Abnormal Modulation of Cholinergic Neurotransmission by Endogenous Nitric Oxide in the Bronchus of Rats with Hyperresponsiveness Induced by Allergen Challenge

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ABSTRACT—The involvement of endogenous nitric oxide (NO) in bronchial cholinergic neurotransmission was compared between normal rats and airway hyperresponsive (AHR) rats. Male Wistar rats were sensitized and repeatedly challenged with dinitrophenylated (DNP)-Ascaris antigen. Twenty-four hours after the last antigenic challenge, enhancements of both the electrical field stimulation (EFS)-induced bronchoconstriction and acetylcholine (ACh) release were observed. NG-Monomethyl-L-arginine (L-NMMA; NO synthase inhibitor, 0.1 mM) augmented the EFS-induced bronchoconstriction and ACh release without affecting exogenously applied ACh-induced bronchoconstriction in normal rats. Interestingly, the augmentative effects of L-NMMA seen in normal rats were not manifested in AHR rats. Sodium nitroprusside inhibited the EFS-induced bronchoconstriction in a concentration-dependent manner; the inhibition was much larger than that of exogenously applied ACh-induced constriction in both normal and AHR rats. Furthermore, dibutryl cGMP (3 mM) inhibited the EFS-induced bronchoconstriction with no effect on the ACh-induced bronchoconstriction in both normal and AHR rats. These findings suggest that endogenous NO may have a modulatory role in bronchial cholinergic neurotransmission in normal rats and that the augmented ACh release in the AHR rats may result from the defect of endogenous NO-induced modulation of cholinergic nerve transmission.

Keywords: Airway hyperresponsiveness, Nitric oxide, Cholinergic neurotransmission, Nitrergic dysfunction, Dinitrophenylated (DNP)-Ascaris

Nitric oxide (NO) may play an important regulatory role in airway functions as an inhibitory non-adrenergic non-cholinergic (NANC) neurotransmitter in many species including humans (1–3). It has been demonstrated that neurally released NO not only relaxes tracheal smooth muscle but also inhibits acetylcholine (ACh) release from tracheal cholinergic nerves in rats and cats (4, 5). Furthermore, excessively produced NO from macrophages, leucocytes and epithelium of airways may take part in airway diseases (6).

Increased ACh release from cholinergic nerves may be an important mechanism that contributes to airway hyperresponsiveness in bronchial asthma (7). The ACh overrelease might be related to dysfunction of the modulatory NO pathway in cholinergic neurotransmission; however, the role of endogenous NO in accelerated cholinergic neurotransmission in airway hyperresponsiveness has not been confirmed.

The purpose of the present study was to determine whether NO has a modulatory effect on bronchial cholinergic neurotransmission in normal rats and whether any alteration of the modulatory effect of NO takes place in airway hyperresponsiveness. We therefore investigated the effects of an NO synthase (NOS) inhibitor, N(G)-monomethyl-L-arginine (L-NMMA), on both the cholinergic bronchoconstriction and amount of released ACh from bronchial tissue evoked by electrical field stimulation (EFS) in normal rats and dinitrophenylated (DNP) Ascaris antigen-induced airway hyperresponsive (AHR) rats. We have also investigated the effects of an NO donor, sodium nitroprusside (SNP), and a cyclic GMP analogue, dibutryl cyclic GMP (db-cGMP), on the EFS-and exogenously applied ACh-induced bronchoconstrictions to assess whether NO may be involved in airway hyperresponsiveness.
MATERIALS AND METHODS

Animals
Male Wistar rats (6 weeks of age, specific-pathogen-free, 170–190 g) were purchased from Charles River Japan, Inc. (Atsugi) and housed under standard laboratory conditions with free access to food and water.

Sensitization and antigenic challenge
Animals were sensitized according to the method of Tada and Okumura (8), and repeatedly challenged with DNP-Ascaris antigen to induce airway hyperresponsiveness as described previously (9, 10). Briefly, animals were sensitized by s.c. injection of DNP-Ascaris antigen (2 mg protein) with Bordetella pertussis (2 × 10^10) as an adjuvant, and 5 days later, were boosted by i.m. injection of DNP-Ascaris antigen (0.5 mg protein). Then 8 days after the first immunization, the animals received 3 times repeated antigenic challenges by inhalation of DNP-Ascaris antigen aerosol for 60 min every 48 hr. The in vitro experiments were performed at 24 hr after the last challenge because it is reported that symptoms of airway hyperresponsiveness (in vivo responsiveness to inhaled ACh, PCA titer and microvascular leakage) in DNP-Ascaris sensitized rats were markedly demonstrated at 24 hr after the last challenge (9).

Tissue preparation
The animals were stunned and exsanguinated. The lungs with the bronchi and trachea were rapidly removed and placed in Krebs-Henseleit solution (K-H solution) of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2, 1.2 mM MgSO_4, 25.0 mM NaHCO_3, 1.2 mM KH_2PO_4 and 10.0 mM glucose. The left main bronchus was isolated to make a 4-mm-long (6–7 cartilages) ring preparation. The ring preparation was suspended by two stainless-steel wires (0.3 mm diameter) passed through the lumen of the ring at a resting tension of 0.5 g in a 10-ml organ bath. The isometrical constriction was measured with a force-displacement transducer (SB-1T; Nihon Kohden Co., Tokyo) and recorded on a polygraph (FBR-252A; Toa Denkoh Co., Tokyo). The medium solution containing indomethacin (10^-6 M) and propranolol (10^-6 M) was maintained at 37°C and gassed continuously with 95% O_2 – 5% CO_2. During the equilibration period, the tissue was washed three to four times every 15 min with K-H solution.

Constrictile response to EFS
EFS was applied from two parallel platinum electrodes mounted on each side of the tissue preparation. Biphasic square wave pulses were delivered for 15-sec periods from a stimulator (SEN-3301, Nihon Kohden Co.) with a supramaximal voltage of 40 V at the source, frequencies between 0.5 to 64 Hz and pulse duration of 0.5 msec. Each 15-sec stimulus was delivered every 7 min, which allowed sufficient time between stimulations to return to its resting tension. Two consistent EFS responses were obtained at each frequency. Tetrodotoxin (10^-6 M), atropine (10^-6 M) or hexamethonium (10^-6 M) was added to the organ bath for 10 min before a series of EFS (0.5–64 Hz) in order to determine whether the EFS-induced bronchoconstriction was a cholinergic response.

Constrictile response to exogenously applied ACh
The concentration-response curve to exogenously applied ACh was constructed cumulatively with a concentration range of 10^-7–10^-3 M. A higher concentration of agonist was successively added after attainment of a plateau response to the previous concentration.

Experimental protocol for constrictile responses to EFS and exogenously applied ACh
L-NMMA was added to the 10-ml organ bath at a final concentration of 0.1 mM for 30 min before EFS or cumulative ACh application. SNP was added at a final concentration of 10^-1–10^-3 M just 2 min before EFS or ACh application. A higher concentration of SNP was added to the same organ bath after washing the previous concentration of SNP for 5 min. In preliminary experiments, repeated treatment with SNP did not reduce the sensitivity to SNP against the EFS- or exogenously applied ACh-induced bronchoconstriction. Furthermore, the amplitudes of EFS-induced bronchoconstriction were substantially equivalent to those of exogenously applied ACh in the normal and AHR rats as follows: ACh at 2 × 10^-6 M for EFS 4 Hz, 2 × 10^-5 M for 8 Hz in normal rats; ACh at 7 × 10^-6 M for EFS 4 Hz, 7 × 10^-5 M for 8 Hz in AHR rats. Db-cGMP (3 mM) was added for 20 min before EFS or ACh application. Effects of SNP and db-cGMP on the EFS- or ACh-induced bronchoconstriction were expressed as a percent change against the tension produced without SNP or db-cGMP.

Measurement of ACh release
The technique used for the measurement of ACh released from the isolated bronchus was based on the method of Baker et al., with some modifications (11). The isolated bronchial ring was suspended in the organ bath filled with K-H solution in the same condition as in the case of tension measurement except for the bath volume of 1 ml. During equilibration for 60 min, the bath solution was changed at 15-min intervals. After equilibration, the tissue was incubated with 0.1 mM ecotiope iodide, a potent irreversible cholinesterase inhibitor, for 15 min to prevent the breakdown of released ACh. The released ACh was extracted with a methanol-hexane mixture and subjected to gas chromatography-mass spectrometry.
ACh by endogenous cholinesterase. After the end of incubation, the bronchial tissue was washed with fresh K-H solution 3 to 4 times to remove ecotiopate. The bath solution was then changed to K-H solution containing L-NMMA (0.1 mM) or the vehicle of L-NMMA as a control. Thirty minutes after L-NMMA treatment, EFS (8 Hz, 40 V, pulse duration of 0.5 msec) was applied to the tissue for 10 min continuously. Ten minutes after the end of EFS, 800 µl of incubated solution was recovered and mixed with 100 µl of 10⁻⁶ M ethylhomocholine (EHC) as an internal standard and 100 µl of 100 mM EDTA-2Na. Then 100 µl of the mixture was subjected to analysis by a high-performance liquid chromatography-electrical detector system. The system consisted of a pump (880-Pu; Japan Spectroscopic Co., Tokyo), a column oven with an injector which had a 100-µl sample loop (960-Co, Japan Spectroscopic Co.), an electrochemical detector (CB-100; Eicom Co., Kyoto), an ACh and choline separation column (AC-GEL, Eicom Co.), and an enzyme reactor column with immobilized acetylcholinesterase and cholinesterase (AC-ENZ, Eicom Co.). The principles of the analytical techniques can be summarized as follows: 1) ACh, choline and EHC in the sample were separated in the separation column; 2) the separated ACh was hydrolyzed to acetate and choline by cholinesterase in the enzyme column, and then choline was oxidized to hydrogen peroxide (H₂O₂) and betaine by choline oxidase in the same column; 3) H₂O₂ produced was oxidized by the applied voltage (450 mV) in the electrochemical detector and resultant generated currents were detected by the electrode (12). The flow rate was 0.6 ml/min and column temperature was maintained at 33°C to improve the efficiency of cholinesterase and choline oxidase. The mobile phase was 0.1 M phosphate buffer (pH 8.5) containing 65 mg/l tetramethylammonium and 200 mg/l sodium decasulphonate. The retention times of EHC and ACh were 12.6 min and 18.2 min, respectively. The calibration curve for the determination of ACh was linear between 1.5 and 10.0 pmol/injection. The lower limit of ACh determination was 1 pmol/injection. L-NMMA (0.1 mM) and K-H solution containing indomethacin (10⁻⁶ M) and propranolol (10⁻⁶ M) did not influence the ACh and EHC peaks.

**Drugs**

N²-O-Monomethyl-L-arginine, L-arginine, sodium nitroprusside, N²,2’-O-dibutyril guanosine 3’:5’-cyclic monophosphate, tetrodotoxin and atropine (Sigma, St. Louis, MO, USA); hexamethonium (Wako Pure Chemicals, Tokyo) and acetylcholine (Daichi Pharmaceutical Co., Tokyo) were dissolved just before use in K-H solution. EHC (Eicom) and ecotiopate iodide (Santen Pharmaceutical Co., Osaka) were dissolved in distilled water and sal-

**RESULTS**

**Change in bronchial response to EFS in AHR rats**

EFS elicited a frequency-dependent constrictile response in normal and AHR rats. The EFS-induced bronchoconstriction was completely abolished by tetrodotoxin (10⁻⁶ M) or atropine (10⁻⁶ M) but not by hexamethonium (10⁻⁶ M) (data not shown), which indicates that the EFS-induced constrictile response is a cholinergic component in nature resulting from postganglionic neural stimulation. In the DNP-Ascaris repeatedly challenged rats, the frequency-response curve was significantly shifted to the left and above (F₁₁,₈₈₁=6.932, P<0.01 by ANOVA) (Fig. 1).

**Effects of L-NMMA on EFS-induced broncoconstriction in normal and AHR rats**

In the normal rat bronchus, pretreatment with L-NMMA (0.1 mM) increased the constrictile response to line at 10 mM and 1 mM, respectively and stored at 4°C.

**Statistical analyses**

All the data are represented as mean values ± S.E. Statistical analyses were performed by repeated measures two way analysis of variance (ANOVA). Individual group comparisons were made by the paired Student’s t-test.

**Drugs**

N²-O-Monomethyl-L-arginine, L-arginine, sodium nitroprusside, N²,2’-O-dibutyril guanosine 3’:5’-cyclic monophosphate, tetrodotoxin and atropine (Sigma, St. Louis, MO, USA); hexamethonium (Wako Pure Chemicals, Tokyo) and acetylcholine (Daichi Pharmaceutical Co., Tokyo) were dissolved just before use in K-H solution. EHC (Eicom) and ecotiopate iodide (Santen Pharmaceutical Co., Osaka) were dissolved in distilled water and sal-

**Fig. 1.** Effect of L-NMMA (0.1 mM) on the constrictile response to EFS of the isolated bronchus of 3 times repeatedly challenged rats with DNP-Ascaris antigen (airway hyperresponsive: AHR) and of normal rats. Each point represents the mean with S.E. from 8 (normal: □), 5 (AHR: ○), 9 (normal with L-NMMA: ■) and 10 (AHR with L-NMMA: ●) experiments. There are significant differences (P<0.01, ANOVA) between AHR and normal groups, and between normal + L-NMMA and normal groups.
EFS and significantly shifted the frequency-response curve for EFS to the left and above ($F_{1,120} = 6.851, P < 0.01$ by ANOVA). The combination of L-NMMA (0.1 mM) with L-arginine (1 mM), however, did not enhance the EFS-induced response (data not shown).

The augmentative effect of L-NMMA on the EFS-induced bronchoconstriction seen in normal rats was not manifested in AHR rats ($F_{1,104} = 3.932$ for L-NMMA vs Control, n.s. by ANOVA) (Fig. 1). L-NMMA (0.1 mM) did not affect the basal resting tension in both normal and AHR rats.

**Effects of L-NMMA on EFS-induced ACh release in normal and AHR rats**

The EFS-induced ACh release from bronchial tissue of AHR rats was significantly increased as compared with that of normal rats (normal rats: 27.4±2.2 pmol/ml, n = 8 and AHR rats: 41.3±3.9 pmol/ml, n = 6, respectively; $P < 0.01$). Basal release of ACh without EFS was below the detection limit (1.5 pmol).

Pretreatment with L-NMMA (0.1 mM) significantly increased the EFS-induced ACh release in normal rats (with and without L-NMMA: 27.4±2.2 pmol/ml, n = 8 and 48.9±3.5 pmol/ml, n = 6, respectively; $P < 0.001$). However, the augmentation by L-NMMA (0.1 mM) of the EFS-induced ACh release disappeared in AHR rats (with and without L-NMMA: 41.3±3.9 pmol/ml, n = 6 and 45.4±2.9 pmol/ml, n = 6, respectively; n.s.) (Fig. 2).

In the present experiments, no difference was observed
Fig. 4. Concentration-response curves to sodium nitroprusside for the constrictile responses of the normal rat bronchus to EFS (4, 8 Hz) and ACh (2×10⁻⁶, 2×10⁻⁵ M). Each point represents the mean with S.E. from 6 (EFS, 4 Hz: ■), 6 (ACh, 2×10⁻⁶ M: □), 5 (EFS, 8 Hz: ○) and 6 (ACh, 2×10⁻⁵ M: □) experiments. **P<0.01 and ***P<0.001 vs ACh (2×10⁻⁶ M). *P<0.05, **P<0.01 and ***P<0.001 vs ACh (2×10⁻⁵ M).

Fig. 5. Concentration-response curves to sodium nitroprusside for the constrictile responses of the AHR rat bronchus to EFS (4, 8 Hz) and ACh (7×10⁻⁶, 7×10⁻⁵ M). Each point represents the mean with S.E. from 7 (EFS, 4 Hz: ■), 6 (ACh, 7×10⁻⁶ M: □), 8 (EFS, 8 Hz: ○) and 6 (ACh, 7×10⁻⁵ M: □) experiments. *P<0.05, **P<0.01 and ***P<0.001 vs ACh (7×10⁻⁶ M). †††P<0.001 vs ACh (7×10⁻⁵ M).
in the weight range of dried bronchial tissue between normal and AHR rats.

Effects of L-NMMA on exogenously applied ACh-induced bronchoconstriction in normal rats

Exogenously applied ACh elicited a concentration-dependent constrictile response in normal rat bronchus. The cumulative concentration-response curve to exogenously applied ACh was not changed in the presence of L-NMMA (0.1 mM) (F[1,641]=4.020, n.s. by ANOVA) (Fig. 3).

Effect of SNP on EFS- or exogenously applied ACh-induced bronchoconstriction in normal and AHR rats

SNP (10^{-7}–10^{-3} M) reduced the EFS- (4 or 8 Hz) and ACh (2×10^{-6} M or 2×10^{-5} M)-induced bronchoconstrictions in a concentration-dependent manner in normal rats, but the extent of inhibition of the EFS-induced bronchoconstriction was significantly higher than that of the ACh-induced bronchoconstriction (Fig. 4) (4 Hz vs ACh (2×10^{-6} M), n=6; 8 Hz vs ACh (2×10^{-5} M), n=5 or 6).

SNP (10^{-7}–10^{-3} M) also reduced the bronchoconstriction in a concentration-dependent manner in AHR rats; the inhibition of the EFS (4 or 8 Hz)-induced bronchoconstriction by SNP was significantly stronger than that induced by ACh (Fig. 5) (4 Hz vs ACh (7×10^{-6} M), n=7 or 6; 8 Hz vs ACh (7×10^{-5} M), n=8 or 6). SNP at even the highest concentration did not affect the basal resting tension in both the normal and AHR rats.

Effect of db-cGMP on EFS- or exogenously applied ACh-induced bronchoconstriction in normal and AHR rats

Db-cGMP (3 mM) significantly inhibited the EFS (4 Hz)-induced bronchoconstriction without affecting the ACh (2×10^{-6} M)-induced bronchoconstriction in normal rats (EFS and ACh: −15.6±2.7%, n=6 and 3.1±2.6%, n=5, respectively; P<0.001).

In AHR rats, db-cGMP (3 mM) also significantly inhibited the EFS (4 Hz)-induced bronchoconstriction without affecting the ACh (7×10^{-6} M)-induced bronchoconstriction as seen in normal rats (EFS and ACh: −14.5±2.8%, n=5 and 7.4±4.2%, n=5, respectively; P<0.01) (Fig. 6). Db-cGMP (3 mM) did not affect the basal resting tension in both normal and AHR rats.

DISCUSSION

In AHR rats, the EFS-induced ACh release was increased as compared to that in normal rats. The ACh overrelease may have resulted in the increased constrictile response to EFS in AHR rats. Chiba and Misawa (13) have, however, shown an increased bronchoconstrictile response to exogenously applied ACh in the same AHR rats. Therefore, the increased constrictile response to EFS seen in AHR rats may be due to both the ACh overrelease and hyperresponsiveness to released ACh of bronchial smooth muscle.

In normal rat bronchus, the pretreatment with L-NMMA increased the EFS-induced constriction and ACh

![Fig. 6. Effect of dibutyryl cGMP (3 mM) on the constrictile responses to EFS (4 Hz) and ACh (2×10^{-6} M) of the normal rat bronchus (left) or to EFS (4 Hz) and ACh (7×10^{-5} M) of the AHR rat bronchus (right). Each column represents the mean with S.E. from 7 (Normal, EFS), 6 (Normal, ACh), 5 (AHR, EFS) and 5 (AHR, ACh) experiments. **P<0.001 vs ACh (2×10^{-6} M) and †P<0.01 vs ACh (7×10^{-5} M).]
release. These results were consistent with that of Sekizawa et al. (4) who demonstrated that L-NMMA increased EFS-induced ACh release from the rat trachea and also with that of Jing et al. (5) who demonstrated that another NO synthase inhibitor, N\textsuperscript{G}-nitro-L-arginine (L-NNA), increased EFS-induced excitatory junctional potential which is an index of cholinergic neurotransmission.

In the present experiment, L-arginine reversed the augmentative effect of L-NMMA on the EFS-induced bronchoconstriction, and L-NMMA neither affected the resting tension nor the exogenously applied ACh-induced bronchoconstriction in normal rats. These findings indicate that the EFS stimulates both ACh release and NO release resulting from activation of NO synthase; the released NO would in turn affect cholinergic nerve transmission rather than bronchial smooth muscle itself. NO synthase in airways is thought to exist in vascular endothelial cells, airway epithelial cells, inflammatory cells and sensory nerves (6). It is also demonstrated that NO synthase coexists with vasoactive intestinal peptide (VIP) in a neuron of ferret trachea (14). The endogenous NO in the rat bronchus may originate from nerve terminal rather than from epithelial cells through NO synthase activation.

Interestingly, the augmentative effects of L-NMMA seen in normal rats on the EFS-induced bronchoconstriction and ACh release were not manifested in AHR rats. These findings suggest that the modulatory role of endogenous NO in cholinergic neurotransmission in airways may be suppressed in the state of airway hyperresponsiveness. We furthermore investigated the effects of exogenously applied NO on cholinergic neurotransmission to determine whether the dysfunction of modulatory NO mechanism seen in AHR rats is due to the lack of responsiveness to NO itself. In both normal and AHR rats, SNP inhibited the EFS-induced bronchoconstriction much more greatly than did the ACh-induced bronchoconstriction whose amplitude was equivalent to that of the constrictile response induced by EFS. Db-cGMP also inhibited the EFS-induced bronchoconstriction much more markedly than did the ACh-induced bronchoconstriction in both normal and AHR rats. These results demonstrate that the endogenously generated NO on cholinergic neurotransmission may possibly fulfill the intended function even in AHR and also suggest that endogenous NO may modulate cholinergic neurotransmission through a cGMP-dependent mechanism.

The reason for the lack of augmentative effects of L-NMMA on cholinergic neurotransmission seen in AHR rats is not yet understood presently. However, it is possible that the function of NO synthase may be disordered in AHR, leading to a reduction of NO generation. Recently, it is demonstrated that NO synthase activity and NO-mediated NANC relaxant response were inhibited by pretreatment with NO donor (15, 16), suggesting that generation of NO may be under autoregulation. It is also demonstrated that inducible NO synthase (iNOS) has been expressed in the airway epithelium of asthmatic patients (17, 18), and the NO generated by iNOS was detectable in the exhaled air (19). It is thus possible that NO synthase may be overregulated by NO excessively generated by iNOS which may be induced in the AHR state. Otherwise, it is also possible that NO may quickly react with superoxide anion (O\textsuperscript{2-}) to become peroxynitrite (ONOO\textsuperscript{-}) (14) which would disturb the normal NO-mediated modulation of neurotransmission. Superoxide anion is reportedly generated in the airway tissues in the asthmatic state (20), and peroxynitrite has a strong cytotoxic effect on the airway (21).

In summary, we demonstrated that endogenous NO may have a modulatory role through a cGMP-dependent mechanism in cholinergic neurotransmission of the normal rat bronchus. The increased ACh release from the bronchus of AHR rats may be attributable to the lack of modulation on cholinergic neurotransmission by endogenous NO to cause AHR.

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