed in the Cl-PD experiment. Therefore, it seems that the stimulated release of NO may be linked to Cl secretion by airway epithelial cells.

Recent molecular cloning and sequencing analyses showed that there are at least three main isoforms of NOS (Nathan, 1992): type I (constitutive NOS, primarily soluble, *e.g.*, brain), type II (inducible NOS, primarily soluble, *e.g.*, macrophage) and type III (constitutive NOS, primarily particulate, *e.g.*, endothelium). Although we did not perform immunohistochemistry for these isoforms using monoclonal antibodies, our present studies showed that NADPH diaphorase activity was stained within rabbit tracheal epithelial cells without stimulation by cytokines or lipopolysaccharide, and that NO release was not altered by tetrodotoxin and atropine. We thus speculate that ISO stimulates airway epithelial Cl secretion through elevation of intracellular cAMP (Welsh, 1986) and that constitutive NOS localized to the epithelial cells rather than neural tissues may be responsible for the release of NO from the tracheal mucosa. Furthermore, it has been shown that constitutive NOS can be stoichiometrically phosphorylated by Ca^{++}/calmodulin protein kinase II and protein kinase A (Bredt *et al.*, 1991) and that vasodilation of coronary artery and ciliary stimulation of airway epithelium induced by *beta* adrenoceptor agonist are associated with the formation of NO (Parent *et al.*, 1993; Jain *et al.*, 1993). We found that application of ISO caused a rapid increase in [NO] in the superfusate. Taken together, stimulation of *beta* adrenoceptor and the concomitant activation of protein kinase A might have phosphorylated airway epithelial constitutive NOS and concomitantly generated NO.

In conclusion, stimulation of *beta* adrenoceptor increases Cl-PD across rabbit tracheal mucosa *in vivo*, an effect that is related to the corresponding generation of NO, probably by airway epithelial cells. Therefore, NO may be acting as a stimulator of Cl secretion, and hence of water secretion, produced by *beta* adrenoceptor agonists in the respiratory tract.

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