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MODULATION OF AIRWAY EPITHELIAL CELL CILIARY BEAT FREQUENCY BY NITRIC OXIDE

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NOS activity has been recently described in airway epithelial cells. Because these cells are often ciliated we hypothesized that NO modulates airway ciliary beating. CBF was measured in cultured BBECs using video microscopy. L-NMMA, a NOS inhibitor, caused a 40% decrease in CBF following pre-stimulation with isoproterenol (8.5 ± 0.3 Hz vs 14.6 ± 0.2 Hz; $p < 0.0001$) which lasted approximately 60 minutes. Similar attenuation in CBF after isoproterenol pre-treatment was observed with another NOS inhibitor, L-NAME. NOS inhibitor-induced CBF slowing was also observed when cells were pre-stimulated with either bradykinin or substance P and was completely reversed by L-arginine or SNP but not by D-arginine. These observations demonstrate a novel NO-dependent mechanism that upregulates ciliary motility in response to stimulation.

Recently it has become clear that NO is an important bioactive molecule that affects many critical cell functions (1). NO is released by an enzyme family of NOS from the substrate, L-arginine. NO has been shown to elicit a number of biological responses in the lung such as pulmonary arterial vasodilatation and bronchodilatation (2, 3). In addition, NO is present in the exhaled air of humans (4). Robbins et al. have recently demonstrated the presence of NOS activity in BBECs (5) . Some of these airway epithelial cells have cilia, which play an important role in host defense by propelling mucus-trapped debris and particles out of the lung. The cellular control of ciliary function, however, is not well

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ABBREVIATIONS:
BBECs, bovine bronchial epithelial cells; CBF, ciliary beat frequency; cAMP, cyclic adenosine 3',5'-monophosphate; cGMP, cyclic guanosine 3',5'-monophosphate; L-NAME, N^G-nitro L-arginine methyl ester; L-NMMA, N^G-monomethyl L-arginine; M199, Medium 199; NO, nitric oxide; NOS, nitric oxide synthase; SNP, sodium nitroprusside; PBS, phosphate-buffered saline.

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understood. Given the local release of NO in the airways and the wide range of biological activities exhibited by NO, we hypothesized that NO modulates ciliary beat frequency in cultured BBECs.

MATERIAL AND METHODS

Preparation and culture of ciliated BBECs. Ciliated BBECs were obtained from fresh bronchi as previously described (6). Briefly, the bronchi from fresh bovine lungs were dissected from alveolar tissue, incubated overnight in 0.1% protease in M199 (GIBCO BRL, Grand Island, NY) and the lumens rinsed repeatedly the next day with M199 containing 10% fetal calf serum (Biofluids, Rockville, MD). The ciliated cell rich aggregates were placed onto petri dishes which were pre-coated with type I collagen gel matrix (Vitrogen 100, Collagen Corp., Palo Alto, CA), and maintained in culture (37 °C; 10% CO_2) for 48-72 hours prior to the experiments.

Determination of CBF. The motion of the actively beating ciliated BBECs was quantified by measuring CBF using phase contrast microscopy and videotape analysis as previously described (6). Using this method, ciliary beat frequencies were measured in a range of 0-15 Hz with an accuracy of ± 0.25 Hz.

Effects of NOS inhibitors on CBF. Prior to performing experiments, the cells were washed with PBS, and 1.5 ml of arginine-free minimum essential media, prepared from the Select Amine Kit (GIBCO), were added. Cells and reagents were equilibrated to 24°C prior to each experiment. All compounds were added in small volumes (10 μl) to the cells at appropriate time intervals. CBF was recorded at baseline (time = 0) and following addition of each compound at various time points. The cells were initially pre-treated with isoproterenol (1 mM), bradykinin (1 μM) or substance P (10 μM). Once CBF stimulation was observed, various concentrations of L-NMMA or L-NAME, in PBS containing 0.1 mM ß-nicotinamide adenine dinucleotide, was added to ciliated BBECs. The experiments were performed over a time period of 90 minutes. Substance P was obtained from Peninsula Laboratories (Belmont, CA) and L-NMMA from Calbiochem Inc.(La Jolla, CA). All other reagents were obtained from Sigma Chemicals Co. (St. Louis, MO).

Measurement of cytotoxicity. Cytotoxicity during these experiments was assessed by assaying lactate dehydrogenase (LDH) and ^{51}Cr release by BBECs into supernatants as previously described (7).

Statistical Analysis. Data are expressed as mean ± one standard error of the mean. Comparison between two groups was performed using the Student's t-test. A p value < 0.05 was considered significant.

RESULTS

Effects of NOS inhibitors on CBF. CBF of BBECs exposed to media alone remained constant over the 90 minute experiment (Fig. 1A). In cells exposed to isoproterenol alone, CBF increased from 12.0 ± 0.5 to 15.0 ± 0.5 hz ($p < 0.01$) within 30 minutes of exposure and remained constant for approximately 60 minutes (Fig. 1B). Addition of L-NMMA induced a rapid decrease in CBF from 14.6 ± 0.2 Hz to 8.5 ± 0.4 Hz ($p < 0.01$). Ciliary slowing was detectable within one minute of adding L-NMMA with maximal slowing occurring by 15 minutes and lasting approximately 60 minutes (Fig. 1C). Similar attenuation of CBF was seen with L-NAME (Fig. 1D).

Concentration dependence of the ciliary-slowing effect of NOS inhibitors. The L-NMMA-induced CBF slowing was concentration-dependent, decreasing CBF from stimulated rates of 15.0 ± 0.5 Hz to 11.0 ± 0.5, 10.0 ± 0.5 and 8.0 ± 0.3 Hz with 0.1, 1.0 and 10 mM L-NMMA, respectively (Fig. 2). A lesser degree of attenuation in CBF was seen with similar concentrations of L-NAME (Fig. 2). In contrast, D-NMMA (10 mM), the inactive enantiomer of L-NMMA, had a very small CBF slowing effect (Fig. 2).

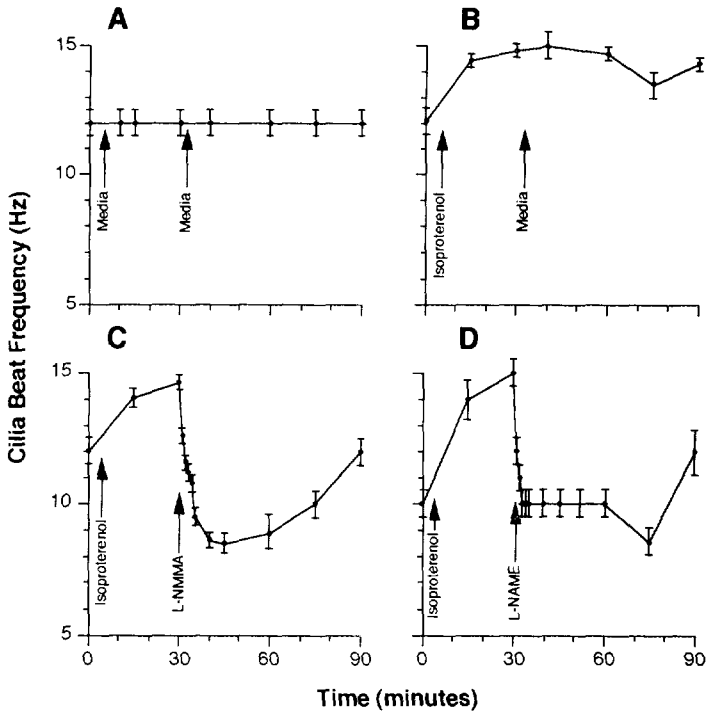

Figure 1. *Effect of NOS inhibitors on CBF in ciliated BBECs.* CBF was measured over a period of 90 minutes. Each point represents the mean ± SEM of 4 different experiments. CBF in each experiment was derived from the mean of at least 5 different cells from different areas of each plate. *A*: CBF measured in a plate to which arginine free media was added at time = 0 and at 30 minutes. *B*: Isoproterenol (1mM) added at time =0 followed by the addition of media at 30 minutes. *C* and *D*: Isoproterenol (1mM) added at time = 0 followed by the addition of L-NMMA (0.1 mM) and L-NAME (10 mM),respectively, at 30 minutes.

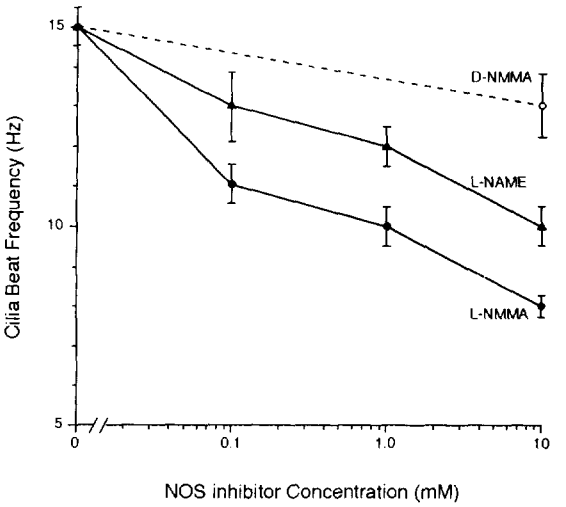

Figure 2. *Concentration dependence of the ciliary-slowing effect of NOS inhibitors.* CBF was measured 10 minutes after exposure to 0.1, 1.0, 10.0 mM L-NMMA (closed circles) and L-NAME (closed triangles), and 10 mM D-NMMA (open circles). These cells were pre-treated for 30 minutes with 1 mM isoproterenol. CBF is representative of the mean ± SEM of at least 5 different cells on each of three plates.

Addition of L-NMMA, in the absence of pre-stimulation with isoproterenol, had no significant effect on CBF. However, pre-treatment with L-NMMA did prevent the CBF stimulation effects of isoproterenol (data not shown).

Effects of NOS inhibitors on BBECs stimulated with other cilia agonists. To determine if L-NMMA-induced ciliary slowing following exposure to isoproterenol was specific only to this agent, ciliated BBECs were pre-stimulated with two other cilia agonists, bradykinin and substance P, which are also known to release NO (8, 9). Pre-treatment with these cilia agonists increased CBF to a level comparable to that observed with isoproterenol (Fig. 3). While the addition of L-NMMA resulted in ciliary slowing following all three agonists, CBF decreased far below baseline in the presence of isoproterenol. In contrast, CBF returned only to baseline values following pretreatment with bradykinin and substance P.

Reversal of NOS inhibitor-induced effects on CBF by L-arginine or SNP. L-arginine and SNP completely reversed L-NMMA-induced CBF slowing (Fig. 4). In contrast, D-arginine, the inactive enantiomer of L-arginine, had no significant effect on L-NMMA-induced CBF slowing. In addition, L-arginine and SNP had no significant effect on CBF in unstimulated cells (data not shown).

Measurement of cytotoxicity. There was no significant differences in the release of LDH or ^{51}Cr from cells exposed to L-NMMA, L-NAME, L-arginine or SNP compared to cells exposed to media alone (data not shown).

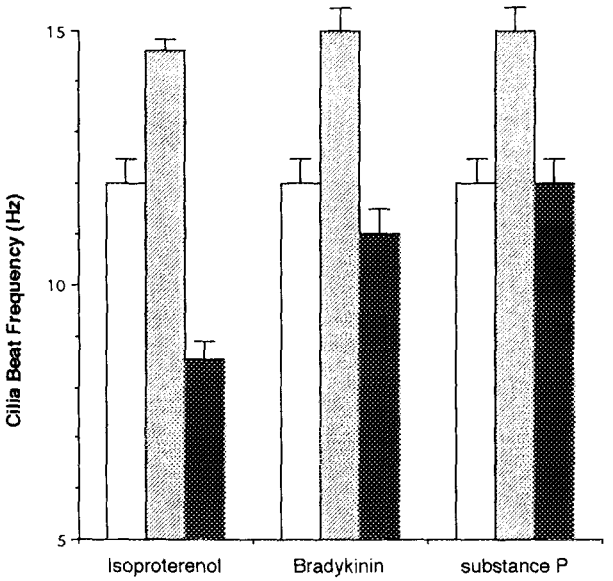

Figure 3. *Effects of NOS inhibitors on cells pre-stimulated with isoproterenol, bradykinin and substance P.* The cells were pre-stimulated with different cilia agonists which included isoproterenol (1 mM), bradykinin (1 μM) and substance P (10 μM). CBF is representative of means ± SEM from one experiment and was derived from the mean of at least 5 different cells from different areas of each plate. The open columns represent CBF obtained from cells exposed to arginine-free media. The hatched columns represent the maximal CBF observed within the 30 minute period following the addition of each agonist. The black columns represent the lowest CBF observed within the 15 minute period following the addition of L-NMMA (0.1 mM).

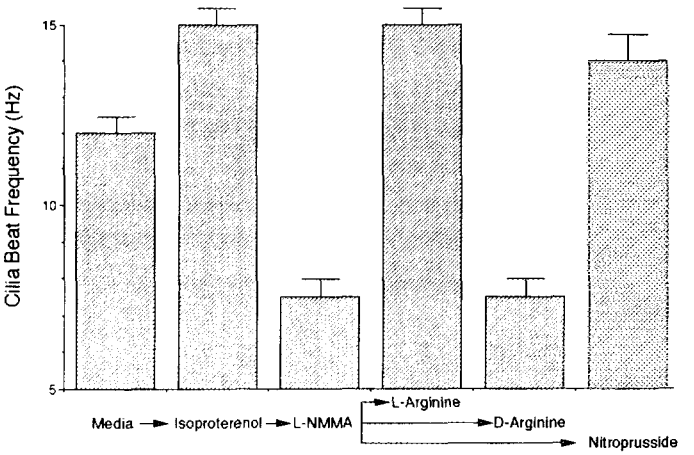

Figure 4. *Reversal of the effects of NOS inhibitors on CBF by L-arginine and NO donors.* Ciliary beat frequency (CBF) was measured at baseline (t = 0 min) and after the sequential addition of isoproterenol (1 mM; t = 30 min) and N^G-monomethyl-L-arginine (L-NMMA; 0.1 mM; t = 40 min). L-arginine (10 mM), sodium nitroprusside (0.1 mM) or D-arginine (10 mM) was then added (t=40 min) and CBF again determined (t = 50 min). CBF represents the mean ± SEM of at least 5 different cells on each of three plates.

DISCUSSION

The results of the present study demonstrate a novel NO-dependent mechanism that upregulates CBF in cultured BBECs. This conclusion is based on the following observations: a) NOS inhibitors slowed CBF following stimulation with three unrelated cilia agonists, two of which are known to release NO; b) these effects were reversed by L-arginine but not D-arginine; c) addition of SNP, an NO donor, was equally effective in reversing NOS inhibitor-induced effects; and d) these effects occurred without any demonstrable cytotoxicity.

We found that L-NMMA and L-NAME induced CBF slowing only when cells were pre-stimulated with cilia agonists. This suggests that resting ciliary motility is not modulated to a significant extent by NO. Further support for this conclusion was the lack of response of CBF to SNP or L-arginine in cells not pre-treated with cilia agonists. Collectively, these data suggest that stimulation of airway epithelial cells leads to NO release which results in an increase in ciliary motility (4, 5, 10-12).

NO appears to play an important role in regulating several biologic functions in the lung including modulation of pulmonary arterial and bronchial smooth muscle tone (2, 3, 12). In addition, NO has been shown to regulate inflammatory responses that are relevant to airway defense mechanisms, such as neutrophil adhesion and chemotaxis, and histamine release from mast cells (13, 14). The possible signal transducing pathways that might modulate NO-induced increase in ciliary motility have not been elucidated in the present study (15-18). It is well established that NO increases cytosolic cGMP concentrations in other systems (1). The observation that substance P and bradykinin, which release NO, both increased CBF is consistent with the hypothesis that NO-induced upregulation of ciliary motility is also mediated through intracellular cGMP. In addition, Boulanger and

Vanhoutte showed that intracellular cAMP may be involved in NO-induced biologic effects (19). Consistent with this observation, we found that the isoproterenol-induced increase in CBF was also attenuated by L-NMMA and L-NAME. Nevertheless, we cannot exclude the possibility that additional mechanisms are involved in upregulation of CBF in ciliated BBECs by the agonists used in the present study.

In summary, our study demonstrates a novel NO-dependent mechanism that upregulates ciliary motility *in vitro*. These findings suggest that NO plays a important role in upregulation of ciliary motility in response to airway stimulation.

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